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OPINION REVIEW

Irreversible electroporation for the management of pancreatic cancer: Current data and future directions

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Abstract

Pancreatic cancer is currently the seventh leading cause of cancer death (4.5% of all cancer deaths) while 80%-90% of the patients suffer from unresectable disease at the time of diagnosis. Prognosis remains poor, with a mean survival up to 15 mo following systemic chemotherapy. Loco-regional thermal ablative techniques are rarely implemented due to the increased risk of thermal injury to the adjacent structures, which can lead to severe adverse events. Irreversible electroporation, a promising novel non-thermal ablative modality, has been recently introduced in clinical practice for the management of inoperable pancreatic cancer as a safer and more effective loco-regional treatment option. Experimental and initial clinical data are optimistic. This review will focus on the basic principles of IRE technology, currently available data, and future directions.

Key Words: Pancreatic cancer; Interventional oncology; Irreversible electroporation; Ablation; Loco-regional treatment; Image-guided treatment

Core Tip: Loco-regional thermal ablative techniques such as radiofrequency, microwave, and cryoablation are rarely implemented for the treatment of inoperable pancreatic cancer due to the increased risk of thermal injury to the adjacent structures. Irreversible electroporation is a promising novel non-thermal ablative modality that could provide a safer and effective ablation *via* the application of electric pulses to damage cell membranes and cell homeostasis resulting in cancer cell necrosis and apoptosis. Experimental and initial clinical data are optimistic, and its potential immunomodulatory effect and synergism with immunotherapy provides are promising.

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INTRODUCTION

Pancreatic cancer is currently the seventh leading cause of cancer death, representing 4.5% of all cancer deaths worldwide. Most importantly, overall prognosis remains extremely poor as approximately 80%-90% of patients suffer from unresectable disease at the time of diagnosis, with a less than 8% relative survival rate at 5 years[1]. Systemic chemotherapy using gemcitabine or more recently FOLFIRINOX regimens, with or without radiotherapy, results in overall survival rates ranging from 9 to 14 mo[2,3]. Moreover, thermal (radiofrequency and microwave) and cryoablative techniques are not commonly employed due to the increased risk of severe trauma to the adjacent major anatomical structures[4]. Irreversible electroporation (IRE) is a promising novel percutaneous, image-guided nonthermal ablative modality that has been recently introduced in clinical practice for the management of pancreatic cancer.

MECHANISM OF ACTION

The phenomenon of IRE was first reported in the 1970s to describe the alteration of transmembrane potential leading to the increased cell membrane permeability, disruption of the dual lipid layer, and the creation of permanent nanoscale defects (nanopores) in the cell membrane following the application of high-voltage pulsed electric fields across the cell[5]. This technique results in failure of the cell homeostasis, electrolyte alteration, and cell death by apoptosis[6-9].

In contrast to necrosis induced by thermal ablative methods, non-thermal apoptotic active cell death does not incite inflammation and enables ablation with minimal distortion of the adjacent tissues. However, since 2006 when the first *in vivo* model of IRE for cancer ablation was reported, several experimental studies have reported solely necrosis or mixed results of both necrosis and apoptosis following the application of IRE[10-13]. According to currently available experimental data, apoptosis has been demonstrated immediately after application of IRE in a murine cancerous pancreas model and at 7 to 14 d in a porcine healthy pancreas model, while necrosis is evident immediately and up to 14 d later. Unfortunately, pathology data on human pancreatic cancer are extremely limited and as the IRE ablative effect is directly correlated to the physical properties of the target tissue, the significant discrepancies between *in vivo* normal/cancer animal models, and human cancer/normal pancreatic tissues. This presents a major limitation regarding our knowledge on the actual effects of IRE[14-16]. Moreover, data indicate that IRE is not homogenously distributed along the target tissue, and various effects are produced with increasing voltage and time.

While apoptosis is certainly occurring in some cells within the treatment zone, Brock *et al*[17] suggest that IRE could initiate multiple types of cell death mechanisms, but the size and shape of the regions in which each type is experienced may vary between clinical treatments depending on differences in pulsing parameters, tissue type, and treatment time. Thus, there may be more than one type of cell death mechanism at play, and may include pyroptosis or necroptosis. Likewise, for cells at the margins of the treatment areas, the response observed may actually be survival signaling in response to reversible electroporation. In theory, this could be taken advantage of and combined with chemotherapy treatments to increase drug delivery, tumor penetration, and treatment of residual cancer cells[17,18].

IRE has significant inherent advantages over thermal ablation for the treatment of pancreatic cancer (Figure 1). Most importantly, IRE does not produce a temperature increase to achieve tumor destruction. Therefore, it does not elicit thermal injury to the superior mesenteric and portal veins, superior mesenteric and celiac arteries, bile duct adjacent nerves, or gastrointestinal structures, which has restrained the use of local thermal ablation treatment. Another significant advantage is the absence of

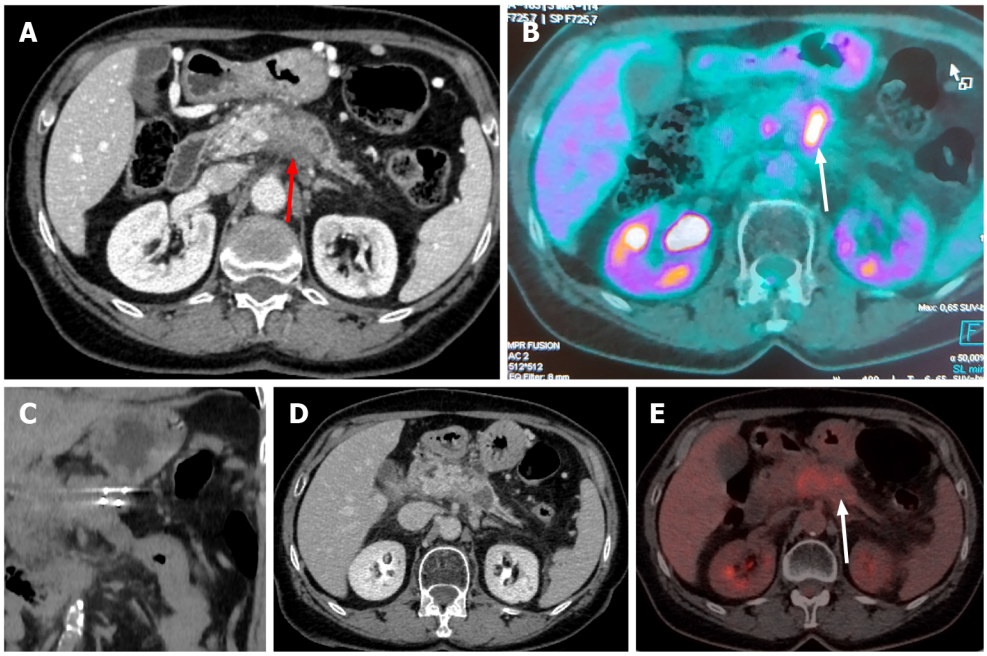

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Figure 1 Pancreatic cancer treated with percutaneous irreversible electroporation. A: Axial computed tomography (CT) showing a lesion (arrow) in the body of the pancreas consistent with ductal adenocarcinoma previously treated with chemo and radiation therapy; B: Positron emission tomography (PET)-CT scan showed the residual active part of the lesion (arrow); C: CT coronal view with 4 parallel electrodes positioned within the lesion; D and E: 3-mo follow-up CT (D) and PET-CT (E) showing complete ablation of the tumor with photopenic area in the site of ablation (arrow).

the "heat sink" effect in which the flow of large blood vessels decreases the thermal ablative effect[19-22]. According to published clinical protocols, the procedure is performed under computed tomography (CT) guidance, general anesthesia (complete muscle paralysis), and electrocardiography synchronization due to the possibility of muscular spasms induced by high-voltage pulses[23].

IRE is induced by electrodes connected to a high-voltage pulse generator. Multiple electrode pairs can be used; the number of electrodes needed and their exact placement is decided during pre-procedural planning. For small tumors measuring up to 2 cm, three electrodes are placed at the periphery of the target lesion, and for lesions between 2 cm and 3 cm, four electrodes are used. However, for lesions > 3 cm, a maximum number of six electrodes is allowed with four or five electrodes at the periphery and one or two at the center of the lesion. An optimal interelectrode distance between 7 mm and 24 mm has been described. The correct positioning of electrodes requires experience often deriving from that with other ablative methods. Skill is also needed in using ultrasound or CT techniques as a guide for positioning the electrodes and avoiding accidental damage to surrounding organs.

With respect to large vessels close to the tumor, a minimum safety distance of 2 mm is recommended to avoid the risk of burn damage. In cases of locally advanced pancreatic cancer with involvement of the mesenteric artery or vein, placing the electrodes parallel to the vessels has proven effective. Following electrode placement, the generator produces short, repeated pulses using predetermined voltage settings to reach a target current of 20-50 A. In clinical practice usually, 90 pulses per treatment cycle are used, with a pulse duration of 70-90 μs, and a voltage setting between 1400-1800 V/cm (maximum capability 3000 V/cm)[2,9,24-26]. According to standard ablation technique protocols, the aim is to create a safe 5 mm tumor-free IRE zone, also referred as A0 ablation (analogous to an R0 surgical resection).

PRE-CLINICAL DATA

To date, only a few reports on IRE for the treatment of pancreatic cancer exist in literature. Some of them reported the use of the technique on animal models (Table 1). These studies have used animal xenografts carrying human pancreatic cancer cells to understand the histological effect of IRE on pancreatic cancer tissue. The results following application of IRE showed evidence of both acute coagulative necrosis and apoptosis of pancreatic cancer tissue followed by fibrosis.

In 2010, Charpentier *et al*[27] reported a pilot study on IRE in a normal pancreas porcine model. They showed the following histological features: Initial active local inflammation, interstitial edema, and significant necrosis (after 7 d) followed by the development of fibrosis. However, these results were not significant for IRE efficacy because normal pancreatic tissues had a very different conductivity than

Table 1 Irreversible electroporation of pancreatic cancer in animal models

Ref.	Animal model	*n*	Tumour location	Histology	Major complication
Charpentier *et al*[27], 2010	Porcine	4	NP	Necrosis	None
Bower *et al*[28], 2011	Porcine	6	Orthotopic PC	Necrosis	None
Joséet al[29], 2012	Mouse	24	Orthotopic PC	Necrosis	None
Fritz *et al*[33], 2015	Porcine	10	Orthotopic PC	Necrosis	None
Su *et al*[30], 2018	Mouse	22	Orthotopic PC	Necrosis	None
Shankara Narayanan *et al*[31], 2018	Mouse	N/A	Subcutaneous/orthotopic PC	Necrosis	None
Lee *et al*[32], 2021	Porcine	N/A	Orthotopic PC	Necrosis	None

N/A: Not available; NP: Normal pancreas; PC: Pancreatic cancer.

pancreatic tumors.

Subsequently, Bower *et al*[28] and José *et al*[29] suggested that IRE could be an effective treatment for locally advanced pancreatic cancer in a mouse model without systemic toxicity and major local complication. Su *et al*[30] concluded that IRE was a safer and shorter operation than traditional ablation and represented a promising new approach for pancreatic cancer. Narayanan *et al*[31] described IRE for a pancreatic cancer mouse model and concluded that this animal model serves as a robust system to study the effects and clinical efficacy of IRE. Also, Lee *et al*[32] demonstrated and confirmed on a porcine model the safety and minimal complications of IRE ablation in pancreatic cancer tissue. Finally, the results of IRE in animal models of the treatment of pancreatic cancer showed the ability to ablate the target cells while preserving the collagen architecture of vascular, biliary, and neuronal structures[12, 28].

CLINICAL DATA

The prognosis of patients with pancreatic cancer not eligible for surgery remains poor despite many chemoradiation protocols. Therefore, different approaches for treatment of this disease are required. Ablation procedures including radiofrequency ablation, microwave ablation, cryoablation, high intensity focused ultrasound, and IRE can offer symptomatic relief, survival benefit, and potential tumor downsizing. Nevertheless, thermal procedures using extreme temperatures can induce injury to the pancreatic duct, bile duct, and adjacent vessels, potentially resulting in fistula formation, bile leaks, and hemorrhage, respectively[34].

IRE is an emerging non-thermal local ablation technique that affects with electricity only target cell membranes and avoids the nearby vessels and vital structures. Therefore, IRE can also be used in tumors positioned near some vital structures or organs more safely compared to other ablative procedures[35]. According to the American Joint Committee on Cancer stage criteria (8th edition), the major current indications for the use of IRE in the treatment of pancreatic cancer are as follows: Locally advanced pancreatic cancer stage II or III (T4N1M0) with ≤ 3 positive regional lymph nodes; tumor size maximal axial diameter ≤ 5 cm; and tumors in patients not candidates for radical resection or who refuse this surgery. IRE also carries some absolute and relative contraindications. It cannot be used if there are metal implants less than 2.5 cm from the ablation area, or in patients with portal vein occlusion, portal hypertension, ascites, bile duct obstruction, or hyperbilirubinemia. Additionally, IRE can also affect myocardial contraction mechanisms and as such cannot be applied in patients with cardiac arrhythmias, previous heart failure, active coronary disease, or recent pacemaker implantation. Finally, IRE cannot be used in patients with epilepsy despite the fact that it not been proven to cause brain stimulation[35].

The indications and contraindications for IRE for the treatment of pancreatic cancer are summarized in Table 2. Martin *et al*[36] and Narayanan *et al*[37] described the first clinical series on the implementation of IRE for the treatment of human pancreatic cancer. Since then, the use of this technique has been widespread[38-40], but to date there is still no defined protocol for the use of IRE in the treatment of pancreatic cancer. Studies showed that IRE was a viable treatment for locally advanced pancreatic cancer or borderline resectable pancreatic cancer because it allowed tumor downstaging, definitive locoregional treatment, or adjuvant treatment following resection[41-43]. In human tumor tissue, IRE induces necrosis as it does in animal cancer models; however, there is no evidence of apoptosis[16]. A series of retrospective and prospective clinical studies on human pancreatic cancer treated with IRE suggested a survival benefit with a median overall survival (OS) up to 30 mo[38,39].

Combined treatments involving IRE, chemotherapy, and immunotherapy can offer a multimodal approach which can limit the disease progression. However, the debate is ongoing with respect to the

Table 2 Indication and contraindications of irreversible electroporation in pancreatic cancer

Indication	Contraindications	
	Absolute	Relative
Biopsy-proven primary and solitary pancreatic tumors	History of ventricular arrhythmias	Atrial fibrillation
Locally advanced pancreatic cancer. Stage II or III (T4N1M0) with regional positive lymph nodes ≤ 3	Implanted cardiac stimulation devices within 1 yr	Coronary artery disease
Tumor size maximal axial diameter ≤ 5 cm	Uncontrollable hypertension	Combined severe stenosis of the common hepatic artery and main portal vein branch
Patients not candidates for radical resection or who refuse the surgical operation	History of epilepsy	Metallic foreign object in the ablation zone
Patients with a predictable OS longer than 3 mo, Karnofsky Performance Score > 50	Congestive heart failure (> NHYA class 2)	Liver failure, portal hypertension, ascites, bile duct obstruction, hyperbilirubinemia
		Irreversible bleeding disorders
		Uncontrolled infections; patients that have received chemo or immunotherapy in the 4 wk prior to treatment

OS: Overall survival; NHYA: New York Heart Association.

timing of multimodal treatment. Some studies that administered IRE before chemotherapy showed only a modest increase in survival; Månsson *et al*[40] reported a median OS of 13 mo. In contrast, studies using IRE after induction chemotherapy reported an increase in survival with a median OS of 27 mo [44]. Despite improvements in radiation therapy, chemotherapy, and surgical procedures over the last 30 years, pancreatic cancer 5-year survival rate remains at 9%.

Recently, the advanced techniques of proton radiation and carbon ion radiation therapies have been used for locally advanced pancreatic cancer with encouraging results. The proton beam offers significant physical advantages over the photon due to the Bragg peak effect with little or no output dose beyond the tumor target, thereby sparing any critical organs adjacent to cancer. Compared to proton radiation, carbon ion radiation offers similar dosimetric characteristics, but it has a substantially different biological property and offers greater biological efficacy in inducing complex DNA damage, leading to an increase in the destruction of cancer cells[44].

Despite the non-thermal effect of IRE, complications related to the production of heat near the electrodes (defined as secondary Joule heating) remain unavoidable[45]. The most common complications following IRE are mild acute pancreatitis, pain, diarrhea, nausea, vomiting, loss of appetite, and delayed gastric emptying. Serious complications after IRE related to the location and size of the pancreatic tumor have also been reported in the literature and include arrhythmia, severe acute pancreatitis, hemorrhage, portal vein thrombosis, bile or pancreatic fistula, gastrointestinal tract perforation, and death[16,45]. In one of the most recent reviews[16], the average rate of serious complications after IRE was 12%, with a maximum reported value of 42%[2]. The size of the tumor is one of the most important factors related to procedure complications; for example, Narayanan *et al*[46] treated patients with tumors up to 8 cm in size and reported one of the highest total complication rates of 62%. Other factors contributing to post IRE complications depend on the team experience, the protocol used, and the type of approach (open *vs* percutaneous)[18]. For example, the average mortality rates have been reported as 2% and 0% for open and percutaneous IRE, respectively[20].

Available IRE protocols in part derive from data gathered from animal studies; however, the pancreas tissue of animals and humans differ significantly in cellular composition and electrical impedance. In addition, available IRE protocols developed to date differ in recommended distance between the electrodes and intensity of applied voltage. Moreover, these protocols vary in the reported individual electrical properties of the tissue being ablated, which can have an impact on the effectiveness of the treatment and on the area of ablation itself[16]. This variation highlights an important knowledge gap, which can be attributed in part to the risks and ethics of *in vivo* human tissue sampling. One way to bridge this gap is to apply IRE to both diseased and healthy perfused human organs. Use of IRE on *ex vivo* perfused pancreas, for example, could help to shape a treatment protocol for the use of IRE in the treatment of pancreatic cancer[16]. Indeed, IRE is not yet widely used in clinical practice because there is a lack of consensus on the optimal IRE treatment protocol and for the approach required to protect adjacent pancreatic tissue[25]. Evaluation of the benefits following IRE are needed in pancreatic tumor tissue in order to establish these appropriate treatment protocols.

Future directions

The main issue surrounding the use of IRE for the treatment of pancreatic cancer is the absence of randomized data. Currently, two major randomized controlled trials (RCT) are recruiting patients in order to provide level 1 safety and efficacy evidence *vs* standard of care. The PAL-PIE study is a United Kingdom-based multicenter RCT that will recruit 50 patients (from up to seven pancreas centers) with locally advanced pancreatic cancer and previous first-line chemotherapy (5-fluorouracil, leucovorin, irinotecan and oxaliplatin) randomized to treatment with IRE (plus chemotherapy if indicated) *vs* chemotherapy alone[47]. The DIRECT study is a randomized, multicenter, controlled, unblinded study to assess the safety and efficacy of the NanoKnife® system for the ablation of unresectable stage III pancreatic adenocarcinoma. This study will randomize over 500 patients with stage III pancreatic cancer to receive IRE plus chemotherapy (a modified FOLFIRINOX regimen) *vs* chemotherapy alone; the estimated completion date is December 2023 (ClinicalTrials.gov Identifier: NCT03899636).

Additionally, following positive initial clinical outcomes, researchers are currently investigating the interesting concept of IRE-induced immune response to cancer[48-51]. IRE has been identified as a potential immunomodulatory therapy due to post-ablation release of intracellular tumoral antigens that act as *in situ* immunization agents resulting in both local and systemic response to remaining cancer cells. Specifically, IRE can remodel the local tumor microenvironment by smoothing the extracellular matrix, alleviating hypoxia, and assisting in the infiltration of immune cells into residual cancer foci. Moreover, the combination of IRE and immunotherapy could incite potent synergistic antitumoral effects[24]. Nevertheless, the mechanisms involved in immunomodulation following IRE in humans remains unclear[52]. However, trials focusing on the potential of IRE combined with immunotherapy to improve prognosis of unresectable pancreatic cancer are ongoing. For example, a pilot multicenter, single-arm phase II trial is currently recruiting patients with metastatic pancreatic ductal adenocarcinoma to investigate whether the combination of IRE treatment of one liver metastasis followed by nivolumab treatment leads to a measurable radiological response (ClinicalTrials.gov Identifier: NCT04212026).

Combination therapy of IRE with chemotherapeutic regiments is also being evaluated as the rim of peripheral sensitivity to chemotherapy produced around central tumor necrosis following IRE typically presents as microscopic peripheral seeding[53]. To this end, several ongoing prospective trials such as the CHEMOFIRE-2 trial (Chemotherapy Followed by Irreversible Electroporation in Patients With Unresectable Locally Advanced Pancreatic Cancer; ClinicalTrials.gov Identifier: NCT04093141) are underway.

Improvements with respect to IRE technology itself are also needed. An interesting technique requiring further investigation is endoscopic IRE, which could provide a solution for patients without safe transabdominal access. A major limitation of IRE is the intent of producing a small ablation zone of approximately 1-1.5 cm, which requires several electrodes to produce the desired A0 ablation. This requirement renders the procedure more technically demanding and time-consuming compared to conventional thermal ablation modalities[54]. Future research should focus on the standardization and optimization of an IRE treatment protocol for the treatment of pancreatic cancer with the goal of providing maximum efficacy without damaging surrounding tissues. It should also aim to refine the parameters of post-treatment radiological assessment for the development of objective and measurable predictors of treatment outcomes following use of IRE.

CONCLUSION

As demonstrated by initial preclinical and clinical data, the unique characteristics of IRE render this non-thermal ablation modality most suitable for the minimally invasive treatment of locally advanced pancreatic cancer. The synergic effect of IRE combined with chemo- or immunotherapy could significantly improve outcomes. Further investigation is required to elucidate its exact mechanism of action, optimize treatment protocols, and provide high-quality comparative clinical data for the management of patients with pancreatic cancer.

FOOTNOTES

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OPINION REVIEW

Acute-on-chronic liver failure: Controversies and consensus

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Abstract

Acute-on-chronic liver failure (ACLF) is a poorly defined syndrome characterised by rapid clinical deterioration in patients with chronic liver disease. Consequences include high short-term morbidity, mortality, and healthcare resource utilisation. ACLF encompasses a dysregulated, systemic inflammatory response, which can precipitate extra hepatic organ failures. Common precipitants include infection, alcoholic hepatitis, and reactivation of viral hepatitis although frequently no cause is identified. Heterogenous definitions, diagnostic criteria, and treatment guidelines, have been proposed by international hepatology societies. This can result in delayed or missed diagnoses of ACLF, significant variability in clinical management, and under-estimation of disease burden. Liver transplantation may be considered but the mainstay of treatment is organ support, often in the intensive care unit. This review will provide clarity around where are the controversies and consensus in ACLF including: Epidemiology and resource utilisation, key clinical and diagnostic features, strategies for management, and research gaps.

Key Words: Acute-on-chronic liver failure; Liver cirrhosis; End stage liver disease; Epidemiology; Mortality

Core Tip: Acute-on-chronic liver failure is characterised by rapid clinical deterioration in patients with chronic liver disease. Consequences include high short-term morbidity, mortality, and healthcare resource use. Heterogenous definitions, diagnostic criteria, and treatment guidelines create further challenges to optimal care. This review summarises epidemiology and resource utilisation, key clinical features, strategies for management, and research gaps.

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INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a well-recognised syndrome of rapid clinical deterioration in those with chronic liver disease (CLD). It is associated with high short-term mortality of 22%-74%[1-3]. The prevalence and impact of ACLF is likely underestimated as there is no consensus definition nor diagnostic criteria[4]. The systemic inflammatory response in ACLF often results in extra-hepatic organ failures and frequently necessitates intensive care unit (ICU) level support[5]. The key management principles in the acute phase of ACLF includes the diagnosis and treatment of underlying triggers such as infection, and provision of organ support[1,6,7]. For those surviving to hospital discharge, there is limited guidance for management in the post-admission period and although comparable evidence is scarce, re-admission rates are likely to be 30%-40%[8,9]. In this article, we review the current areas of controversies and consensus with regards to ACLF epidemiology, economic impact, clinical manifestations, diagnostic criteria, and management principles.

EPIDEMIOLOGY

In European populations, the CANONIC study[1] demonstrated 30% prevalence amongst patients admitted with cirrhosis, of which ACLF was the presenting complaint in 20% of hospital admissions using the European Association for the Study of the Liver criteria. In North America, one study demonstrated a 24% prevalence using the North American Consortium for the Study of End Stage Liver Disease criteria, which includes bacterial infection as a criterion[10]. In a study of 565 patients who underwent liver transplantation in Shanghai, China; 41% had ACLF according to Asian Pacific Association for the Study of the Liver (APASL) criteria[11].

The primary aetiology of underlying CLD and the precipitant for ACLF is likewise reflective of local region and definitions. Historically, chronic hepatitis B through vertical transmission has been the most frequent cause of CLD, particularly in Asian populations from which the APASL criteria was derived (49%-59%)[12,13]. Globally, alcohol use disorder has likely overtaken chronic hepatitis B as the most common aetiology for CLD and precipitant of ACLF[13]. The most common precipitating events for ACLF in order of frequency are bacterial infections, alcohol excess, and hepatitis B reactivation[14,15]. The latter remains the most common precipitant for ACLF in Asia[12]. The absence of an identifiable precipitant in up to 40% of patients is a significant contributor to the diagnostic uncertainty and variability in criterion applied[1].

ECONOMIC IMPACT

There is limited data on the total economic burden of ACLF, although this has been explored for cirrhosis and chronic liver disease in Australia[16] and internationally[17-19]. Current cost estimations are extrapolated from cirrhosis populations and mostly reported from the healthcare payer/provider perspective[6]. The indirect costs of ACLF such as lost productivity and disability may be significant, but its value has not been extensively quantified[20]. Whilst hospital-based costs or direct healthcare related costs can be theoretically analysed through application of diagnostic criteria or International Classification of Disease coding to health service records, there is a paucity of data on indirect costs related to disability, impact on carers, and premature mortality.

Direct healthcare costs are related to the number of organ failures, need for ICU support, and total length of stay. Each of these direct cost components are disproportionately higher in ACLF compared to decompensated cirrhosis alone[7]. A population-based cohort study in Thailand demonstrated a 3.5-fold average cost of hospitalization for ACLF compared to hepatic decompensation, using the North

American Consortium for the Study of End-Stage Liver Disease (NACSELD) definition of ACLF of two or more extrahepatic organ failures in patients with cirrhosis[7]. A national inpatient database study from the United States similarly demonstrated increasing annual liver-related hospital expenditure between 2001 and 2011. Inpatient costs increased 2-fold for cirrhosis to $9.8 billion and 5-fold for ACLF to $1.7 billion[6]. The global trend of an increasing prevalence of CLD and incidence of ACLF worldwide[17,21] will further compound the economic burden for healthcare systems.

There are no proven cost-effective interventions for ACLF treatment, which is primarily supportive care in addition to addressing underlying aetiologies and precipitants[19]. Treatments evaluated include intravenous human albumin transfusions which have not demonstrated a mortality benefit in ACLF[22] and variable mortality benefit in hepatic decompensation[23,24]. Indirect ACLF-related costs have been captured in large studies on the global burden of CLD. These suggest a 1.5% contribution to all disability-adjusted life years in 2016, which was more pronounced in countries with a lower socio-demographic index[17,25]. This data may overlook discrepancies in outcomes in countries such as Australia with a higher socio-demographic index as a proxy of development, where Indigenous Australians with cirrhosis have disproportionately higher rates of hospital re-admission and death than non-Indigenous Australians[26]. To reduce healthcare-related costs, interventions for ACLF must achieve the Holy Grail of reduced short-term mortality, length of stay, readmission, and organ failure. Additionally, early use of prognostic scores such as NACSELD-ACLF[10] or chronic liver failure (CLIF)-C ACLF[27] should be routinely applied to accurately predict those who with a poor prognosis and may be better suited to palliative care and thus reduce ineffectual resource allocation.

CLINICAL MANIFESTATIONS AND DIAGNOSTIC CRITERIA

There is no universally accepted set of diagnostic criteria for ACLF, with variable criteria identified by four major international hepatology associations[28,29]. Lack of a consensus remains problematic with the potential for delayed or missed diagnoses, and challenges in applying evidence-based treatment. There is clinical consensus that ACLF is a distinct syndrome to acute hepatic decompensation, however, patients may initially present with clinical features of a decompensating event including worsening of abdominal ascites, jaundice, gastrointestinal bleeding and hepatic encephalopathy (HE)[30]. Features of bacterial infection, such as urinary tract infection, pneumonia, or spontaneous bacterial peritonitis, with may also be present[5]. Organ failure is a hallmark of ACLF and can include renal failure and manifestations of this (such as uremia, acidosis, oliguria), respiratory and circulatory failure[2]. Beyond these non-specific clinical manifestations, the regionally relevant set of diagnostic criteria diverge in the exact thresholds and subtypes of how and what they classify as ACLF.

The *World Gastroenterological Organisation* (WGO) has proposed criteria to identify clinical, prognostic, and pathophysiologic subtypes[3] and define ACLF as an independent syndrome. Five requirements have been stipulated including: (1) Distinction from acute liver failure; (2) distinction from hepatic decompensation; (3) definition of pathophysiology; (4) definition of specific clinical signs and laboratory tests to confirm diagnosis and exclude other disease; and (5) a validated scoring system to assess severity. A system categorising ACLF into three subtypes is shown in Table 1.

The APASL criteria includes a serum bilirubin level ≥ 50 mg/L and International Normalized Ratio (INR) ≥ 1.5 complicated by ascites and/or encephalopathy within 4 wk in a patient with previously diagnosed or undiagnosed chronic liver disease or cirrhosis[31].

The European Association for the Study of the Liver (EASL) and the CLIF consortium definition requires concomitant organ failure and provides prognostication guidance according to the grading of severity[1,27]. ACLF is explicitly excluded in the absence of extra-hepatic organ failure, defined as renal failure with serum creatinine ≥ 2.0 mg/dL or single non-kidney organ failure with HE to meet criteria for low grade ACLF[1]. ACLF severity grading and criteria are summarised in Table 2, with the scoring system and organ system involvement shown in Table 3. The 28-d mortality is graduated from 23.3% in grade 1 to 75.5% in grade 3[1]. Most patients meeting ACLF criteria in the latter cohort required intensive care unit support, highlighting the greater disease severity and associated resource utilisation in this diagnostic system.

The NACSELD criteria was developed as a bedside tool to predict 30-d survival in hospitalised patients with cirrhosis with decompensation in the context of infection[11,32]. The NACSELD-ACLF is defined as two or more of the following organ failures: Brain failure (West-Haven grade 3 or 4 encephalopathy), renal failure (need for renal replacement therapy), respiratory failure (need for bilevel positive airway pressure or mechanical ventilation), and shock (the need for vasopressor support, mean arterial pressure < 60 mmHg, or a reduction of > 40 mmHg in systolic blood pressure from baseline despite adequate fluid resuscitation). Validation studies have demonstrated that the NACSELD-ACLF predicts survival in infected and uninfected hospitalised patients with cirrhosis, and similarly to EASL criteria, demonstrates that the number of organ failures strongly predicts survival[10].

The WGO clinical sub-types were proposed early in the identification of ACLF as a distinct clinical entity and are a useful bedside tool. However, WGO criteria have limited correlation with prognosticating mortality and resource use[3]. The other three definitions (APASL, CLIF-C ACLF and

Table 1 World Gastroenterological Organisation definitions of acute-on-chronic liver failure subtypes[30]

Type A-noncirrhotic	Type B-compensated cirrhosis	Type C-decompensated cirrhosis
Acute flare of noncirrhotic CLD resulting in liver failure including hepatic encephalopathy	Rapid deterioration of previously well-compensated cirrhosis after major insult such as hepatitis (drug, viral, alcoholic), infection, or surgery	Rapid deterioration in those with previous hepatic decompensation

CLD: Chronic liver disease.

Table 2 The European Association for the Study of the Liver and chronic Liver Failure Consortium grading of acute-on-chronic liver failure severity[12,25]

ACLF Grade	Criteria
No ACLF	No organ failure or; one organ failure (liver, coagulation, circulatory, respiratory) with serum creatinine < 1.5 mg/dL and no HE or single cerebral failure and serum creatinine < 1.5 mg/dL
Grade 1	Single kidney failure or single liver, coagulation, circulatory, or respiratory failure + serum creatinine 1.5-1.9 mg/dL and/or HE I-II or single cerebral failure (HE III-IV) + serum creatinine 1.5-1.9 mg/dL
Grade 2	2 organ failures
Grade 3	3 or more organ failures

HE: Hepatic encephalopathy; ACLF: Acute-on-chronic liver failure.

Table 3 Defining organ/system failure using Chronic Liver Failure-Acute-on-Chronic Liver Failure Sequential Organ Failure Assessment scoring[12]

Organ system	Parameter	Score = 1	Score = 2	Score = 3
Liver	Serum bilirubin (mg/dL)	< 6	6-12	> 12
Kidney	Serum creatinine (mg/dL)	< 2	2.0-3.5	≥ 3.5 or renal replacement therapy
Brain	West-Haven grade	0	I-II	III-IV
Coagulation	INR	< 2.0	2.0-2.5	≥ 2.5
Circulation	MAP (mmHg)	≥ 70	< 70	Vasopressors
Respiratory	PaO2/FiO2	> 300	≤ 300 and > 200	≤ 200
	OR SpO2/FiO2	> 357	> 214 and ≤ 357	≤ 214

INR: International Normalized Ratio; MAP: Mean arterial pressure, mmHg millimeters of mercury; PaO2: Partial pressure of arterial oxygen; FiO2: Fraction of inspired oxygen; SpO2: Pulse oximetric saturation.

NACSELD) have better correlation with mortality, primarily due to correlation with objective biochemical parameters, and organ failures with respect to the CLIF-C ACLF criteria. The APASL criteria require the presence of CLD but not necessarily cirrhosis, and that the acute precipitating event must be liver-related[33]. Conversely, CLIF-C ACLF criteria stipulates the presence of underlying cirrhosis, extra-hepatic organ failures and the acute precipitating event can be of non-hepatic origin[32]. Therefore, ACLF populations identified using APASL criteria may include more patients with hepatic decompensation, who may not have the same short-term mortality and economic burden as those with ACLF defined otherwise by EASL. The NACSELD criteria incorporates organ failures but does not specify values for pulse oximetry or arterial blood gases to guide ventilation and therefore is potentially more vulnerable to subjectivity compared to EASL criteria. Recent clinical guidelines published by Bajaj *et al*[34] suggest that none of the current sets of criteria are adequate to inform management. In summary, organ failure appears to be an important marker of mortality in ACLF and is a component of diagnostic criteria for two of the four major definitions described. Standardisation of ACLF definition and management protocols is a critical unmet clinical need. It is the cornerstone to prompt diagnosis, evidence-based management, and reduced population heterogeneity in the research setting.

MANAGEMENT PRINCIPLES

Despite high short-term mortality[35,36], ACLF management is primarily supportive and focuses on reversing organ failure. The key pathophysiologic drivers of systemic inflammation and paradoxical immunoparesis have no specific therapy at present[36], so addressing the precipitating factors, prevention and management of end-organ complications, and targeted organ support constitute the foundations of care[4,31]. However, more than 30% of patients still progress to multiorgan failure and death within 30 d of diagnosis. Model for End-Stage Liver Disease including serum sodium (MELD-Na) and Child-Pugh Scoring systems have limited prognostic integrity as they do not account for the cerebral, respiratory or circulatory dysfunction that accompanies ACLF[5,31].

The APASL ACLF Research Consortium (AARC) ACLF score is a prognostic model constructed from the AARC database. It incorporates five variables, including lactate, grade of HE, INR, bilirubin and serum creatinine levels[37] to stratify patients into grade 1 (score of 5-7), grade 2 (score of 8-10) or grade 3 ACLF (score of 11-15), with 28 day mortality rates of 12.7%, 44.5%, and 85.9% respectively[30]. Grade I ACLF are said to have potential recovery, grade II require intensive monitoring and grade III need immediate intervention and consideration of transplantation[30]. Scores of > 10 should be considered for transplant. This has been predominantly validated in an Asian population. Alternate scoring systems include the CLIF-ACLF SOFA score and the CLIF-Consortium ACLF scores (CLIF-C ACLF). These dynamic scoring systems allow for better prognostication of 28-d mortality rate, hence assisting stratification of ACLF[38-40]. The CLIF-C ACLF score incorporates the CLIF-C Organ Failure score (bilirubin, creatinine, INR, West-Haven grade for encephalopathy, mean arterial pressure and PiO2/FiO2 ratio), along with age and white cell count[5,41]. This has been validated as a prognostic tool in ACLF[2,40], with emerging evidence for its use in guiding treatment options. A CLIF-C ACLF score > 70 represents a subgroup in whom defining limitations of care and futility is important given their very poor predicted outcomes[30,39,41].

Increasingly there appears to be a role for liver transplantation (LT) in a select cohort of these critically ill patients. The percentage of LT performed for ACLF varies significantly between transplant centres and even within regions[4,41,42], which reflects the considerable debate around the concept of liver transplant as a therapeutic strategy. The median transplant-free mortality rate in ACLF is 30%-40% at 28-d[1,40], increasing to 75% for grade 3 ACLF[1], and 40%-60% overall at 6 mo[40,42]. A recent consensus[43,44] developed by 35 international experts from North America and Europe suggested that contraindications to transplant include PaO2/FiO2 < 150 mmHg, noradrenaline dose > 1 μg/kg/min and/or serum lactate > 9[43]. Those who recover from their initial ACLF event are at high risk of recurrent and more severe ACLF in the future[45]. Whilst at this stage transplantation in advanced ACLF is the only curative intervention available, it is associated with higher postoperative complications and longer ICU and hospital stays compared to other indications[30]. Whilst scoring systems are useful in defining timing for LT escalation and features suggesting futility, clinical and ethical challenges remain in the referral and activation of appropriate candidates.

Common precipitating events in ACLF include bacterial infection, alcoholic hepatitis, gastrointestinal bleeding, HE, and reactivation of hepatitis B in endemic regions[41,46]. In European cohorts, bacterial infection is the most common precipitant[30,41], including spontaneous bacterial peritonitis, urinary tract infections and pneumonia[30]. Bacterial infection also predicts the development of organ failure in ACLF[31], hence early detection and treatment of infection are imperative. ACLF patients have higher rates of multi-drug resistant bacteria and demonstrate a lower infection resolution rate[12]. Antimicrobial choice should incorporate local guidelines and involve prompt initiation of empiric broad spectrum antibiotics whilst awaiting sensitivity profiles[4,31,44].

Acute Kidney Injury (AKI) is a frequent feature of ACLF and considered a strong predictor of poor survival in the short and long term[36,47,48]. There is significant overlap between hepatorenal syndrome AKI (HRS AKI) and non-HRS AKI in ACLF. Isolated HRS is believed to only represent a fraction of ACLF renal complications[38]. The management of renal dysfunction in ACLF requires the exclusion of reversible causes, including nephrotoxic contributors, and optimising circulating blood volume to ensure adequate renal perfusion[38,49]. Volume expansion with intravenous albumin and continuous intravenous terlipressin is recommended for those meeting HRS-AKI criteria[49]. Continuous terlipressin infusion is preferable to bolus regimes due to the improved tolerability and reduction in adverse effects[38,49]. Noradrenaline is a possible alternative to terlipressin, with a 2016 meta-analysis of four studies (154 patients) demonstrating no superiority with regards to survival in patients treated with terlipressin *vs* noradrenaline[50]. Renal replacement therapy has historically been restricted to patients with AKI who fail the above methods and have clinical or laboratory indications as per the general AKI guidelines, and this has been translated to the ACLF population given the lack of validated data around this specific cohort[38,49]. There are also unanswered questions regarding the specific benefit of rapid correction of electrolyte abnormalities and hyperammonemia in the ACLF cohort[38].

HE in ACLF is associated with higher mortality, correlating with increasing grades of HE[51]. Management involves identification and treatment of precipitants as well as specific measures for reducing hyperammonaemia and systemic inflammation[31]. Treatment of concomitant infection, drugs and electrolyte abnormalities must always be considered and excluded[38]. Cerebral imaging should be

performed to exclude an alternative cause of altered neurology, especially given the increased risk of bleeding and clotting in this cohort[38]. Ammonia lowering therapies are the cornerstone for managing HE with lactulose as the first line agent (oral, nasogastric or rectal preparations) followed by Rifaximin as second-line therapy[38]. Continuous veno-venous haemofiltration use in acute liver failure has been associated with clinically significant reductions in serum ammonia levels and is a recognised treatment for HE in these patients[52]. There have been no large randomised controlled trials to elucidate the role for haemofiltration or haemodialysis in lowering serum ammonia levels in ACLF[38].

Variceal and other types of bleeding can precipitate ACLF and should be managed similarly to those with decompensated CLD. Non-selective beta-blockers should be continued even in patients with ascites[30,31,53]. Their use is thought to reduce systemic inflammation and have favourable effects beyond their potential haemodynamic benefits[31,53] and should only be ceased in those with haemodynamic instability[44]. Circulatory failure should be managed with volume expansion, and if haemodynamic instability persists, the use of vasopressors, aiming for a mean arterial pressure of ≥ 65 mmHg[44]. Bleeding in the ACLF cohort is predominantly secondary to portal hypertension whilst spontaneous haemorrhage is rare[54]. Historic plasma-based coagulation tests are poor predictors of bleeding in chronic liver disease[55,56]. Newer viscoelastic assays, such as thromboelastography and rotational thromboelastometry assess whole blood, which may be superior and preferential to standard laboratory testing in clinical practice but their role in ACLF management is poorly defined[38,57].

CONCLUSION

ACLF is a distinct and severe clinical entity, separate from hepatic decompensation, with high short-term mortality, healthcare resource utilisation, and poorly defined treatment goals. Clinical diagnosis and management are limited by variable definitions and diagnostic criteria. Future focuses for research should including investigating and defining specific clinical and biomarkers for prognostication and classification of ACLF subtypes, standardisation of prognostic scores for both clinical management and population stratification in clinical trials, and further evidence to support the role for liver transplantation in a well-defined cohort most likely to demonstrate long term benefit.

FOOTNOTES

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REVIEW

Liver injury in COVID-19: Clinical features, potential mechanisms, risk factors and clinical treatments

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic has been a serious threat to global health for nearly 3 years. In addition to pulmonary complications, liver injury is not uncommon in patients with novel COVID-19. Although the prevalence of liver injury varies widely among COVID-19 patients, its incidence is significantly increased in severe cases. Hence, there is an urgent need to understand liver injury caused by COVID-19. Clinical features of liver injury include detectable liver function abnormalities and liver imaging changes. Liver function tests, computed tomography scans, and ultrasound can help evaluate liver injury. Risk factors for liver injury in patients with COVID-19 include male sex, preexisting liver disease including liver transplantation and chronic liver disease, diabetes, obesity, and hypertension. To date, the mechanism of COVID-19-related liver injury is not fully understood. Its pathophysiological basis can generally be explained by systemic inflammatory response, hypoxic damage, ischemia-reperfusion injury, and drug side effects. In this review, we systematically summarize the existing literature on liver injury caused by COVID-19, including clinical features, underlying mechanisms, and potential risk factors. Finally, we discuss clinical management and provide recommendations for the care of patients with liver injury.

Key Words: Liver injury; COVID-19; Clinical feature; Risk factor; Treatment and

management strategy

Core Tip: A growing body of evidence suggests that patients with coronavirus disease 2019 (COVID-19) may experience varying degrees of liver injury. The characteristics and mechanisms of liver injury associated with COVID-19 are not fully understood. In this review, we summarized the clinical features, mechanisms, and management strategies of liver injury associated with COVID-19. Moreover, we collected all the information about high risk factors for liver injury from COVID-19, which is of significance and help for further study of liver damage related to severe acute respiratory syndrome coronavirus 2.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. Since the first outbreak in late December 2019 in China, it has unleashed a matchless public health crisis worldwide. The COVID pandemic has been going on for nearly 3 years, and there is still no end in sight. Initially, it was considered solely an atypical pneumonia until patients started to show signs of multiorgan involvement[1]. Now we know that the effects of COVID-19 on the body are extensive. In addition to the respiratory system, almost all systems in the body, including the circulatory system, cardiovascular system, urinary system, gastrointestinal and hepatobiliary system, endocrine system, nervous system, ophthalmic system, and skin system can be affected[2,3]. SARS-CoV-2 virus mainly affects the respiratory system, causing common symptoms such as fever, fatigue, cough, and dyspnea. Relatively, diarrhea, myalgia, hemoptysis, and sore throat are less common[4]. Other reports show that liver dysfunction is a common manifestation of COVID-19 and is associated with higher mortality[5]. It is worth mentioning that the incidence of liver injury in severe COVID-19 cases can reach 93%[6]. However, the exact mechanism of how COVID-19 impairs liver function remains unclear. This comprehensive literature review is aimed at providing useful guidance for diagnosis, risk factor identification, and management of liver injury associated with COVID-19.

CLINICAL FEATURES OF LIVER INJURY IN COVID-19

Liver injury is mainly manifested as abnormal liver function (ALF) indexes. Alterations in hepatocyte damage biomarkers (HDBs), such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT), are commonly used to evaluate COVID-19-related liver injury[6,7]. In some cases, elevated lactate dehydrogenase (LDH), hypoproteinemia, prolonged prothrombin time, total bilirubin (TBil), and direct bilirubin (DBiL) are also used to assess liver function in COVID-19 patients[8-10].

In COVID-19 patients, transaminase elevations are usually mild [1-2 times the upper limit of normal (ULN)][9]. These changes in laboratory values may persist for a long time, even after hospital discharge. ALF was defined as at least one test HDB exceeding the ULN. Xu *et al*[10] evaluated the proportion of patients with abnormal HDBs, and found on admission ALT 13.2%, AST 8.5%, ALP 2.0%, GGT 7.4%, LDH 37.6%, TBiL 4.0%, DBiL 7.8%, and albumin 10.1%, and peak during the hospitalization ALT 29.4%, AST 17.5%, ALP 2.6%, GGT 13.4%, LDH 49.4%, TBiL 10.1%, DBiL 18.0%, and albumin 30.6%. In another study, the proportion of patients with at least one of the HDBs and TBil exceeding the ULN for the first time immediately after hospitalization, before discharge, a median of 14.0 d after discharge, and 1 year after discharge was 32.2%, 45.8%, 54.8%, and 28.8%, respectively[11]. In addition, a single-center prospective cohort study found that the proportion of patients with any ALF was 25.1% at 1 mo, 13.2% at 3 mo, 16.7% at 6 mo, and 13.2% at 12 mo after discharge[12]. Based on these data, long-term monitoring of liver enzymes may be warranted in patients with a history of COVID-19.

AST is generally considered to be less specific for liver injury than ALT due to additional extra-hepatic production[13,14]. Nevertheless, in liver damage, elevated AST levels appear earlier, and the increase in AST levels at admission is usually more pronounced than ALT levels. In cases of severe

COVID-19, however, ALT levels typically rise rapidly, exceed the ULN value and peak within 10-15 d of admission. Subsequently, ALT levels remained stable in all patients with liver injury and then gradually decreased with longer hospital stay. ALT is a more effective indicator of liver injury in COVID-19 patients with severe manifestations[15]. However, if serum AST and LDH levels are elevated but ALT levels remain normal, other causes of elevated liver biochemical responses rather than liver injury should be considered, such as myositis (especially AST > ALT), cardiac injury, ischemia, and cytokine release syndrome (CRS)[16].

The reported prevalence of liver injury in COVID-19 patients varied widely across studies, ranging from 4.8% to 78%[17]. This is mainly due to a variety of factors, including dynamic changes in liver function, small sample sizes, different admission criteria, lack of adjustment for baseline chronic liver disease (CLD), use of different HDBs, and inconsistent definitions of "liver injury"[10,18-20]. Notably, almost all studies were conducted on hospitalized patients, ignoring non-hospitalized patients, thus resulting in unclear overall morbidity (Table 1).

LIVER INJURY AND PROGNOSIS

COVID-19 patients with moderate or severe liver injury (SLI) have an increased risk of admission to the intensive care unit (ICU), disease progression, and death compared with patients without elevated liver chemistries[9,19,23]. Cai *et al*[6] have reported that patients with liver injury have a 9-fold greater risk for developing severe COVID-19. In a retrospective cohort study, when compared with moderate liver injury (2-5 ULN) and no/mild liver injury (< 2 ULN), COVID-19 patients with SLI (ALT > 5 ULN) had more severe clinical outcomes, including higher ICU admission rates (69% *vs* 42% *vs* 16%), intubation (65% *vs* 38% *vs* 13%), renal replacement therapy (33% *vs* 15% *vs* 7.5%), and mortality (42% *vs* 23% *vs* 21%). Among SLI patients, 70% required vasopressors, 12% received inotropes, 39% were paralyzed, 10% were proned, and 2.8% required extracorporeal membrane oxygenation[19].

Changes in liver function are predictors of severity and mortality in patients with COVID-19[5,23]. Abnormal liver biochemical parameters are closely related to an increased risk of mortality in critically ill patients with COVID-19. The levels of ALT, AST, GGT, LDH, TBil, and DBil in severe patients were significantly higher than those in mild-moderate patients. Conversely, severe patients had significantly lower albumin levels than non-severe patients[5,20]. In a study of 151 hospitalized patients, 5 liver injury parameters, ALT, AST, TBil, DBil, and indirect bilirubin, were identified as notable prognostic factors, while total protein, albumin, ALP, GGT, and total bile acid appeared to be less related to prognosis[25]. In other studies, low albumin is also a marker of severe infection and poor prognosis[10,26]. Lei *et al*[15] emphasized the association of ALF tests, especially AST and TBil, with higher mortality. They observed that AST was more frequently elevated than ALT in severe patients. However, elevated ALP and peak ALT were significantly associated with discharge to hospice and death[19,27].

ABDOMINAL IMAGING FINDINGS

Possible imaging signs of liver damage on computed tomography (CT) scans of the hepatobiliary system include hepatomegaly, decreased liver density, periportal edema, fat stranding around the gallbladder, portal lymphadenopathy, and dilated gallbladder and bile ducts[28,29]. Portal venous gas can be seen in patients with mesenteric ischemia, especially in critically ill patients[30]. CT-quantified liver density can be assessed by the liver-spleen attenuation ratio, which correlates with the severity of liver injury. A common manifestation of liver damage caused by COVID-19 is homogeneous or heterogeneous low density of the liver. Liver hypodensity is more common in critically ill cases[28]. Ultrasound can be easily performed in COVID-19 patients to help identify liver damage quickly and effectively. The most frequent sonographic finding is hepatomegaly with increased parenchymal echogenicity, followed by biliary disease, including gallbladder sludge and distention, gallbladder wall thickening, mural hyperemia, intraluminal mud, and pericholecystic fluid[29-31]. Portal venous gas suggests mesenteric ischemia. Further, gallbladder cholestasis is common in critically ill patients of COVID-19[30]. Collectively, imaging of liver injury can reveal changes in liver density, gallbladder and bile duct dilation, portal pneumatosis and/or mesenteric ischemia.

PROPOSED MECHANISMS OF LIVER INJURY

The pathological basis of liver injury following COVID-19 infection is puzzling and not fully understood. Studies suggest that direct cytotoxicity, hypoxic hepatitis, cytokine storm syndrome, exacerbation of preexisting liver disease, and drug-induced liver injury (DILI) may be major mechanisms of COVID-19-related liver injury.

Table 1 Criteria, grading, and incidence of abnormal liver function or injury

Ref.	Sample size	Study type	Criteria and grading of ALF or injury	Comments
Salık *et al*[5]	533	Retrospective study	Liver biochemical parameters: ALT, AST, and TBiL > ULN. Liver injury: ALT and/or AST > 3 ULN, and/or TBiL > 3 ULN	NA
Cai *et al*[6]	417	Retrospective, single-center study	ALF: > ULN. Liver injury: ALT and/or AST > 3 ULN, ALP, GGT, and/or TBiL > 2 ULN	76.3% had ALF and 21.5% had liver injury during hospitalization
Fan *et al*[8]	148	Retrospective, single-center study	Increased levels of ALT, AST, GGT, ALP, and total bilirubin	37.2% had ALF at hospital admission
Kulkarni *et al*[9]	20874	Meta-analysis	ELC: AST or ALT > ULN. SLI: Any elevation of enzymes > ULN and bilirubin over 2 ULN	ELC: 23.1% at initial presentation. 24.4% developed ELC during the illness
Xu *et al*[10]	1003	Retrospective cohort study	Mild liver injury: 1-2 ULN. Moderate liver injury: 2-5 ULN. Significant liver injury: > 5 ULN	Most patients with abnormal liver function parameters had mild elevations (1-2 ULN) at admission and peak hospitalization
Hundt *et al*[13]	1827	Retrospective observational cohort study	ELC: AST, ALT, ALP, TBiL, albumin: > ULN	ELC at pre-hospitalization (AST 25.9%, ALT 38.0%, ALP 56.8%, and TBiL 44.4%). Admission (AST 66.9%, ALT 41.6%, ALP 13.5%, and TBiL 4.3%). Peak hospitalization (AST 83.4%, ALT 61.6%, ALP 22.7%, and TBiL 16.1%)
Balderramo *et al*[14]	298	Multicenter study	ALEx2: The elevation of at least one of the following: TBil, ALT, AST, GGT, or ALP > 2 ULN	During admission, 29.2% out of 298 patients presented ALEx2
Phipps *et al*[19]	6913	Retrospective cohort study	Mild: ALT 1-2 ULN. Moderate: ALT between 2-5 ULN. Severe: ALT > 5 ULN	Among patients who tested positive, 45% had mild, 21% moderate, and 6.4% SLI
Wang *et al*[21]	156	Retrospective, 2-centers study	Elevated aminotransferases	41.0% patients with elevated aminotransferases
Liu *et al*[22]	245	Retrospective, single-center study	Mild liver dysfunction: AST ≥ ULN. Moderate liver dysfunction: AST ≥ ULN combined with any parameter being greater than the ULN values of ALT, GGT, and TBiL. Severe liver dysfunction: AST ≥ ULN combined with ALT ≥ 3 ULN and/or GGT, TBiL ≥ 2 ULN	43.7% experienced mild liver dysfunction, 40.4% experienced moderate liver dysfunction, and 20.4% experienced severe liver dysfunction
Chaibi *et al*[23]	281	Retrospective cohort study	ALF: AST, ALT, GGT, ALP or TBil > ULN	36.3 % had liver dysfunctions. Only a minority of patients (6.4%) had perturbations above 5 times the ULN
Shousha *et al*[24]	547	Multicenter cohort study	Liver injury: Transaminase > 3 ULN	26% and 32% of patients had elevated ALT and AST, respectively. 4.91 and 3.70%patients, respectively, had AST or ALT elevation > 3 ULN

ALEx2: Abnormal liver enzymes over twice the upper limit of normal; ALF: Abnormal liver function; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ELC: Elevated liver chemistries; DBiL: Direct bilirubin; GGT: Gamma-glutamyl transferase; NA: Not available; TBil: Total bilirubin; ULN: Upper limit of normal.

Direct cytotoxicity

The dual blood supply to the liver may be a route of infection. It is speculated that retrograde liver infection occurs after intestinal infection with SARS-CoV-2[32,33]. It is known that the S protein of SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor to facilitate virus entry into host cells. ACE2 receptors are widely expressed in multiple organs, including the liver[34]. Although the expression of ACE2 is much lower in hepatocytes compared to type 2 pneumocytes, its expression levels are similar in cholangiocytes and type 2 pneumocytes[35], indicating that the hepatobiliary system is a potential target organ of SARS-CoV-2.

SARS-CoV-2 RNA has been reported to be detectable in the liver of COVID-19 patients. Electron microscopy also revealed larger numbers of coronavirus particles in the livers of these patients[21,36]. Postmortem liver biopsies showed typical coronavirus particles in the cytoplasm and typical viral infection lesions, such as mitochondrial swelling, endoplasmic reticulum dilation, and decreased glycogen granules. Besides, massive hepatocyte apoptosis and some binuclear hepatocytes were also observed[21].

Cytokine storm syndrome

Cytokine storm refers to the rapid and massive production of various cytokines in body fluids, which plays an important role in acute respiratory distress syndrome and multiple organ failure. The liver

cannot escape the cytokine storm. The pathogenesis of cytokine-mediated liver injury may stem from inflammation, altered coagulation, and activation of the renin-angiotensin-aldosterone system, culminating in microvascular insult, hepatocyte damage, and perpetuation of inflammation[37]. It has been reported that plasma levels of interleukin (IL)-2, IL-6, IL-7, IL-10, interferon (IFN)-γ, granulocyte colony-stimulating factor, IFN-inducible protein-10, monocyte chemoattractant protein-1, recombinant macrophage inflammatory protein 1 alpha, and tumor necrosis factor alpha (TNF-α) were higher in severe COVID-19 patients than in mild and moderate cases[38,39].

The IL-6 signaling complex causes deleterious changes in hepatic sinusoidal endothelial cells and may promote blood clotting. This may be a possible mechanism behind liver injury in these patients [40]. Animal experiments have demonstrated that TNF-α has a moderate contribution to ALT elevation, necroinflammation, and apoptosis[41]. The role of other cytokines in liver injury in COVID-19 patients still requires further study.

Hypoxia, endotheliitis, and coagulation dysfunction

Patients with COVID-19, especially with severe manifestations, may have varying degrees of hypoxemia. Interestingly, some of them have no experience with breathing difficulties[42]. *In vivo* and *in vitro* studies have observed the occurrence of hepatic ischemia and hypoxia, hepatic cell death, and inflammatory cell infiltration[43]. Moreover, studies have found that SARS-CoV-2 enters endothelial cells, destroys vascular endothelium, and causes diffuse endothelial inflammation that can rapidly induce vasoconstriction and procoagulant tendency[44,45].

Spiezia *et al*[46] found that COVID-19 patients with acute respiratory failure presented with severe hypercoagulability rather than consumptive coagulopathy. In these patients, plasma levels of fibrinogen and D-dimer were significantly elevated and a marked hypercoagulable thromboelastometry profile was observed. Rampotas and Pavord[47] examined 20 random blood films from COVID-19 patients receiving invasive ventilation and observed the presence of platelet aggregates and macrothrombocytes, indicating increased platelet activity.

Reactivation of pre-existing liver disease

Liu *et al*[48] evaluated hepatitis B virus (HBV)-DNA viral load in 19 hospitalized patients with COVID-19. They found that three patients had HBV reactivation (HBVr) and one patient had a high HBV-DNA viral load throughout the hospital stay. This study suggests that COVID-19 patients with pre-existing chronic HBV infection, with or without corticosteroids use, may be at risk for hepatitis B reactivation. In a review, Perrillo *et al*[49] divided the drugs that induce HBVr into three categories. High-risk drugs are anticipated to induce HBVr in > 10% of cases, moderate-risk drugs are anticipated to induce HBVr in 1%-10% of cases, and low-risk drugs are anticipated to induce HBVr in < 1% of cases. Moderate/high-dose corticosteroid therapy for ≥ 4 wk is a high-risk factor for HBVr. Anthracycline derivatives are moderate/high-risk drugs. Moderate-risk drugs include TNF-α and other cytokine inhibitors, integrin inhibitors, tyrosine kinase inhibitors, and ≥ 4 wk of low-dose corticosteroid therapy. Therefore, patients receiving any of these drugs for COVID-19 are at risk of inducing HBVr and its complications.

DILI

Various potentially hepatotoxic drugs such as remdesivir, lopinavir, azithromycin, hydroxychloroquine, acetaminophen, antibiotics, and corticosteroids are thought to induce liver injury[50,51]. In some cases, the extent of liver damage depends on the dose[52]. Antiviral drugs have been used against SARS-CoV-2, examples of such antivirals are remdesivir, lopinavir-ritonavir, and others. They have all been documented to be potentially hepatotoxic. Although some small-scale trials have reported ALT/AST elevations with remdesivir, most clinical trials have not shown significant hepatotoxicity in the treatment of COVID-19[53]. Lopinavir/ritonavir and remdesivir have similar hepatotoxicity profiles [54].

Dexamethasone, used for hypoxic respiratory failure in patients with COVID-19, is known to induce the elevation of liver enzymes, increase hepatic lipid peroxidation, and decrease antioxidant activity [55]. The liver-damaging effects of azithromycin and acetaminophen have been proven for many years [56,57]. Acetaminophen, an analgesic and antipyretic drug widely used for mild-to-moderate pain and fever, may cause dose-dependent hepatotoxicity[52].

RISK FACTORS FOR LIVER INJURY

Studies have shown that the incidence of liver injury in severe/critically ill patients is much higher than the incidence in moderate cases[17,58]. Apparently, male sex, older age, and higher body mass index are also associated with liver damage from COVID-19[6,17,58,59]. Besides, coexisting diseases such as hypertension, diabetes, cardiovascular disease, malignancy, and some liver diseases may all be risk factors for liver damage[60,61]. Currently, the susceptibility of children and pregnant women to liver injury is not fully understood.

Male sex

Multiple studies show that men with COVID-19 have an increased risk of liver damage[6,17,59,62]. Among younger patients, men also have higher odds of severe pneumonia, acute kidney injury, and acute liver injury than women. However, among elderly patients, there was no difference in the likelihood of poor outcomes between men and women[62].

Possible mechanisms are attributed to the activity of sex hormones and X-linked genes and differential regulation of innate and adaptive immune responses to viral infection. Compared with women, men have higher circulating levels of ACE2 and ACE2 levels in the lungs. Moreover, testes have much higher levels of ACE2 than ovaries. Additionally, men have lower expression of protective cytokines but higher levels of pro-inflammatory cytokines and chemokines[62].

Elderly

In a study of 900 patients with COVID-19, those aged 40-69 were at particularly high risk of liver injury and liver-related death. COVID-19-related deaths were more frequent in patients 40-69 years and ≥ 70 years of age with elevated AST levels. Although only a small proportion (1.7%) of patients without prior liver disease also died from liver-related causes, severe liver impairment and acute liver failure are rare but important complications of COVID-19[63]. Liver dysfunction is associated with poor prognosis in elderly patients with higher mortality due to liver cell damage[64].

Liver transplant

According to recent reports, liver transplant (LT) patients have a higher incidence of COVID-19, possibly due to long-term immunosuppression. Despite the increased risk of acquiring COVID-19, LT patients have lower mortality rates than matched general individuals[65]. In another study, the prevalence of COVID-19 in LT patients was 6.05%, twice that of the general population of the same age, possibly due to higher susceptibility to the virus[66]. Verbeek *et al*[67] suggested that organ transplantation should be avoided in patients with active infection and respiratory symptoms because of the risk of COVID-19 progression and subsequent organ failure, as well as the risk of exposure to the virus for transplant operators.

Furthermore, patients with LT are at high risk for hepatic decompensation and increased mortality, and may suffer from severe extrahepatic sequelae of COVID-19[68,69]. Due to lack of evidence that LT children are at a greater risk of contracting COVID-19, routine withdrawal of immunosuppressive drugs is not recommended for LT children or patients with autoimmune liver disease[70]. Generally, LT recipients do not appear to have an increased risk of death following COVID-19 infection compared to the matched general population[71].

CLD

The most common cause of CLD is nonalcoholic fatty liver disease (NAFLD), followed by HBV infection, alcohol-related liver disease, and hepatitis C virus infection[72]. Liver injury and pre-existing CLD are significantly associated with disease severity and mortality in COVID-19 patients[73,74]. Yang *et al*[75] found that CLD is independently associated with COVID-19 severity and mortality, especially in a male-dominated elderly population. However, some studies believe that liver injury is indeed an independent predictor of key outcomes, but CLD and HBV infection status are not significant comorbidities of COVID-19[73,74,76].

Similar to other CLDs, metabolically associated fatty liver disease (MAFLD) has been shown to have longer viral shedding, a higher risk of disease progression, a higher all-cause mortality, and higher COVID-19-related mortality than patients without MAFLD[72,77]. Compared with other causes of CLD, patients with autoimmune hepatitis have a worse prognosis for COVID-19[78,79].

In adult studies, certain populations, such as those with cirrhosis, nonalcoholic steatohepatitis, and liver cancer, have been found to have an increased risk of severe COVID-19 and a poorer prognosis[69,80-82]. In adults with COVID-19, cirrhosis is a risk factor associated with worse outcomes. A large survey of 220727 patients found that COVID-19 infection in patients with cirrhosis was associated with a 2.38-fold risk of death, while cirrhosis in CLD patients with COVID-19 was associated with a 3.31-fold risk of death[83]. These results suggest that cirrhotic patients with COVID-19 infection are associated with an increased risk of all-cause mortality. Zecher *et al*[84] concluded that there were no differences in age, sex, autoimmune liver disease, and cirrhotic status between COVID-19 and non-COVID-19 cases.

Children with CLD, including obese children with suspected or confirmed NAFLD, may be at an increased risk for COVID-19 infection and severe COVID-19[70,85]. Children with CLD may experience decompensation of end-stage liver disease during COVID-19 infection[70]. Compared with LT recipients, children with CLD, including children with end-stage liver disease, are more likely to be hospitalized and require intensive care[86]. However, in the study by Di Giorgio *et al*[87], the susceptibility of different pediatric patient groups to COVID-19 infection was similar, and underlying liver disease may not increase the risk of severe COVID-19. The inconsistency between these findings may be related to the different sample sizes collected.

Obesity

Cumulative evidence support obesity as a risk factor for severe COVID-19 and related death, directly or indirectly increasing inflammation, hypercoagulability, and mechanical obstruction[88]. Obesity has emerged as a strong independent determinant of increased risk of morbidity and mortality in patients infected with COVID-19. In addition, data suggest that visceral obesity and hyperglycemia in non-diabetic and diabetic patients may also be significant independent risk factors for severe COVID-19[89]. In another study, patients aged 40-69 had a higher prevalence of obesity (44.4%), suggesting that a certain proportion of patients with hepatic steatosis in this age group may be predisposed to COVID-19-related liver damage[63,78,90]. Furthermore, one study showed that > 50% of COVID-19 cases in patients with underlying hepatic steatosis were severe, with a mortality rate of 17%[91].

Diabetes mellitus

Diabetes mellitus, whether due to insufficient pancreatic beta cells or peripheral insulin resistance, is considered a risk factor for COVID-19 infection. Numerous studies have shown that new-onset hyperglycemia, ketoacidosis, and diabetes are frequently observed in patients with COVID-19[88,89,92,93]. Individuals with diabetes often have associated comorbidities, such as obesity, hypertension, and cardiovascular disease, as well as diabetic complications, including chronic kidney disease, vascular disease, and related immune dysfunction, all of which put them at risk for infectious complications[94]. In a study of 458 patients with COVID-19 and diabetes, those with liver injury and chronic kidney disease had significantly higher mortality rates than other complications[95]. In other words, chronic kidney disease and liver disease are the two main contributors to the rise in mortality among patients with diabetes and COVID-19.

Malignancy

Hepatocellular carcinoma (HCC) has been identified as a predictor of poor prognosis in COVID-19 patients[72,76]. HCC is often associated with cirrhosis, suggesting that decreased immunity may increase the risk of severe COVID-19, and that infection with COVID-19 can exacerbate pre-existing liver disease, complicating cancer management[96]. Furthermore, COVID-19 vaccination is recommended for LT candidates and patients with CLD or HCC as they are susceptible to severe COVID-19[68]. Overall, cancer patients are considered to be at high risk of developing severe COVID-19 due to comorbidities and immunosuppressive status, especially among those who have recently received chemotherapy or had surgery within a month[96,97].

Hypertension

In a study of 300 patients with COVID-19, 33.2% were diagnosed with hypertension at admission. These hypertensive patients displayed higher levels of Troponin T and creatinine near hospital discharge[93]. Notably, the proportion of hypertensive patients in severe COVID-19 was significantly higher, and the mortality rate of severe patients was higher[93]. In addition, high blood pressure may increase the risk of liver damage following COVID-19 infection in elderly patients without pre-existing CLD[73].

It has been reported that hypertensive patients have a higher probability of liver damage and a poorer prognosis. The underlying mechanism may be related to the activation of the renin-angiotensin system and the damage of ACE2-positive cholangiocytes and hepatocytes, which further lead to cholangiocyte and hepatocyte-associated disorders[69,81,98,99]. ACE2-stimulating drugs for high blood pressure have been hypothesized to increase the risk of fatal COVID-19. Fang *et al*[100] reported that patients using ACE2-elevating drugs for hypertension, diabetes or heart disease are at increased risk of COVID-19 infection.

Pregnancy

Pregnant woman with COVID-19 have significantly decreased blood lymphocytes, increased neutrophils, and elevated C-reactive protein and TBil levels[101]. In the study by Deng *et al*[102], the incidence of liver injury in pregnant women infected with COVID-19 was 29.7%. Despite a higher frequency of ICU admissions, in-hospital mortality was lower among pregnant patients compared with non-pregnant patients with COVID-19 viral pneumonia, at 1.1% for pregnant women and 3.5% for non-pregnant women. Pregnancy is not an independent risk factor for in-hospital mortality in COVID-19 patients[103]. In the study by Tunç *et al*[104], COVID-19-related hospitalization rates were 24.1% in the first trimester, 36% in the second trimester, and 57.3% in the third trimester; there was no significant relationship between pregnancy duration and the need for ICU admission.

However, pregnant women may have many comorbidities, including hypertension, chronic lung disease, diabetes, and obesity, compared with non-pregnant women[103]. Pregnant patients with COVID-19 and chronic complications such as hypertension and diabetes have an increased risk of developing inflammation and liver damage[101]. Pregnant women taking antiviral drugs have several options, including continuing treatment, stopping or switching to safer drugs. Patients with high pretreatment ALT or less than 1 year of treatment prior to pregnancy have a high risk of severe hepatitis flares after cessation of antiviral agents[105].

The perinatal outcomes of all reported cases were reassuring, with 98% live births, 78% full-term births without neonatal complications, and a 20% neonatal ICU admission rate. The stillbirth rate was as low as 1.7%, and the neonatal mortality rate was 0.8%. No vertical transmission was found in 98.4% of neonates[106,107].

Children

Children with COVID-19 infection often have minimal or no increase in liver enzymes[60]. COVID-19 may impair liver function, usually resulting in transient and moderate elevations in liver markers without significant impairment of hepatic synthesis. COVID-19-infected patients with elevated ALT are at risk for a more severe disease course, including longer hospital stay and ICU stay[85]. Compared with adult patients, pediatric patients have relatively lower rates of lymphopenia, higher inflammatory markers, and possible thrombocytopenia[108].

MANAGEMENT OF LIVER INJURY IN PATIENTS WITH COVID-19

Liver injury in mild cases of COVID-19 is usually transient, self-limiting, and reversible without treatment. However, some COVID-19 patients who present with liver injury may become critically ill and require medical attention[16]. The cause of liver injury should be analyzed and judged in all patients. Initial screening includes careful review of preexisting liver disease, exposure to hepatotoxins (alcohol, drugs, herbs, and chemicals), hypoxia, and circulation status (Table 2). Liver function indicators including ALT, AST, TBil, DBiL, albumin, prothrombin activity, and international normalized ratio should be closely monitored[109,110].

Prophylactic use of hepatoprotective and enzyme-lowering drugs is not recommended[109]. Theoretically, reducing viral load with antiviral therapy is the most effective way to reduce organ damage. However, there is currently a lack of clinical data to support it, and more attention is paid to antiviral drug-related liver injury. This may be one reason for the lack of particularly effective antiviral drugs until recently.

The management of liver injury from COVID-19 is largely empirical and mainly supportive. Patients with severe liver damage associated with COVID-19 should be treated with hepatoprotective, anti-inflammatory, and jaundice-reducing agents such as glycyrrhizic acid, polyene phosphatidyl choline (PPC), bicyclol, and vitamin E[111,112]. Glycyrrhizic acid can effectively inhibit the replication and cytopathic effect of coronavirus without obvious cytotoxicity to host cells[113]. Glycyrrhizin has anti-inflammatory properties that may offer protection against liver disease[109]. PPC may be a drug that enhances the hepatoprotective function through glutathione and magnesium isoglycyrrhizinate[114].

Currently, there is no specific treatment for critically ill patients with COVID-19. Effective suppression of the host's uncontrolled immune response during cytokine storm may be a critical step in preventing disease progression and reducing mortality[115,116]. Anakinra is an IL-1 receptor antagonist that blocks the release of IL-β. A study concluded that early anakinra treatment is associated with significantly lower ICU admissions and mortality in patients with moderate/severe COVID-19[117]. Successful anakinra therapy includes treatment duration ≥ 10 d, dose ≥ 100 mg, intravenous administration, and early initiation of therapy[118]. Canakinumab is a human monoclonal anti-IL-1β specific antibody. Studies have shown that canakinumab therapy provides rapid and durable improvement in oxygenation levels, reduced proinflammatory markers and reduced need for mechanical ventilation resulting in better outcomes[119,120].

IL-6 is one of the key mediators of cytokine storm-induced damage[121]. Currently, there are two main types of IL-6 inhibitors that target IL-6 itself (siltuximab) or its receptors (tocilizumab and sarilumab)[115]. IL-6 levels drop after administration of siltuximab, suggesting that the inhibitor may reduce CRS and mortality[122]. The literature supports the early use of tocilizumab as it has been observed to lower mortality in adults with COVID-19 pneumonia[123,124] and achieve better clinical recovery at day 28[125]. In another study, clinical improvement and mortality were not statistically different between tocilizumab and standard treatment[125]. The reason may be a higher risk of bacterial or fungal infection in patients within tocilizumab application[123,124,126]. Sarilumab is a high-affinity anti-IL-6 receptor antibody. In a phase II, open-label, randomized, controlled clinical trial of hospitalized patients with COVID-19, early use of sarilumab was safe and associated with a trend for better outcomes[127]. However, in some other studies, the efficacy of sarilumab in hospitalized patients with moderate-to-severe COVID-19 has not been established[128-130]. Inhibition of IL-6-mediated signaling may not be sufficient to reduce CRS, and the answer may lie in combination therapy and interfere with other related pathways. So far, conflicting results hinder efforts to use IL inhibitors to combat COVID-19 infection[131].

Anti-TNF therapy has also shown conflicting results. In a case-cohort study, patients treated with anti-TNF-α inhibitors were hospitalized less frequently[132]. This was a systematic review and meta-analysis of COVID-19 and outcomes in patients with inflammatory bowel diseases (IBD). Compared with patients on corticosteroids, those on anti-TNF-α therapy had a lower risk of hospitalization and ICU admission. Moreover, similar results were seen in patients treated with anti-TNF-α compared to

Table 2 Treatments of liver injury in coronavirus disease 2019

Mechanisms of liver damage	Treatments	Caution	Ref.
Hepatocellular injury	Hepatoprotective, anti-inflammatory, and jaundice-reducing agents	Preventive administration is not recommended	[109, 111, 112]
Cytokine storm syndrome	Continuous renal replacement therapy. IL-1 inhibitor, IL-6 inhibitor, TNF inhibitor	IL-1 or IL-6 inhibitors could reduce inflammation; however, they have a potential to cause DILI and worsen clinical conditions	[109, 139, 140]
DILI	Prompt discontinuation or reduction of doses of suspected triggers. Medication reconciliation is important. Discontinue all non-vital therapy, redundant types/doses, modify course duration	Requires a trade-off between therapeutic effects and side effects	[109]
Reactivation of pre-existing liver disease	Continue treatment for hepatitis B and hepatitis C if already on treatment	Difficulty distinguishing between new-onset liver injury and reactivation of pre-existing liver disease	[16, 109]
Hypoxic hepatitis	Circulation and respiratory support	Higher PEEP, which may be needed to improve oxygenation, may affect cardiac output, decreasing hepatic arterial flow, thus enhancing arterial dysfunction	[139, 140]

DILI: Drug-induced liver injury; IL: Interleukin; PEEP: Positive end-expiratory pressure; TNF: Tumor necrosis factor alpha.

patients treated with mesalamine[133]. Colonic ACE2 expression was downregulated after anti-TNF-α therapy in IBD patients[134], but no liver-related data have been reported. In another meta-analysis and systematic review of 84 studies, no difference was found in the risk of hospitalization in patients receiving anti-TNF-α therapy compared to patients not receiving anti-TNF-α therapy[135]. Foods rich in vitamins, minerals, polyphenols, and other bioactive compounds may decrease inflammatory pathway activity and prevent liver damage in COVID-19 patients[136].

Corticosteroids have a dual effect. They have been associated with DILI, especially at high doses, however they are used to treat drug-induced cholestatic hepatitis and DILI associated with hypersensitivity reactions[137,138]. The only specific antidote for acute DILI remains N-acetylcysteine for acetaminophen poisoning. Glycyrrhizin, ursodeoxycholic acid, and silymarin have been used for decades to treat DILI, but success remains anecdotal[138]. The most effective treatment for suspected DILI is to discontinue drug therapy before progression to irreversible liver failure, which results in spontaneous recovery in approximately 90% of cases[139].

CONCLUSION

Nearly 3 years later, there is still no sign that the COVID-19 pandemic is over. COVID has long-term devastating effects involving multiple organs. Particular attention should be given to liver injury associated with COVID-19. There is growing evidence that liver injury is a typical long-term effect of COVID-19, especially in critically ill cases, and may require monitoring after the patient is discharged. The exact incidence and underlying mechanism of liver damage are not well known. Fortunately, most patients with mild liver damage recover without special treatment. However, SLI is believed to worsen the prognosis and increase mortality from COVID-19. Increased research efforts are needed to identify those patients at higher risk of complications, better definition of liver injury, better understanding of the pathophysiology, and effective therapies.

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REVIEW

COVID-19 and liver injury: An ongoing challenge

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Abstract

The new coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified in December 2019, in Wuhan, China. The virus was rapidly spread worldwide, causing coronavirus disease 2019 (COVID-19) pandemic. Although COVID-19 is presented, usually, with typical respiratory symptoms (*i.e.*, dyspnea, cough) and fever, extrapulmonary manifestations are also encountered. Liver injury is a common feature in patients with COVID-19 and ranges from mild and temporary elevation of liver enzymes to severe liver injury and, even, acute liver failure. The pathogenesis of liver damage is not clearly defined; multiple mechanisms contribute to liver disorder, including direct cytopathic viral effect, cytokine storm and immune-mediated hepatitis, hypoxic injury, and drug-

induced liver toxicity. Patients with underlying chronic liver disease (*i.e.*, cirrhosis, non-alcoholic fatty liver disease, alcohol-related liver disease, hepatocellular carcinoma, *etc*.) may have greater risk to develop both severe COVID-19 and further liver deterioration, and, as a consequence, certain issues should be considered during disease management. The aim of this review is to present the prevalence, clinical manifestation and pathophysiological mechanisms of liver injury in patients with SARS-CoV-2 infection. Moreover, we overview the association between chronic liver disease and SARS-CoV-2 infection and we briefly discuss the management of liver injury during COVID-19.

Key Words: COVID-19; Liver injury; Cytokine storm; Hypoxic hepatitis; Drug-induced liver injury; Chronic liver disease

Core Tip: Liver injury is a common feature in coronavirus disease 2019 (COVID-19) patients and was associated with disease severity and prognosis. Multiple pathophysiological mechanisms are responsible for liver injury, including direct viral effect, cytokine storm, hypoxia and drug hepatotoxicity, however, further research is needed, in order, for them, to be clearly defined. Patients with underlying chronic liver disease may be more susceptible to severe acute respiratory syndrome coronavirus 2 infection; nevertheless, evidence is still limited. It is necessary to know the mechanisms of liver injury, the clinical manifestations and the effect of COVID-19 in underlying liver disease, in order to design appropriate management programs.

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INTRODUCTION

In December 2019, severe acute respiratory syndrome coronavirus 2, (SARS-CoV-2), causing respiratory infection in humans, was detected in Wuhan, China[1]. The new coronavirus was spread worldwide, resulting in coronavirus disease 2019 (COVID-19) outbreak. On March 11, 2020, the World Health Organization declared COVID-19 as a global pandemic[2]. As of September 2022, over 603 million confirmed cases and over 6.4 million deaths have been reported worldwide[3].

Most COVID-19 patients present with typical respiratory symptoms (*i.e.*, cough, dyspnea) and fever. However, abnormal liver function is often developed in patients with COVID-19, and liver injury has been related with severe disease[4,5]. Liver damage ranges from mild asymptomatic elevation of liver enzymes to severe liver injury, while a few cases of acute liver failure have also been reported[6,7].

The aim of this review is to present the prevalence and clinical manifestations of liver injury in COVID-19, to overview the potential pathophysiological mechanisms leading to liver damage and to summarize the existing literature for patients with COVID-19 and underlying chronic liver disease. Furthermore, the management of liver complications during SARS-CoV-2 infection is also briefly discussed.

PREVALENCE AND RISK FACTORS

Numerous studies have focused on liver injury induced by COVID-19 infection. However, the definition of liver injury in COVID-19 patients has not been clearly established yet. Some researchers defined it, as any increase of liver enzymes above the upper limit of normal (ULN), while others, as an increase, at least 2 or 3 times above the ULN[8-12]. Moreover, the different statistical time points across the studies, could also affect the incidence of liver injury[8]. As a consequence, the prevalence of liver damage varies across studies. Wang *et al*[13] conducted a retrospective study and found that the 41% of 156 COVID-19 patients had abnormal liver function, while, Fan *et al*[10] demonstrated that 55 out of 148 patients (37.2%) had elevated liver enzymes on admission. In a recent retrospective study of 228 patients, without chronic liver disease, 29.4% had abnormal liver function on admission; the rate increased to 56.3% during hospitalization[14]. Cai *et al*[15] defining liver injury as alanine transaminase (ALT) or

aspartate aminotransferase (AST) 3 times higher than ULN or alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin (TBIL) 2 times higher than ULN, observed that 41% of patients had abnormal liver tests and 5% had liver injury on admission. During hospitalization, patients with abnormal liver tests and patients with liver injury increased to 76.3% and 21.5%, respectively. Ding *et al*[16], also, demonstrated the same trend of liver function, in a large retrospective cohort study of 2073 patients. On admission, 46.2% and 5.1% had abnormal liver tests and liver injury, respectively. Yet, during hospitalization, the incidence increased to 61.8% and 14.3%, respectively. Across several meta-analyses, the pooled prevalence of liver injury ranged between 19% and 27.4%[4,5,17]. Kulkarni *et al*[18], in their meta-analysis, found that the pooled incidence of abnormal liver enzymes at initial presentation, only slightly increased during the course of disease (from 23.1% to 24.4%). Wu *et al*[19] observed a similar trend; the pooled incidence of elevated liver tests on admission and during hospitalization was 27.2% and 36%, respectively.

Liver injury has been associated with severe COVID-19 disease[4,5,19-21]. Chen *et al*[22] demonstrated that patients with deranged liver function had higher risk of systemic inflammatory response syndrome (53.5% *vs* 41.3%, $P = 0.007$) and higher mortality rate (28.9% *vs* 9.0%, $P < 0.001$). In another retrospective study, elevated AST (> 3-fold ULN) was associated with higher risk of mechanical ventilation and death[23]. Moreover, Wang *et al*[24] found that the levels of aminotransferases were significantly higher in ICU patients compared to non-ICU patients (ALT: 35 *vs* 23, normal range 9-50 U/L, $P = 0.007$ and AST: 52 *vs* 29, normal range 5-21 U/L, $P < 0.001$). Kumar *et al*[4], in their meta-analysis, confirmed that liver injury was higher in patients with severe COVID-19 disease, compared to non-severe COVID-19 disease (44.63% *vs* 20.02% respectively). Furthermore, Mao *et al*[5] conducted another meta-analysis and found that patients with severe COVID-19 infection exhibited a higher risk for abnormal liver function, including increased AST and ALT. Finally, in a recent meta-analysis of 15 studies, patients with deranged liver function and/or histopathological findings of liver disease, presented a significantly higher risk of poor COVID-19 outcomes[21]. Across several studies, other risk factors for liver injury were found to be male gender, higher BMI, older age, severe lung disease and underlying chronic liver disease[11,15,25].

CLINICAL MANIFESTATIONS

In most cases, liver injury is presented as elevated liver enzymes without specific symptoms and signs. The elevation of AST, ALT and/or TBIL is a very common manifestation in COVID-19 patients, while increased GGT and/or ALP is a less usual feature, observed in later stages of the disease[6,7]. The elevation of the aminotransferases is usually mild; their level is mostly < 5 times ULN[26]. Furthermore, liver injury in COVID-19 has been noted to be transient, while hepatic biochemical tests return to normal within 2-3 wk[6]. Severe liver injury, with aminotransferases > 20 times ULN, has been observed in 0.1% of COVID-19 patients on admission and in 2% during hospitalization, while acute liver failure, induced by COVID-19, has been reported in extremely rare cases[27,28]. Febrile hepatitis, acute cholecystitis and hepatic artery thrombosis are, also, rare clinical presentations of COVID-19[29-31]. Moreover, in some cases reports, it is suggested that SARS-CoV-2 triggered a *de novo* development of immune-mediated liver disease, such as autoimmune hepatitis and primary bile cholangitis[32-35]. Interestingly, cholangiopathy, characterized by cholestasis and structural abnormalities of bile duct, has been reported in post-COVID-19 patients, who recovered from severe and critical disease[36,37].

MECHANISMS OF LIVER INJURY

The pathogenesis of live injury in COVID-19 disease is still unclear. According to the available literature, the underlying mechanisms of liver injury are multifactorial and mainly, include direct viral cytopathic damage, immune-mediated hepatitis, caused by cytokine storm, hypoxia and ischemic injury and drug-induced liver toxicity. The possible pathophysiologic mechanisms of liver injury are presented in Figure 1.

Direct cytopathic effect of SARS-CoV-2

Liver is a potential target of direct SARS-CoV-2 infection. Existing literature suggests that the new coronavirus could be detected in the liver and indicates typical histological lesions related to viral infection[38]. Indeed, a series of small sample size studies demonstrated that SARS-CoV-2 RNA and viral particles are detectable in the liver of patients with COVID-19[13,39-43]. Furthermore, in a recent cohort study of 45 autopsy cases, virus RNA was detected in 69% of cases[44].

SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) to invade into host cells, while cell entry is facilitated by transmembrane serine protease 2 (TMPRSS2) and paired basic amino acid cleaving enzyme (FURIN)[45,46]. Single-cell RNA sequencing analysis revealed that ACE2 is expressed among different cell types in liver; in parallel, TMPRSS2 and FURIN are, also, expressed in liver cells[47-49].

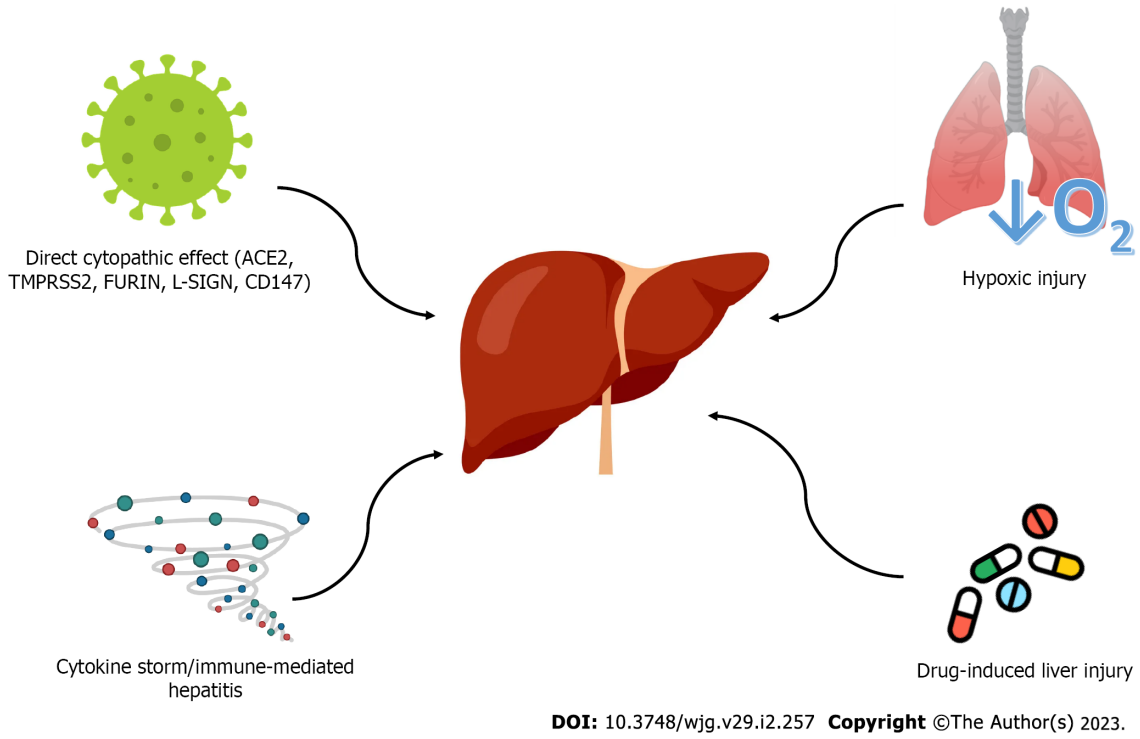

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Figure 1 Mechanisms of liver injury in coronavirus disease 2019. ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2; FURIN: Paired basic amino acid cleaving enzyme; SIGN: Specific intercellular adhesion molecule-3-grabbing non-integrin.

The above evidence indicates that liver tissue could be susceptible to COVID-19 infection. Yet, the expression of ACE2 in bile duct cells is 20-fold higher than the expression level in hepatocytes[50]. Despite the high expression of ACE2 in cholangiocytes, which would be associated with cholestatic injury (*i.e.*, elevated levels of GGT and ALP), most studies found that hepatocellular damage is the most common pattern in COVID-19 patients (*i.e.*, elevated levels of ALT and AST)[17,51,52]. Therefore, alternative molecular pathways for liver infection cannot be excluded. The liver/Lymph node-specific intercellular adhesion molecule-3-grabbing integrin, a liver-specific capture receptor, and CD147, a receptor highly expressed in inflamed and/or pathogen-infected tissues, have been proposed as alternative receptors or enhancer factors, mediating in the SARS-CoV-2 cellular entry in the liver. Moreover, antibody-dependent enhancement may be responsible for liver infection[53]. Instead of neutralizing the virus completely, suboptimal non-neutralizing antibodies, attached to Fc receptor, promote viral entry into the liver cells[53]. In addition, existing evidence suggests that inflammatory signals [*i.e.*, interleukin-6 (IL-6), type 1 interferon] and hypoxia, related to SARS-CoV-2 infection, could result to hepatocyte regeneration, compensatory hyperplasia and upregulated expression of ACE2, leading to potentially increased hepatic susceptibility to SARS-Cov-2[45,53].

Despite that virus particles have been observed in hepatocytes and molecular pathways of virus invasion have been suggested, further evidence is needed to clearly establish the role of direct viral infection in liver injury.

Immune-mediated liver injury

COVID-19 infection can trigger uncontrolled immune response, called cytokine storm, which is characterized by exaggerated activation of immune cells and massive production of inflammatory mediators [54,55]. Indeed, pro-inflammatory cytokines [*i.e.*, IL-1β, IL-2, IL-6, IL-8, tumor necrosis factor-α, interferon-α (IFN-α), IFN-γ, granulocyte-macrophage colony-stimulating factor] were increased in severe COVID-19 disease[56]. Cytokine storm generates a process leading to tissue damage and even multiorgan failure[57]. Due to its anatomical location, liver is highly exposed to circulating cytokines, and thus, prone to inflammatory-mediated injury[58]. Furthermore, viral-induced $CD8^+$ T cells provoke the activation of Kupfer cells, resulting to T cell-mediated hepatitis[58].

Several studies have demonstrated a correlation between liver injury and increased levels of inflammatory mediators in COVID-19 patients. In a recent cohort study of 192 patients, increased IL-6 and IL-10 Levels and decreased number of $CD4^+$ T cells were identified as independent risk factors for severe liver injury[59]. Likewise, in another retrospective cohort study, inflammatory markers, such as IL-6, CRP and ferritin, were significantly higher in patients with liver injury[60]. Huang *et al*[61], conducting a retrospective study of 2623 patients, found a positive correlation between IL-6 and liver enzymes (*i.e.*, AST, ALT, and GGT), indicating that COVID-19-induced cytokine storm leads to hepatotoxicity. In

addition to that, Liao *et al*[62], suggested that, apart from IL-6, IL-2 and IL17A were also key inflammatory factors triggering liver damage.

Hypoxia-reperfusion injury

The liver is a highly aerobic organ, and, thus, it is remarkably susceptible to hypoxia[38]. Patients with COVID-19 can be complicated with respiratory failure, acute heart failure and systemic stress, causing low oxygen saturation level and/or decreased systemic arterial pressure. As a consequence, arterial perfusion and oxygenation of the liver can be reduced, leading to hepatic ischemia and hypoxia-reperfusion injury[38,63]. Furthermore, systemic inflammatory response, through microvascular dysfunction and microthrombosis, could worsen liver hypoxia[38]. Hepatic venous congestion, caused by heart failure, or high positive end-respiratory pressure, used in patients with respiratory failure, can, also, lead to hypoxic damage in the liver cells[58].

Hypoxic injury involves a biphasic process; ischemic cell damage and reperfusion-associated inflammatory response. Lipid accumulation, glycogen consumption, mitochondrial damage and increased reactive oxygen species and their peroxidation products lead to cell death, during ischemia[53]. Following ischemic injury, reperfusion induces activation of immune response and release of pro-inflammatory cytokines, resulting in further cell damage[53].

In a retrospective cohort study, hepatocellular injury pattern in COVID-19 patients was associated with hypoxia[64]. Likewise, Fu *et al*[65], in a more recent multicenter retrospective study, confirmed that patients with hypoxia were more likely to have abnormal liver function.

Drug-induced liver injury

The liver plays a crucial role in drug metabolism. Several drug metabolites induce liver cell apoptosis/necrosis and can lead to liver damage. Drug-induced liver injury (DILI) is often detected by liver enzymes tests, using the following thresholds: (1) ALT > 5 times ULN; (2) ALP > 2 times ULN; and (3) ALT > 3 times ULN and TBL > 2 times ULN[66]. Based on ALT/ALP ratio, DILI pattern can be defined as hepatocellular, cholestatic or mixed. DILI can also be intrinsic, which is dose-dependent and predictable, or idiosyncratic, which is unpredictable, with variable latency period[66]. Concerning prognosis, DILI ranges from mild to severe or even fatal, with approximately 10% of patients requiring liver transplantation[66].

At present, many drugs have been used to treat COVID-19 patients, such as corticosteroids, antiviral agents, immunoregulatory factors and antibiotics, leading to potential hepatotoxicity. Systemic corticosteroids, especially dexamethasone, were widely prescribed to both outpatients and hospitalized patients with COVID-19. Despite that, the prolonged use of corticosteroids is related to side effects (*i.e.*, infections, hyperglycemia), DILI is uncommon[67]. Corticosteroids have been associated with liver steatosis, hepatomegaly, worsening non-alcoholic fatty liver disease (NAFLD) and exacerbating HBV reactivation, however, existing literature is limited[67,68]. With regards to COVID-19, Yip *et al*[69] found that the use of corticosteroids was an independent factor of liver injury. However, this association could be explained by the fact that patients with more severe disease received corticosteroids.

Remdesivir is an inhibitor of viral RNA-dependent RNA polymerases, used in COVID-19 disease. Among its side effects, remdesivir can cause hepatotoxicity, manifested as elevated AST and ALT[67]. In most studies, 10%-50% of patients developed mild-to-moderate increase of aminotransferases, while levels > 5 times ULN were reported in 9% of patients in clinical trials[70]. Subsequently, remdesivir is contraindicated in patients with ALT > 5 times ULN or severe liver dysfunction[71]. The elevation of aminotransferases is generally reversible without clinically apparent hepatic dysfunction[67].

Tocilizumab, a humanized anti-interleukin-6 receptor (IL-6R) monoclonal antibody, is indicated in hospitalized COVID-19 patients with rapid respiratory deterioration[67]. Elevation of aminotransferases has been reported, but it is generally transient, dose-dependent, without significant liver complications [67,72]. Anakinra, an IL-1 inhibitor, has, also, been used in severe COVID-19, but hepatotoxicity is an extremely uncommon side effect. In addition to that, liver enzymes levels did not significantly differ between anakinra and placebo in clinical trials[67,73].

More recently, nirmatrelvir/ritonavir has been prescribed in COVID-19 patients, early in the course of infection, as a post-exposure protection. In clinical trials, elevation of aminotransferases was uncommon or mild in nirmatrelvir/ritonavir group and did not differ from placebo group. However, clinical data are still limited and further evidence is needed[74].

Table 1 presents the most studied drugs for COVID-19 and the existing evidence concerning their hepatotoxicity.

CHRONIC LIVER DISEASE AND COVID-19

Most studies have not provided sufficient data about the prevalence of underlying chronic liver disease (CLD) in COVID-19 patients. However, in a meta-analysis of 73 studies including 24299 COVID-19 patients, the pooled prevalence of CLD was estimated to be at 3%[75]. Patients with CLD may, already, have liver damage and SARS-Cov-2 infection is an additional "hit" to the liver, leading to further liver

Table 1 Evidence of hepatotoxicity of most studied and used drugs in coronavirus disease 2019

Drug	Mechanism of action	Characteristics of LI	Risk of DILI	DILI pattern
Corticosteroids[126]	Anti-inflammatory	Hepatomegaly, steatosis; triggering/worsening NAFLD; reactivation HBV (prolonged administration)	Low	Hepatocellular or mixed
Remdesivir[70]	Antiviral; active inhibitor of viral RNA-dependent RNA polymerases	Mild-to-moderate ALT and AST elevations; Elevation > 5 times ULN in 9% (resolved with discontinuation)	Moderate	Hepatocellular
Tocilizumab[72]	Anti-IL-6 receptor monoclonal antibody	Elevation of ALT and AST; no reports of severe LI or HBV reactivation (in COVID-19 trials)	Moderate	Hepatocellular
Anakinra[73]	IL-1 inhibitor	ALT elevation in < 1%; No association with HBV reactivation	Low	Hepatocellular
Nirmatrelvir/ritonavir [74]	Antiviral; Inhibitor of the main protease of SARS-CoV-2/protease inhibitor and potent inhibitor of the enzyme CYP 3A4	Mild ALT and AST elevation; no reports of clinical apparent LI; limited data	Low	Hepatocellular
Molnupiravir[127]	Antiviral; prodrug of the ribonucleoside analogue N-hydroxycytidine	Mild ALT and AST elevation; no reports of clinical apparent LI; limited data	Low	Hepatocellular
Low-molecular-weight heparins[128]	Anticoagulant	Mild ALT and AST elevation; LI with rapid onset and rapid recovery, without clinical symptoms	Low	Hepatocellular
NSAIDs[129]	Anti-inflammatory	Mild, transient and asymptomatic elevation of liver enzymes; more common in obese patients with comorbidities; reports of acute hepatitis (idiosyngratic, prolonged administration)	Moderate	Hepatocellular, cholestatic or mixed
Acetaminophen[130]	Analgesic and antipyretic	Dose-dependent; transient and asymptomatic elevation of ALT and AST; acute hepatitis and/or acute liver failure in overdose	High	Hepatocellular

DILI: Drug-induced liver injury; ALT: Alanine aminotransaminase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus; IL-6: Interleukin-6; IL-1: Interleukin-1; LI: Liver injury; NAFLD: Non-alcoholic fatty liver disease; ULN: Upper limit of normal; NSAID: Nonsteroidal antiinflammatory drugs.

functional impairment[76]. Although patients with stable CLD, without cirrhosis, are not more susceptible to severe COVID-19, those with cirrhosis, alcoholic liver disease (ALD), hepatocellular carcinoma (HCC) and NAFLD may be in a greater risk for severe disease with liver injury and poor outcome[6,76-78].

Cirrhosis

Patients with cirrhosis may be more susceptible to SARS-CoV-2 infection, due to their immunodeficient status, referred as cirrhosis-associated immune dysfunction[79]. In several studies, COVID-19 patients with cirrhosis presented worse prognosis, compared to patients without cirrhosis[80-85]. In a large multicenter study, including 745 COVID-19 patients with CLD (386 with and 359 without cirrhosis), cirrhotic patients exhibited higher mortality rate, compared to those without cirrhosis (32% *vs* 8%, $P < 0.001$)[82]. Mortality was correlated with the stage of liver cirrhosis; 19% in Child- Pugh class A, 35% in class B, and 51% in class C. A similar trend was also observed in the rates of ICU admission, mechanical ventilation and renal replacement therapy. In the same study, it was noted, that the main cause of death was respiratory failure (71%) followed by liver complications[82].

Moreover, COVID-19 patients with cirrhosis are in increased risk for acute decompensation and acute-on-chronic liver failure (ACLF)[78]. Sarin *et al*[86], conducting a multicenter cohort study, found that 20% of patients with compensated cirrhosis developed acute decompensation or ACLF during COVID-19 disease, while 57% of patients with decompensated cirrhosis had further liver complications. Acute decompensation is a common clinical feature in cirrhotic patients during SARS-CoV-2 infection, usually presented as new or worsening ascites or hepatic encephalopathy[82]. Interestingly, liver complications can also be developed and in the absence of typical symptoms of respiratory system[82,85].

Non-alcoholic fatty liver disease

Patients with NAFLD usually have other comorbidities, such as diabetes mellitus, obesity, hypertension and chronic cardiac disease, which are common risk factors for severe COVID-19[77]. Consequently, it is challenging to define an independent effect of NAFLD on COVID-19 and evidence from concomitant studies is controversial. More particularly, some studies did not prove an association between NAFLD and worse COVID-19 outcomes[87-89]. On the other hand, numerous observational studies demonstrated that NAFLD is related to more severe SARS-CoV-2 infection, while three meta-analyses

confirmed this association[90-99]. Despite multiple confounding factors, NAFLD was considered as an independent risk factor for severe COVID-19. Hashemi *et al*[91] found that NAFLD was an independent risk factor for ICU admission and mechanical ventilation in COVID-19 patients. In a retrospective case-control study, NAFLD was associated with COVID-19 severity, irrespective of metabolic syndrome[90]. Furthermore, Sachdeva *et al*[97], in their pooled analysis, reported that NAFLD was a predictor of COVID-19 severity, even after adjusting for obesity.

Alcoholic liver disease

Although the existing evidence is limited, few studies demonstrated that ALD is related to increased COVID-19 mortality. In a multicenter cohort study of 867 COVID-19 patients, reported that ALD is an independent risk factor of higher mortality[100]. Likewise, Marjot *et al*[82] identified independent association between ALD and COVID-19 mortality. Mallet *et al*[101], also, found that ALD is a risk factor of day-30 mortality after COVID-19. The exact mechanism leading to the aforementioned correlation is not clear. However, ALD-related immune dysregulation and low nutritional status may have a negative impact on the course of SARS-CoV-2 infection[7,79].

Viral hepatitis

The influence of viral hepatitis on COVID-19 severity and COVID-19-related liver injury has not been clearly established. COVID-19 patients with chronic hepatitis B (CHB) may have prolonged virus shedding and infection[48]. Furthermore, during SARS-CoV infection, replication of hepatitis B virus (HBV) was found to be enhanced, inducing more severe liver injury; similar enhancement could be noted during SARS-CoV-2 infection[102]. Wang *et al*[103], in a retrospective cohort study of 437 patients, found that those with co-infection SARS-CoV-2/HBV had higher risk of severe disease and mortality. Likewise, Zou *et al*[104] reported that COVID-19 patients with CHB and liver injury were more prone to poor outcomes. Nevertheless, other studies did not demonstrate the above associations. Chen *et al*[105] found no difference in terms of liver function and disease severity between COVID-19 patients with HBV and those without co-infection. Guan *et al*[106] also suggested that CHB does not affect COVID-19 outcome, as only one of 23 patients with CHB developed severe disease. In addition, Yip *et al*[107] demonstrated that current and past HBV infection were not related to higher risk of liver injury or mortality.

Due to extended use of immunosuppressive drugs for COVID-19 treatment (*i.e.*, tocilizumab), potential re-activation of HBV should be taken into consideration. Although the immunosuppressive therapies are short-term and results of clinical trials are contradictory, there are some clinical case reports of HBV re-activation in COVID-19 patients after administration of these immunosuppressive agents[108].

Of note, COVID-19 pandemic has disrupted the progress in the global hepatitis C virus (HCV) elimination program, resulting in delays in diagnosis and HCV therapy, which could extend the direct COVID-19-related morbidity and mortality in these patients[109].

Autoimmune liver disease

Although immunosuppressive therapy, used in patients with autoimmune liver diseases, could be associated with higher risk of severe disease, there is no evidence that patients with autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC) are more prone to SARS-CoV-2 infection[6,77]. In a phone-based survey, there was no difference in percentage of COVID-19 diagnosis in patients with autoimmune liver diseases and the general population. Most of patients reported a favorable disease outcome in the same survey[110]. Data derived from three multinational registries (SECURE-Cirrhosis, COVID-Hep and ERN RARE-LIVER) revealed that patients with AIH had increased risk of hospitalization compared to patients with other CLD, but there was no difference in adverse outcome, including ICU admission and death, despite the immunosuppressive treatment[111]. However, a recent retrospective study of 254 patients with COVID-19 and AIH demonstrated that baseline treatment with corticosteroids or azathioprine was associated with COVID-19 severity[112]. Evidence for patients with PBC and PSC is limited and no defined association with COVID-19 severity has been established yet[7].

Hepatocellular carcinoma

COVID-19 patients with may have a high risk for poor outcomes. Due to chemotherapy/immunotherapy, HCC patients are immunosuppressed, and, subsequently, vulnerable to severe SARS-CoV-2 infection[102]. Furthermore, most HCC patients have an underlying CLD (*i.e.*, cirrhosis, ALD *etc.*), and as a result, they are already identified as a high-risk group[102]. However, the corresponding literature is limited. A small retrospective study of 28 cancer patients with COVID-19, including 2 HCC patients) found that these patients had worse prognosis compared to general population[113].

VACCINATION AGAINST SARS-COV-2 IN CHRONIC LIVER DISEASE

Different types of SARS-CoV-2 vaccines have been developed, such as mRNA vaccines, adenoviral-vectored vaccines and inactivated vaccines. In general, patients with CLD may exhibit lower immune response to vaccination; according to previous studies, rate of seroconversion after HBV vaccine and cell-mediated immunity were reduced in cirrhotic patients[114,115]. Regarding efficacy, although trials of both mRNA vaccines included few patients with underlying CLD, they reported significant efficacy in the subgroup with coexisting comorbidities[116,117]. Of note, in a large retrospective cohort study of cirrhotic patients, a single mRNA vaccine dose appeared to reduce not only rates of SARS-CoV-2 infection, but also, rates of hospitalization and mortality[118]. With regard to safety, none of vaccine contain living virus, and subsequently, they can be used even in immunosuppressed patients[119]. Moreover, no significant liver-associated side effects have been reported in the vaccinated population [120]. Given that benefits outweigh the potential risks, European Association for the Study of the Liver (EASL) and American Association for the Study of Liver Diseases (AASLD) recommend that patients with CLD should be vaccinated against SARS-CoV-2[121,122].

MANAGEMENT OF LIVER INJURY IN COVID-19

Liver injury in COVID-19 is usually mild and resolves spontaneously without any special treatment [77]. If present, hypoxia and circulatory failure should be regulated with standard symptomatic support (*i.e.*, oxygen therapy, intravenous fluids) in order to prevent further liver damage[45,123]. If liver injury persists, underlying chronic liver disease should be suspected[124]. With regard to DILI, there are no specific management guidelines. Discontinuation or dose's reduction of suspected medication is the most effective treatment in case of DILI, as the only available antidote is N-acetylcysteine for acetaminophen overdose[67]. In the case of severe COVID-19, benefits and risks have to be weighed in order to decide discontinuation of systematic treatment. This dilemma hardly arises for pharmaceutical agents which need short administration, such as remdesivir and tocilizumab[67]. Standard guidelines and supportive therapy should be followed for management of acute liver failure[67].

Regarding chronic liver diseases, comprehensive recommendations related to COVID-19 management have been published by EASL-ESCMID and AASLD[124,125]. Cirrhotic patients with acute decompensation or ACLF have to be tested for COVID-19, even without any other symptom[125]. Patients with HBV or HCV and SARS-CoV-2 coinfection should continue antiviral therapy, while in COVID-19 patients with chronic, occult or resolved HBV, who receive immunosuppressive agents (*i.e.*, tocilizumab, corticosteroids), clinicians have to consider and prevent potential HBV re-activation[124,125]. In COVID-19 patients with AIH, discontinuation or reduction of immunosuppressive agents is not recommended. Reduction is considered in special cases, such as severe COVID-19 and bacterial/fungal co-infection, or severe lymphopenia[124,125].

CONCLUSION

Liver abnormalities are common in COVID-19 patients, especially in patients with severe and critical disease. The pathogenesis of liver injury may be multifactorial involving direct cytopathic viral effect, inflammatory storm, hypoxic/hypoperfusion injury and drug hepatotoxicity. Liver injury is usually mild and transient; however, some cases of severe liver injury and acute liver failure have been reported. Although, patients with stable chronic liver disease are not more vulnerable to SARS-CoV-2 infection, patients with cirrhosis, ALD, NAFLD and HCC have higher risk for severe COVID-19 and liver damage. Specific management issues should be taken into consideration during COVID-19 treatment in patients with underlying CLD. Further investigation is needed in order to clarify the association between SARS-CoV-2 and liver dysfunction, in terms of prognosis, pathophysiology and treatment.

FOOTNOTES

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REVIEW

Advancing the precision management of inflammatory bowel disease in the era of omics approaches and new technology

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Abstract

There is great heterogeneity among inflammatory bowel disease (IBD) patients in terms of pathogenesis, clinical manifestation, response to treatment, and prognosis, which requires the individualized and precision management of patients. Many studies have focused on prediction biomarkers and models for assessing IBD disease type, activity, severity, and prognosis. During the era of biologics, how to predict the response and side effects of patients to different treatments and how to quickly recognize the loss of response have also become important topics. Multiomics is a promising area for investigating the complex network of IBD pathogenesis. Integrating numerous amounts of data requires the use of artificial intelligence.

Key Words: Inflammatory bowel diseases; Precision management; Multiomics; Artificial intelligence

Core Tip: Inflammatory bowel diseases (IBDs) exhibit different pathogeneses and clinical manifestations. Making precise and appropriate therapeutic decisions according to the condition of each patient remains challenging. We summarize the clustering strategies, the approaches used to apply multiomics and artificial intelligence to IBD precision management.

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INTRODUCTION

Since it was first proposed in 2011[1], the concept of precision medicine has become increasingly popular and attracted much attention. Great progress has been made, especially in the treatment of cancer. Precision medicine typically refers to the use of targeted therapy based on etiology and mechanism. The essence of the idea involves classifying individuals with common characteristics into the same subgroup using specific clinical features, treatment features and prognoses. Thus, this strategy should actually combine a wide array of data, including clinical, genetic and environmental information, as well as multiple types of biomarkers[2]. These efforts would add to the objectivity and flexibility of treatment decision-making.

Inflammatory bowel disease (IBD) is a group of intestinal disorders of unknown etiology characterized by inflammation that arises from a complex interaction between genetic and environmental factors and immune responses[3]. An increasing number of studies have reported on the great heterogeneity of IBD patients in terms of pathogenesis, clinical manifestation, response to treatment and prognosis, and IBD is currently regarded as a continuous spectrum of diseases[4]. The introduction of biologics has greatly improved the quality of life of IBD patients, which also embodies precision medicine to some extent. However, due to the complexity of pathogenesis, targeting only immune pathways without addressing the genome or microbiome may result in limited success[5]. The treatment strategies used are largely based on evidence from clinical trials, which typically do not stratify patients with enough precision. Additionally, the frequency of treatment may be indicated for a certain population, but this approach might not be the most suitable for an individual. Compared to the oncology field, there is still much room for precision medicine development in IBD.

In this review, we discuss the strategies used to categorize IBD patients and biomarkers for identifying these subgroups. We suggest that applications of multiomics and artificial intelligence (AI) approaches could facilitate the precision management of IBD patients (Figure 1).

THE HETEROGENEITY OF IBD AND CLUSTERING STRATEGIES FOR IBD PATIENTS

Phenotype refers to the traits that can be observed in patients, and deep phenotyping plays a key role in the progress of precision medicine[2]. In other disease contexts, there is also the concept of endotype, which is defined as the molecular mechanism underlying the visible phenotype[6]. However, clustering phenotypes and endotypes remains difficult in the context of IBD due to heterogeneity.

The Montreal classification is the most widely used clinical classification of IBD and considers age at diagnosis, location and behavioral factors[7]. The characteristics and natural history of IBD seemed to vary depending on the age of onset[8]. Very early onset IBD (VEO-IBD), defined as IBD occurring in those who are diagnosed under the age of 6 years and sometimes exhibiting a more aggressive disease pattern, seemed to have stronger genetic triggers with less environmental influence[9]. In addition, complications or extraintestinal organ involvement can also be used to identify some unique subsets of IBD patients. For example, IBD patients who experienced complications with primary sclerosing cholangitis (PSC) have been shown to exhibit higher rates of colectomy, cancer and death than non-PSC-IBD patients[10]. However, current clinical classification is far from the precise identification of IBD phenotypes.

Some specific genetic factors have been found to determine disease progression, which is difficult to assess by clinical manifestations. Next-generation sequencing has been used to identify more than 100 monogenic causes that could manifest as IBD-like phenotypes. The genes involved in monogenic IBD disorders are generally classified into six categories according to the mechanisms: Epithelial barrier defects; T-cell and B-cell defects; hyperinflammatory and autoinflammatory disorders; phagocytic defects; immunoregulation defects; and others[11]. For example, mutations in the IL-10 pathway could lead to neonatal or infantile VEO-IBD with severe enterocolitis and crissum disease by impairing IL-10-

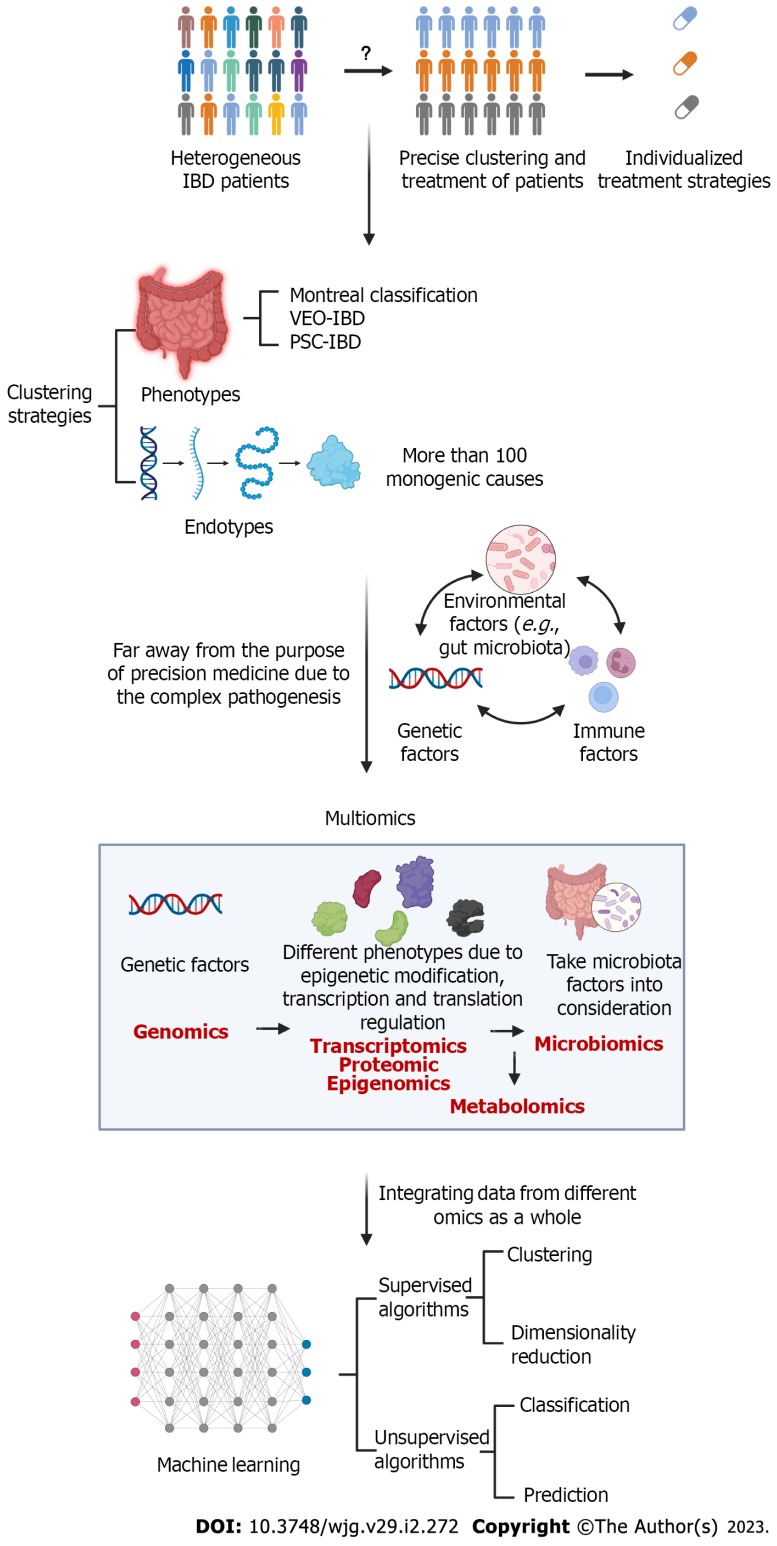

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Figure 1 Flow gram of the precision management strategies of inflammatory bowel disease. IBD: Inflammatory bowel disease; PSC: Primary sclerosing cholangitis; VEO: Very early onset.

mediated control of inflammatory responses involving IL-1 and IL-23. Mutations in *CYBB* could lead to chronic granulomatous disease characterized by intestinal inflammation and autoimmune disease due to impaired antimicrobial activity caused by defects in NADPH oxidase[12,13]. Conventional treatment in patients with the subsets of IBD that are largely driven by genetic factors often exhibit unsatisfactory efficacy, and these patients have poor prognosis. These various mechanisms that underlie the effects of monogenic mutations also have some crossover with the mechanisms involved in sporadic and multifactorial IBD, which reflects the divergence and convergence of the mechanisms. For precision treatment of IBD, strategies should not be limited to therapies targeting upstream etiology; therapy

based on more superficial mechanisms should also be pursued.

The etiology of monogenic causes, which account for only a small percentage of IBD cases, is complex, but sporadic IBD involves many more factors. More than 260 risk loci have been identified to be associated with sporadic IBD by genome-wide association studies (GWASs)[14], yet these loci only explained approximately 20% of the genetic heritability in complex adult-onset IBD[15]. This finding is easy to understand because there are also environmental, microbiota or other factors involved in the pathogenesis of IBD. IBD cannot be classified by a single factor, but the application of biomarkers can aid in the advancement of the precision management of IBD to some extent.

BIOMARKERS

Due to the rising incidence of IBD and the inconvenience of endoscopy, there is an urgent need for noninvasive, accessible and cost-efficient biomarkers. Precision medicine should cover the whole management process of IBD patients, including the early identification of patients at potential risk for disease progression and enabling appropriate adjustments in response to ongoing assessments of treatment efficacy. Such a strategy should be a highly sophisticated process, not just the endpoint of a single stratification approach[16]. Accordingly, we reviewed two categories of biomarkers (mainly from serum and feces) for IBD: Those used to identify disease progression risk and activity and those used to predict treatment responses.

Identifying disease progression risk and activity

C-reactive protein (CRP) is the most widely used serum biomarker for inflammation in IBD[17]. It reflects both clinical disease activity and endoscopic inflammation in IBD patients[18]. Additionally, the level of CRP is not influenced by treatments and thus is also suitable for monitoring treatment response [19]. However, it is not a specific biomarker, and its levels might be elevated in other diseases, including noninflammatory conditions. Additionally, up to 25% of Crohn's disease (CD) patients with endoscopically proven activity could not be identified by CRP[20]. Fecal calprotectin (FC) is another important noninvasive biomarker widely used in clinical practice. In the assessment of endoscopically defined disease activity in IBD, FC analysis exhibits higher sensitivity in the context of both ulcerative colitis (UC) and CD, especially in UC[21,22]. In particular, FC analysis could be used in the early prediction of relapse risk 6 and even 12 mo in advance[23]. A meta-analysis reported 78% sensitivity and 73% specificity when using FC at remission to predict IBD relapse, with cutoffs varying between 120 μg/g and 340 μg/g[24]. However, this biomarker also faces the problem of limited specificity; inflammation in the gut that is not associated with IBD, such as during infection, necrotizing enterocolitis and drug-induced enteropathy, could confound the results[25]. For patients with borderline FC levels, combining FC analysis with other metrics, such as clinical activity indices or CRP levels, could help the assessment [26]. The levels of serum calprotectin (SC), as an indirect marker of inflammatory activity in UC[27], can indicate the involvement of other extraintestinal organs. Another well-accepted fecal biomarker is fecal lactoferrin (FL), the levels of which could also reflect IBD activity and be used to predict disease relapse. Unlike the analysis of FC levels, the advantage of FL is its specificity[28], and combined analysis of these biomarkers might result in better assessment. In addition, some secondary biomarkers measured using simple laboratory tests, such as the CRP-albumin ratio, neutrophil-lymphocyte ratio (NLR), platelet-lymphocyte ratio (PLR) and lymphocyte-monocyte ratio (LMR), can also be used to infer the activity of IBD[29,30].

In recent years, an increasing number of novel serum and fecal biomarkers with application potential have been discovered. The levels of leucine-rich glycoprotein, a glycoprotein that is also related to IBD activity, could be elevated in patients with normal CRP levels during the active period of UC[31]. Some serum antibodies resulting from autoimmunity and loss of immune tolerance to microbial antigens have been considered in the diagnosis and assessment of IBD[32]. For example, anti-*Saccharomyces cerevisiae* antibody (ASCA) and perinuclear anti-neutrophil cytoplasmic antibody (pANCA), which are antibodies of microbial antigens and autoantibodies, respectively[33], are two extensively studied antibodies with high specificity for IBD[34]. They could help identify potential CD patients five years before diagnosis when combined with the analysis of other protein markers[35]. In addition to enabling diagnosis, a higher ASCA titer was related to more aggressive fibrosis and stenosis and internal penetrating disease behaviors[36], while the pANCA titer changed with the activity of UC[37]. In addition, cytokines such as granulocyte colony-stimulating factor were associated with endoscopically active disease[38], while IL-6 and IL-2 Levels could also be used to predict the course of disease relapse 12 mo in advance in quiescent CD patients[39]. Circulating noncoding RNAs, including microRNAs (miRNAs) and long ncRNAs (lncRNAs), also play a role in IBD, and the analysis of miRNAs might help monitor disease activity and stricture phenotypes[32]. Other newly emerging biomarkers for disease progression risk and activity include cathelicidin[40], trefoil factor 3[41], and 25-hydroxyvitamin D3[42]. Several extracellular matrix (ECM) components and growth factors are important biomarkers indicating intestinal fibrosis and stenosis[32]. The analysis of fecal biomarkers, cytokines and other indicators of inflammation could also help with the identification of IBD activity[43]. Fecal myeloperoxidase, another

biomarker related to neutrophil inflammation in addition to FC, was recently reported to accurately indicate endoscopic activity in IBD and predict the disease course during follow-up[44]. However, all these novel biomarkers are mainly used in research and remain far from clinical use (Table 1).

Predicting treatment responses

The mainstream therapeutic drugs for IBD include aminosalicylates (ASAs), glucocorticoids (GCs) and immunosuppressive agents[45]. The treatment of IBD has greatly advanced since the recent introduction of biologics, including tumour necrosis factor-α (TNF-α) inhibitors (such as infliximab and adalimumab), integrin inhibitors (such as vedolizumab and etrolizumab) and IL-12/IL-23 inhibitors (such as ustekinumab). However, selecting among these therapies largely depends on the clinical characteristics, comorbidities, and patients' preferences or concerns, and this decision-making process lacks a uniform objective standard for indicating the path of treatment. Thus, effective biomarkers for predicting treatment responses are urgently needed.

For 5-ASA, a multicenter prospective cohort study in pediatric UC patients developed a predictive model with initial clinical activity and early treatment response to 5-ASA to predict long-term corticosteroid-free remission[46]. The baseline FC level and UC endoscopic index of severity could be used to predict the early outcome of GCs treatment[47].

Clinical responses to biologics are even more varied. However, the application of biologics is usually tried in a certain order by experienced physicians without effective biomarkers used to influence the selection of different kinds of biologics, which is of concern in research. Some laboratory test results are taken into consideration. For example, a clinical trial revealed that using CRP and FC levels in combination with clinical symptoms could result in better clinical and endoscopic outcomes than considering only clinical symptoms[48]. These downstream indicators of active inflammation could suggest a response to anti-inflammatory TNF-α inhibitors, but they are still not enough for accurate prediction of the likelihood of remission in a given patient in a real clinical environment[49]. Thus, we need to explore more biomarkers that could reveal the molecular heterogeneity of patients treated with different biologics.

Existing biologics can be briefly classified into two groups according to underlying mechanisms: inhibitors of cytokines and inhibitors of lymphocyte migration. TNF-α is considered to be a downstream inflammatory pathway effector in multiple immune-related diseases[50]. It is rational to speculate that IBD patients with increased TNF-α levels might have a good response to anti-TNF-α agents. Detecting membrane-bound TNF (mTNF) by endoscopy with the aid of fluorescence labeling has been used to successfully predict the efficacy of anti-TNF-α treatment[51]. Another study also reached this goal by measuring the TNF production capacity of peripheral blood mononuclear cells (PBMCs)[52]. A lack of response to anti-TNF-α therapy might indicate the activation of other inflammatory pathways. Baseline levels of serum oncostatin M, a member of the IL-6 family that might mediate inflammation in another manner, have been reported to be elevated in anti-TNF-α nonresponders and could be used to predict the efficacy of this treatment[53,54]. In addition, low triggering receptor expressed on myeloid cells 1 (TREM1) expression in both whole peripheral blood samples and intestinal biopsy samples, which indicated a complete macrophage autophagy pathway, could be used to predict a good anti-TNF response in IBD patients[55]. Antibodies including anti-drug antibodies (ADA), pANCA and anti-OmpC (*Escherichia coli* outer membrane porin) were also found to be associated with the response to infliximab[56]. In the aspect of relapse after discontinuation of anti-TNF therapy, mucosal TNF gene expression and IL1RL1-transcripts might play a role[57].

The IL23/Th17 pathway is also a central cytokine pathway involved in IBD in addition to TNF-α. The levels of IL-22 and IL-17, the downstream factors involved in this pathway, are potential molecular predictors of the response to IL-23 blockers[58]. Another category of biologics for IBD is integrin inhibitors, which act by inhibiting gut-selective lymphocyte homing. The most widely used integrin inhibitor, vedolizumab, works by blocking the binding of the α4β7 integrin heterodimer on lymphocytes to MAdCAM1 on the gut[59]. Higher expression levels of α4β7 on T, B, and NK cells as well as the presence of $\alpha4\beta7^+$ intestinal mucosa cells could be used to predict responses to vedolizumab[60,61]. However, the predictive role of serum α4β7, VCAM-1 and ICAM-1 remains controversial[62,63]. In addition, higher IL-6 and IL-8 Levels have been reported to be associated with the response to vedolizumab[64] (Table 2).

Sometimes, simply by assessing early responses to biologics, we can judge the potential future efficacy to some extent[65]. Therapeutic drug monitoring (TDM) is another tool for the assessment of biologic therapeutic outcomes based on the findings that drug concentrations correlate with biologic efficacy. However, due to the long time required for detection and the lack of an instructive reference range, there is still no consensus for the use of TDM[66,67].

FUTURE OF PRECISION MANAGEMENT IN IBD: MULTIOMICS

In addition to simple serum and fecal biomarkers, emerging high-throughput analytical technologies offer opportunities for the improved management of IBD. Omics strategies, often including genomics,

Table 1 Biomarkers of serum or feces identifying disease progression risk and activity

Sample	Biomarker	Outcome	Characteristic
Serum	CRP[17-20]	Monitor disease activity and mucosal healing	Widely used and low-cost; lack of specificity for intestinal inflammation; relatively low sensitivity
	SC[27]	Disease burden, prognosis, and relapse	More representative of systemic inflammation
	LRG[31]	Monitor disease activity and mucosal healing	More correlated to activity in UC than CRP
	Serum antibodies		
	ASCA[35,36]	More aggressive fibro stenosing and internal penetrating disease behaviors	CD specificity
	pANCA[35,37]	UC disease activity	UC specificity
	Cytokines		
	G-CSF, IL-1Ra, PDGF-BB[38]	Endoscopically active disease	-
	IL-6, IL-2[39]	Predict disease relapse in quiescent CD	-
	Noncoding RNAs[32]	Monitor disease activity and stricture phenotypes	-
	ECM components[32]	Intestinal fibrosis and stenosis	-
	Growth factors[32]	Intestinal fibrosis and stenosis	-
	Cathelicidin[40]	Mucosal disease activity in UC, risk of intestinal stricture in CD, and clinical prognosis in IBD	-
	Trefoil Factor 3[41]	Monitor disease activity	-
	Vitamin D[42]	Disease activity	-
	Secondary biomarkers (CRP-albumin ratio, NLR, PLR, LMR)[29,30]	IBD activity	Easy to obtain; fluctuates greatly
Feces	FC[21-26]	Monitor disease activity and mucosal healing; early prediction of relapse risk	Higher sensitivity than CRP; confounding of non-IBD gut inflammation
	FL[28]	Monitor disease activity and predict disease relapse	Higher specificity than fecal calprotectin
	MPO[44]	Endoscopic activity in IBD and predict the disease course during follow-up	-

ASCA: Anti-*Saccharomyces cerevisiae* antibody; CD: Crohn's disease; CRP: C-reactive protein; ECM: Extracellular matrix; FC: Fecal calprotectin; FL: Fecal lactoferrin; G-CSF: Granulocyte colony-stimulating factor; IL-1Ra: Interleukin 1 receptor antagonist; IBD: Inflammatory bowel disease; LMR: Lymphocyte-monocyte ratio; LRG: Leucine-rich alpha-2 glycoprotein; MPO: Myeloperoxidase; NLR: Neutrophil-lymphocyte ratio; pANCA: Perinuclear anti-neutrophil cytoplasmic antibody; PDGF-BB: Platelet-derived growth factor BB; PLR: Platelet-lymphocyte ratio; SC: Serum calprotectin; UC: Ulcerative colitis.

transcriptomics, proteomics, metabolomics, epigenomics and microbiomics, have completely transformed the trajectory of medicine. These different omics approaches might also provide some insights into IBD from different perspectives.

Genomics

Genomics aims to characterize and quantify all the genetic information of an organism. Numerous variations in factors of genetic susceptibility involved in many complex diseases have been identified. Different genes have been reported to associate with IBD severity and activity. *NOD2/CARD15* is the most classic CD-related gene found in Western countries[68,69] and is also associated with stricturing behaviors and the need for operation[70,71]. However, it was not found to be related to CD development in East Asian cohorts[72]. Regarding UC, a GWAS developed a risk score based on 46 single nucleotide polymorphisms to identify medically refractory UC that needed colectomy[73]. Regarding therapeutic efficacy, HLA-DQA1*05 carriage was reported to be associated with ADAs for TNF-α inhibitors in CD and suggested the need for combination therapy[74]. In addition, polymorphisms in *TLR2*, *TLR4*, *TLR9*, *TNFRSF1A*, *IFNG*, *IL6*, *IL1B*, *TNF-α* and apoptosis-associated genes (Fas ligand and caspase-6) were also potential genetic biomarkers for the anti-TNF treatment response[75-77]. HLA-DQA1-HLA-DRB1, nudix hydrolase 15 and thiopurine-S-methyltransferase variants were

Table 2 Biomarkers predicting treatment responses

Type of agent	Biomarker	Sample	Outcome
5-ASA	Initial clinical activity, early treatment response of 5-ASA[46]	-	Predict long-term corticosteroid-free remission
GCs	FC, UCEIS[47]	Feces	Predict the early outcome of GCs treatment
Biologics			
TNF-α inhibitors	mTNF[51]	Endoscopy	Predict anti-TNF-α efficacy
	TNF production capacity of PBMCs[52]	Blood	Predict anti-TNF-α efficacy
	OSM[53,54]	Serum	Upregulate in anti-TNF-α non-responders
	TREM1 expression[55]	Blood and intestinal biopsies	Predict anti-TNF-α efficacy
	Antibodies: ADA, pANCA, anti-OmpC [56]	Serum	Associate with the response to infliximab
	Mucosal TNF gene expression and IL1RL1- transcripts[57]	Intestinal biopsies	Predict long-term remission after discontinuation of anti-TNF-α therapy
IL-23 inhibitors	IL-22, IL-17[58]	Serum	Predict anti-IL-23 efficacy
Integrin inhibitors	α4β7 on T, B, and NK cells[60,61]	Blood and endoscopy	Predict responses to vedolizumab
	α4β7, VCAM-1, ICAM-1[62,63]	Serum	Remain controversial
	IL-6, IL-8[64]	Serum	Associate with the response to vedolizumab

ADA: Anti-drug antibodies; ASA: Aminosalicylates; FC: Fecal calprotectin; GCs: Glucocorticoids; ICAM-1: Intercellular adhesion molecule-1; mTNF: Membrane-bound tumour necrosis factor; OmpC: *Escherichia coli* outer membrane porin; OSM: Oncostatin M; pANCA: Perinuclear anti-neutrophil cytoplasmic antibody; PBMC: Peripheral blood mononuclear cell; UCEIS: Ulcerative Colitis Endoscopic Index of Severity; VCAM-1: Vascular cell adhesion molecule-1; TREM1: Triggering receptor expressed on myeloid cells 1.

found to be associated with thiopurine-related adverse events[53]. However, the utility of genomics alone is limited due to the complexity of IBD pathogenesis.

Transcriptomics/proteomics/epigenomics: From genes to phenotypes

In exploring the pathogenesis of complex diseases such as IBD, the limitations of using a single genomics approach are becoming increasingly apparent. The same gene variation might lead to distinct phenotypes by epigenetic modification and transcriptional and translational regulation. Transcriptomics, proteomics and epigenomics could better reflect the gene expression profiles, which combine genetic and environmental factors and thus perform better than simple genomics.

In particular, numerous transcriptomic studies have provided insights into the prediction of IBD progression. Researchers found that the expression of ECM accumulation-associated genes in the ileum was associated with stricturing behaviors in pediatric CD patients. After combining age, race, disease location, and antimicrobial serology factors, they established a competing-risk model that reached a specificity of 71%[78]. However, ileum samples are difficult to access, which poses a barrier to the utility of this approach. Studies on blood samples are thus warranted. Transcriptional profiling of circulating $CD8^+$ T cells successfully distinguished patients with a risk of aggressive disease mainly based on the expression of genes involved in T-cell responses[79]. Furthermore, this classification was also feasible for use in whole blood samples with transcriptional signatures based on 17 genes[80]. The transcriptional risk score, which represented the summation of risk alleles for CD from ileum or blood samples, could be used to identify patients who would progress to complicated disease[81]. Regarding treatment, UC patients could be clustered into different groups with distinct transcriptomic profiles of the rectum and showed different responses to anti-TNF therapy[82].

Unlike transcriptomics, the use of proteomics in the context of IBD is still in its infancy. Some studies have sought to detect proteins involved in early inflammatory mechanisms of IBD, and some proteome analyses have been performed in studies with small sample sizes to investigate the differentiation of disease behavior as well as the prediction of response to biological treatment[83,84]. In the Proteomic Evaluation and Discovery in an IBD Cohort of Tri-service Subjects (PREDICTS) study, a series of protein biomarkers involved in the complement cascade, lysosomes, innate immune response, and glycosaminoglycan metabolism along with some antibodies were able to be used to predict potential CD patients 5 years in advance[35]. Another study revealed that MMP10, CXCL9, CXCL11, and MCP1 were upregulated in UC patients before disease onset[85]. However, there is still a long way to go before these approaches are applied clinically.

Epigenetic mechanisms mainly include DNA methylation, histone modifications, and noncoding RNAs. The complement of methylated DNA in the genome is called the methylome. Epigenetic alterations have been detected when IBD patients were compared with healthy individuals[86]. The number of epigenomic studies investigating IBD subgroup identification is still limited. The earliest finding observed in this area involved the assessment of cancer risk in the context of UC[87]. In future research, epigenomics studies might provide useful biomarkers for the early detection of cancer development in UC patients.

Microbiomics: Perspective of the environment

All of the above strategies provide omics analysis of the host. As mentioned previously, the gut microbiota, as an environmental factor, also plays an important role in the pathogenesis of IBD. Due to the convenience of fecal sample collection, microbiomics is promising for monitoring and managing IBD patients. The microbiota might also be able to be used to predict relapse risk. For example, a deficit in some bacterial groups or species, such as *Faecalibacterium prausnitzii* and *Bacteroides*, could be a predictive factor for relapse of CD after ileal resection or infliximab cessation[88,89]. Another study revealed that *Ruminococcus* and *Veillonella* were associated with stricturing and penetrating complications, respectively[78]. A recent prospective study classified CD patients into different subgroups with different clinical relapse risk based on microbiota[90]. Additionally, microbial analysis revealed distinct microbiota compositions between patients with different responses to anti-TNF-α therapy[84,91] as well as anti-integrin therapy[92]. Furthermore, manipulation of the microbiota might be a direction for IBD treatment.

However, this method is easily influenced by environmental factors such as diet and confounded by the causal relationship between microbiota and IBD; thus, its reliability is questionable. Recent findings are still at a superficial stage of providing simple differences in microbial abundance, and there has been a lack of in-depth analysis of microbial networks and microbiota-host interactions, as well as solid and effective prediction models.

Metabolomics

Metabolomics generally includes serum and fecal metabolomics. As a combination of host metabolic factors and environmental gut microbiota factors, it is also a potential technique for use in future IBD research and clinical practice. Several studies have applied metabolic profiling for the diagnosis and classification of IBD[32]. A serum metabolomics study identified altered lipid and amino acid metabolism in parallel with CD activity[93]. Short-chain fatty acids (SCFAs) have been widely validated to be beneficial metabolites[94], among which butyrate is one of the most important. Studies have confirmed that fecal SCFA levels were reduced during active IBD[95]. Butyrate levels were associated with the efficacy of azathioprine, TNF-α inhibitors and integrin antibodies[92,96,97]. Other metabolites, such as bile acids and tryptophan, are also worth studying for future use[94].

APPLICATION OF ARTIFICIAL INTELLIGENCE FOR INTEGRATED OMICS

Due to the complexity of IBD pathogenesis, interpretation of a single set of omics data often fails to provide insight into complex biological phenomena; thus, the omics approaches discussed above must be considered as a whole. Integrating multiple omics strategies into a network would contribute to the elucidation of the pathway involved in pathogenesis and facilitate the identification of different subgroups and the optimization of therapy regimens in IBD. The analysis of different molecules, including at the genomic, transcriptomic, proteomic, microbiome, epigenetic and metabolomic levels, could be performed simultaneously, and the results could be further integrated into multiomics models [98]. By this approach, we could obtain more insight into disease pathogenesis, identify more promising predictive biomarkers and facilitate early diagnosis[99]. Some multiomics projects are ongoing and are investigating IBD heterogeneity to improve precision management[53].

These high-throughput data need to be modeled by AI algorithms with the aid of advanced computational techniques. Machine learning is a subset of AI where machines can learn from experience provided by the data without the need for programming. Machine learning includes supervised and unsupervised algorithms. Supervised algorithms are often used for classification or prediction using example data, while unsupervised algorithms are often used for clustering according to similarity[100]. These approaches could be well applied to address the need for patient clustering and predictions and the detection of novel biomarkers. Progress made in machine learning has benefited the integrated analysis of multiomics data; these strategies mainly include concatenation-, model- and transformation-based methods[101]. In addition, deep neural networks have been used in the integration of multiomics data for the prediction of drug efficacy in cancer therapy[102], which indicates progress may be made in the context of IBD.

However, due to the obscure nature of machine learning, the robustness of the models established is sometimes uncertain. Thus, testing in independent cohorts and even clinical trials are needed before this approach is employed in a clinical setting. Additionally, products that are easy to implement in clinical

settings need to be developed from research.

CONCLUSION

The pathogenesis of IBD remains uncertain, which challenges the clustering and precision management of patients. Genetic, environmental and immune factors are all involved in the complex process of IBD development. Thus, the future direction of IBD management may largely rely on the development of multiomics analysis. Numerous data processing workflows require the help of AI.

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REVIEW

Screening and interventions to prevent nonalcoholic fatty liver disease/nonalcoholic steatohepatitis-associated hepatocellular carcinoma

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Abstract

Liver cancer is the sixth most commonly diagnosed cancer worldwide, with hepatocellular carcinoma (HCC) comprising most cases. Besides hepatitis B and C viral infections, heavy alcohol use, and nonalcoholic steatohepatitis (NASH)-associated advanced fibrosis/cirrhosis, several other risk factors for HCC have been identified (*i.e.* old age, obesity, insulin resistance, type 2 diabetes). These might in fact partially explain the occurrence of HCC in non-cirrhotic patients without viral infection. HCC surveillance through effective screening programs is still an unmet need for many nonalcoholic fatty liver disease (NAFLD) patients, and identification of pre-cirrhotic individuals who progress to HCC represents a substantial challenge in clinical practice at the moment. Patients with NASH-cirrhosis should undergo systematic HCC surveillance, while this might be considered in patients with advanced fibrosis based on individual risk assessment. In this context, interventions that potentially prevent NAFLD/NASH-associated HCC are needed. This paper provided an overview of evidence related to lifestyle changes (*i.e.* weight loss, physical exercise, adherence to healthy dietary patterns, intake of certain dietary components, *etc.*) and pharmacological interventions that might play a protective role by targeting the underlying

causative factors and pathogenetic mechanisms. However, well-designed prospective studies specifically dedicated to NAFLD/NASH patients are still needed to clarify the relationship with HCC risk.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Hepatocellular carcinoma; Risk stratification; Lifestyle interventions; Prevention

Core Tip: Nonalcoholic fatty liver disease (NAFLD) is a public health problem, especially in developed countries. This condition, depending on certain associated risk factors, can ultimately lead to liver cirrhosis and hepatocellular carcinoma (HCC). Having the necessary tools and knowing the characteristics of patients in whom the disease progresses more quickly, effective monitoring programs can be developed. Primary prevention of NAFLD/nonalcoholic steatohepatitis (NASH)-associated HCC relies on controlling the main modifiable risk factors. Some pharmacological (*e.g.*, metformin, statins, aspirin) and non-pharmacological interventions (weight loss, physical exercise, healthy diet, avoiding heavy drinking and smoking) might have protective effects. Herein, we emphasized the need for continued investigations to find the optimal methods for NAFLD/NASH-associated prevention.

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INTRODUCTION

Primary liver cancer was estimated to be the fourth leading cause of cancer-related deaths and the sixth most commonly diagnosed cancer in 2018 worldwide, with hepatocellular carcinoma (HCC) comprising most cases (75%-85%)[1]. The main risk factors for HCC are chronic infection with hepatitis C virus (HCV) and hepatitis B virus (HBV), and non-viral factors, such as heavy alcohol drinking or nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) and associated metabolic disorders [like type 2 diabetes mellitus (T2DM), obesity], which have emerged as important determinants of the disease[2]. In fact, while the incidence and mortality rate related to viral-associated HCC is decreasing lately, NAFLD/NASH has become a major cause of cirrhosis and HCC[3]. This is relevant considering the increased prevalence of NAFLD, which affects about one-quarter of the adult population worldwide[4]. Even if the HCC risk is lower in NAFLD patients compared to the HCV-infected patients, it is still seven times higher in comparison with the general population[5,6].

Besides NASH/advanced fibrosis and cirrhosis, several other risk factors for HCC have been identified (Figure 1). Among these, T2DM appears to be strongly and independently associated with both NAFLD/NASH and HCC[7]. In fact, the prevalence of NAFLD in patients with T2DM is twice as high, and the prevalence of NASH is about seven-ten times higher, while the risk of HCC is 2.0-2.5 fold higher than in the general population[4,8,9]. Some studies suggest that a longer duration of diabetes can increase the risk of HCC[10]. The underlying mechanisms that link T2DM to HCC are complex and not fully elucidated, but insulin resistance, chronic inflammation, lipotoxicity, and oxidative stress may play a substantial role by promoting DNA damage, angiogenesis, cellular growth and proliferation, and decreasing cellular apoptosis[11-13]. In fact, insulin resistance seems to play an important role in HCC development (through associated proinflammatory, vasoactive, and pro-oxidative environment), and it might explain in part the occurrence of HCC in non-cirrhotic NAFLD patients[6,13].

Although not unanimous, the overall evidence is suggestive of an increased risk of HCC in individuals with obesity [as evaluated by the body mass index (BMI)][7,14,15]. A case-control study performed in the United States has identified obesity in early adulthood (mid-20s to mid-40s) as a significant HCC risk factor [odds ratio (OR): 2.6, 95% confidence interval (CI): 1.4-4.4], as each unit of increased BMI was associated with a 3.89-mo decrease in age of HCC diagnosis ($P < 0.001$)[16]. Moreover, obesity in childhood (ages 7-13-years-old) was reported to be associated with higher HCC risk later in life in a retrospective cohort comprising 285884 Danish children[17]. Visceral obesity appears to be particularly significant as an HCC risk, regardless of BMI[18,19]. The gut-liver axis seems to play an important role in the obesity-associated HCC, as gut microbiota creates a tumor-promoting microenvironment by transferring its metabolites/components, which further trigger the release of proinflammatory cytokines (like tumor necrosis factor alpha, interleukin-1β, interleukin-6, *etc.*), suppress the anti-tumor immunity, and modify the bile acid metabolism[20-22].

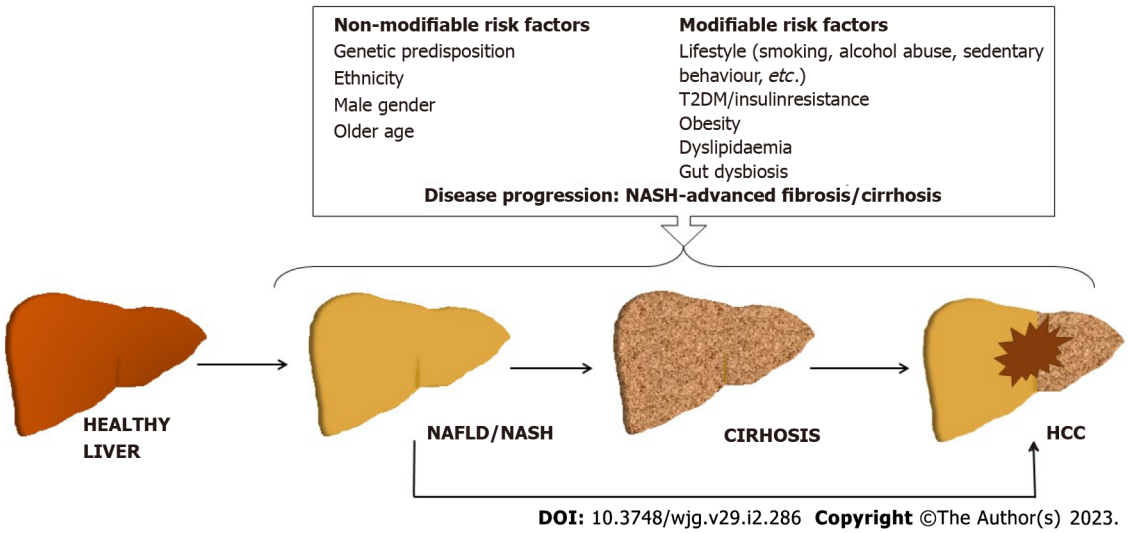

Figure 1 Risk factors for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis -associated hepatocellular carcinoma. HCC: Hepatocellular carcinoma; NAFLD: Nonalcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; T2DM: Type 2 diabetes mellitus.

Other risk factors for NAFLD-related HCC are male sex, older age, smoking, genetic predisposition (*i.e. PNPLA3* polymorphism, *etc.*) (Figure 1)[6,23-27]. In addition, dyslipidemia, ethnicity, intestinal dysbiosis, and sedentary lifestyle may also contribute (Figure 1)[3,6,26,28-30]. Apparently, the presence of multiple risk factors increases the risk of liver cancer synergistically[6].

In fact, multiple hits drive the development of the NAFLD/NASH-associated HCC through activation of various metabolic, endocrine, and immunological pathways (*i.e.* increased free fatty acids levels/impaired lipid metabolism, hyperinsulinemia, oxidative stress, endoplasmic reticulum stress, and hyperleptinemia and increased production of proinflammatory cytokines, altered immune response, release of pro-fibrinogenic mediators, *etc.*) on a background of genetic/epigenetic alterations [31].

RISK STRATIFICATION AND PREDICTION

The goal of HCC surveillance in NAFLD/NASH patients is to reduce the HCC-related mortality by promoting early tumor detection[32]. Controversy still exists regarding which NAFLD patients would benefit most from the HCC surveillance[6]. In NAFLD patients, the risk of liver-related and all-cause mortality raises exponentially with higher fibrosis stage (from F1 to F4)[33]. The meta-analysis by Dulai *et al*[33] indicated that compared to F0, the rate ratio of the all-cause mortality was 1.58 (in stage 1), 2.52 (in stage 2), 3.48 (in stage 3), and 6.40 (in stage 4), and the same trend was seen for liver-related mortality. However, it should be noted that the evolution of fibrosis is not linear, as it progresses and regresses in about 20%-30% of patients over 5 years[34]. Among patients with NAFLD, those with cirrhosis are at greatest risk, with an annual HCC incidence rate of 10.6/1000 person-years (PY) compared to 0.08/1000 PY in patients without cirrhosis[5]. Furthermore, HCC incidence rates are higher in patients with decompensated cirrhosis than in those with compensated cirrhosis[28].

Nevertheless, NAFLD patients without cirrhosis are still at risk of developing HCC. The analysis of data obtained from a cohort of 1500 patients with HCC showed that about 13% of them did not have cirrhosis, and patients with NAFLD had a five-fold increased risk of developing HCC in the absence of cirrhosis compared with those with HCV-related HCC[35]. A lower proportion of patients with NAFLD-associated HCC presented cirrhosis than patients with HCV- or alcohol-related HCC (58% *vs* 85.6% and 72.4%, respectively)[35]. The same was basically shown by the meta-analysis by Tan *et al*[36] (61 studies; 94636 patients). They reported that NAFLD-related HCC patients were more likely to be non-cirrhotic (38.5% *vs* 14.6%, $P < 0.0001$) and had larger tumor diameters ($P = 0.0087$)[36]. Moreover, these patients had undergone surveillance in a lower proportion than patients with HCC secondary to other causes (32.8% *vs* 55.7%, $P < 0.0001$)[36].

Poor HCC surveillance is a significant problem for patients with NAFLD, and in fact, identification of pre-cirrhotic NAFLD individuals with high HCC risk remains a significant challenge at the moment. A prospective multicenter study performed in Italian secondary care centers that included 756 patients with NAFLD- or HCV-related HCC has shown that HCC was diagnosed though regular ultrasound/specific surveillance in a lower proportion of NAFLD patients compared to HCV patients (47.7% *vs* 63.3%, $P < 0.0001$), resulting in a more advanced HCC burden at diagnosis in the former group[37]. Similarly, the analysis of data from the United States Veterans Administration HCC cohort study

showed that more patients with NAFLD-HCC did not benefit from HCC surveillance 3 years before diagnosis (43.3%) compared to patients with alcohol abuse- or HCV-related HCC (40.2%, and 13.3%, respectively)[38].

Solid data and guidance regarding risk stratification in non-cirrhotic NAFLD patients who might benefit from HCC surveillance are limited, and specific recommendations in this area are urgently needed due to the growing epidemic of NAFLD[39].

How to perform the screening?

Liver biopsy remains the "gold standard" for the diagnosis of NASH, but it cannot be routinely used in practice as a screening method to diagnose NAFLD, given its multiple limitations: It is expensive, the procedure is subject to interpretation errors, and it is potentially associated with adverse effects such as pain, bleeding, and infection[40,41]. The emergence of non-invasive methods for quantifying fibrosis and their validation has led to a decrease in the need for liver biopsies[42-44]. The Asia-Pacific and the American Gastroenterology Association guidelines agree that the combined use of serum tests and imaging tools may provide more reliable information than using either method alone[30,45]. The American Association for the Study of Liver Diseases guideline also consider the non-invasive methods as first-line tests for the investigation of fibrosis, but it does not recommend a specific diagnostic algorithm or follow-up strategies[43].

Identification of fibrosis and risk stratification is an essential step for HCC surveillance, as the guidelines clearly recommend screening in patients with cirrhosis, while patients with advanced fibrosis (F3) might also undergo surveillance based on individualized evaluation.

Currently, the primary imaging method for HCC detection is ultrasound (US)[40,46,47]. However, recent studies have highlighted the limitations of this examination[28]. For example, a study comprising 941 patients with cirrhosis who underwent ultrasonography reported that 20% of examinations had an inadequate quality to exclude images showing possible focal points[48]. Therefore, other methods (computer tomography, magnetic resonance imaging) might be used[45,47]. For these two investigations, the follow-up interval is not clearly established nor are the benefits of the association with measurement of alpha-fetoprotein (AFP) levels[49].

It has been questioned whether the addition of AFP quantification to routine biannual US examinations would increase the detection rate of HCC during screening. However, only about three-quarters of HCC patients are AFP positive[50]. In fact, the probability of having elevated AFP levels (*i.e.* > 10 ng/mL) in patients with early NAFLD-associated HCC without cirrhosis and normal transferase levels was only 17.5%-24.0% compared with 86.5%-90.5% in patients with viral-associated cirrhosis and advanced HCC with increased transaminases values, as showed in an Italian study that included 4123 HCC patients[51]. The use of AFP measurement as a screening tool for HCC might result in earlier diagnosis, but it does not seem to improve the mortality rate[52]. The European guidelines do not currently recommended AFP as a surveillance parameter for the detection of HCC in patients with NAFLD. The American guidelines recommend ultrasound screening with or without AFP, while Asia Pacific Society guidelines recommend screening with AFP[30,47,53]. Other biomarkers such as microRNAs (*e.g.*, miR-34a, miR-221) are under investigation for early detection of HCC but need further validation[54].

HCC screening in low risk NAFLD-patients

Most patients screened in a primary care setting have a low risk of clinically significant liver fibrosis, defined as having a Fibrosis-4 (FIB-4) score < 1.3, liver stiffness measurement (LSM) < 8.0 kPa on transient elastography, or a liver biopsy fibrosis stage of F0–F1[44,55-57]. Systematic HCC screening may not be prudent and is currently not recommended by the American Association for the Study of Liver Diseases, American Gastroenterology Association, and the European Association for the Study of the Liver in non-cirrhotic NAFLD patients (Figure 2)[42-44].

HCC screening in indeterminate risk-NAFLD patients

An estimated 30%–40% of screened patients have an indeterminate risk of clinically significant (advanced) liver fibrosis, defined as FIB-4 score of 1.3-2.67 and/or a LSM of 8.0-12.0 kPa on transient elastography[44,55-57]. The estimated incidence of HCC in non-cirrhotic NASH seems to be too low to justify systematic screening[58]. Apparently, non-cirrhotic NAFLD patients with multiple features of metabolic syndrome are at higher risk of HCC development and need special attention[5,35]. It is argued that additional triggers (such as active inflammation and fibrosis) are needed to promote carcinogenesis[6].

The current guidelines recommend the referral of these patients to a hepatologist for further evaluation by magnetic resonance elastography or liver biopsy[30,42,43]. The decision should be taken by mutual agreement and on the basis of an individual assessment (presence/absence of comorbidities, degree of fibrosis, *etc.*)[44]. The European Association for the Study of the Liver guidelines recommend that patients with liver disease and advanced fibrosis (F3) might be considered for HCC surveillance based on the individual risk, while the Asian Pacific and American Association for the Study of Liver Diseases clinical practice guidelines do not provide a specific recommendation for surveillance in

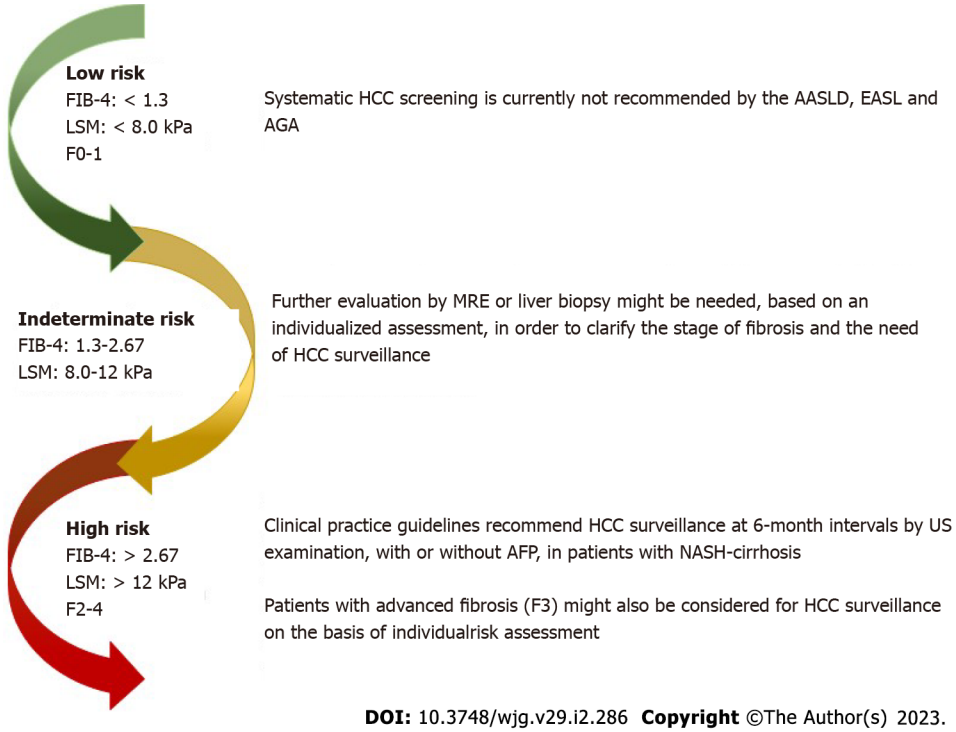

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Figure 2 Recommended hepatocellular carcinoma screening approach in nonalcoholic fatty liver disease/nonalcoholic steatohepatitis patients according to the risk category. AASLD: the American Association for the Study of Liver Diseases; AFP: Alpha-fetoprotein; AGA: American Gastroenterology Association; EASL: The European Association for the Study of the Liver; FIB-4: Fibrosis-4; HCC: Hepatocellular carcinoma; LSM: Liver stiffness measurement; NASH: Nonalcoholic steatohepatitis; MRE: Magnetic resonance elastography; US: Ultrasonography.

patients with NAFLD without cirrhosis[30,43].

HCC screening in high risk-NAFLD patients

Nearly 10% of screened patients have a high risk of advanced liver fibrosis, defined as a FIB-4 score > 2.67, LSM > 12.0 kPa, or a liver biopsy showing clinically significant liver fibrosis (≥ F2)[44,55-57]. Patients with cirrhosis are at the highest risk for HCC. The meta-analysis and meta-regression by Orci *et al*[59] involving 470404 patients showed that the incidence rate of HCC was 0.03/100 PY in patients with NAFLD at a pre-cirrhotic stage and 3.78/100 PY in those with cirrhosis, while in patients with cirrhosis undergoing regular screening for HCC, it was 4.62/100 PY.

Some data suggested that HCC surveillance might not be associated with improved clinical outcomes. For example, a matched case-control study from the United States Veterans Affairs health system failed to find an association between screening (by US, AFP, either test, or both tests) and rate of HCC-related mortality[60]. However, the lack of benefit may have not been related to the failure of surveillance but rather to other causes, such as underuse of HCC treatment or applying surveillance in patients who were not candidates for HCC treatment. On the other hand, a meta-analysis of 59 cohort studies indicated that HCC surveillance was associated with improved early HCC detection, receiving curative therapy, and survival in patients with cirrhosis but with heterogeneity in pooled estimates[32]. Thus, available data is in favor of HCC surveillance in patients with cirrhosis, although it still needs further confirmation[32].

Surveillance programs by regular US (and AFP) in patients with compensated cirrhosis are cost effective[61]. In fact, cost-effectiveness analyses indicate that HCC screening should be considered for patients with Child-Pugh A and B (compensated) cirrhosis and decompensated liver cirrhosis patients waiting for liver transplantation[47].

In patients with NASH-cirrhosis, all three liver study societies recommend the use of an HCC surveillance program at 6-mo intervals, with US exams, with or without AFP[30,42,43]. The same was endorsed by the recommendations of American Gastroenterology Association Clinical Practice[40].

INTERVENTIONS TO PREVENT NAFLD/NASH-ASSOCIATED HCC

The NAFLD/NASH-associated HCC primary prevention relies on controlling the main modifiable risk factors, *i.e.* obesity, T2DM/insulin resistance, gut dysbiosis, disease activity/fibrosis (disease progression), that have been associated with activation of various oncogenic pathways finally leading to

hepatocarcinogenesis[62]. There is no clearly effective intervention for HCC prevention available for NAFLD/NASH patients at the moment, although some pharmacological and non-pharmacological approaches might indeed be useful by addressing the predisposing factors/causes and underlying pathogenetic mechanisms (Table 1)[63].

Weight loss and bariatric surgery

Weight loss through lifestyle intervention represents the cornerstone for NAFLD management, as it has been associated with the regression of steatosis, steatohepatitis, and even fibrosis (for > 10% weight loss) [64]. The analysis of two randomized controlled trials (RCTs) indicated that each lost kg was associated with a 7% increase in odds of obtaining NASH resolution without worsening of fibrosis, and a 5% increase in odds of obtaining fibrosis improvement without worsening of NASH[65]. The meta-analysis by Koutoukidis *et al*[66], which included 2588 NAFLD subjects who underwent weight loss interventions (through behavioral programs, pharmacotherapy, or bariatric surgery), indicated that these interventions were associated with the improvement of liver steatosis, histologic NAFLD activity score (NAS) and presence of NASH (OR: 0.14, 95%CI: 0.04-0.49) but not of fibrosis.

Preliminary results from a retrospective analysis of a database containing 72 million unique patients reported that weight loss medications reduced the risk of HCC in obese populations (OR: 0.07), with orlistat and liraglutide showing statistically significant decreases (OR: 0.13, and 0.35, respectively)[67]. In addition, the meta-analysis by Ramai *et al*[68], which included data from major databases, indicated that bariatric surgery was associated with a reduced risk of HCC (pooled unadjusted OR: 0.40), although with high heterogeneity (I2: 79%).

Although there is limited evidence regarding the impact of weight loss on the risk of developing HCC, it is intuitive and reasonable to encourage overweight/obese patients to decrease their weight. Weight loss is associated with the improvement of metabolic health (or indirect outcomes, such as the decrease of insulin resistance, inflammation, oxidative stress, *etc.*), which may translate into liver health benefits[69-72]. However, a recent RCT in patients with NAFLD demonstrated that a modest weight loss (-4 kg) through a calory-restricted diet was accompanied by reduction in transaminases, but the liver steatosis grade or the markers of oxidative stress were not significantly changed in comparison with the controls[73]. Thus, the liver benefits still have to be demonstrated by prospective data, which should clarify the direct effect of weight loss on long-term NAFLD/NASH progression and primary HCC prevention.

The effects of bariatric surgery on liver outcomes might be explained in part by weight loss, although other contributing mechanisms cannot be excluded [*e.g.*, increase in glucagon-like peptide-1 (GLP-1) concentrations after intervention][74]. The meta-analysis by Lee *et al*[75], which analyzed the data of 32 cohort studies (n = 3093 liver biopsies from NAFLD patients with obesity that underwent bariatric surgery) showed that surgical intervention resulted in an absolute percentage BMI reduction of 24.98% (from 48.68 ± 2.92 to 34.2 ± 3.53 kg/m^2). This was accompanied by steatosis resolution in 66% of patients as well as the resolution of inflammation (in 50% of patients), ballooning degeneration (in 76%), and fibrosis (in 40%)[75]. However, 12% of the subjects presented new or worsening fibrosis after the intervention[75].

The more recent meta-analysis by Ramai *et al*[68] included nine studies (19514750 patients) and reported that bariatric surgery was associated with a reduced risk of HCC (pooled unadjusted OR: 0.40, 95%CI: 0.28-0.57 and adjusted OR: 0.63, 95%CI: 0.53-0.75). So far there is no clear indication regarding the type of surgical intervention that would be most beneficial in terms of liver health.

Lifestyle changes

A large prospective cohort study has demonstrated the association of unhealthy lifestyle (assessed by a composite score comprising BMI, alternative Mediterranean diet, alcohol intake, smoking, and sleep duration) with the risk of HCC: Higher composite scores (5, 6, 7, 8) representing healthier lifestyle were associated with a lower risk of HCC (0.67, 0.61, 0.49, and 0.13, respectively; $P_{trend} < 0.0001$) compared with lower scores (0-4) over a mean follow-up of 17.7 years[76]. As unhealthy lifestyle is associated with a higher risk of NAFLD/NASH and HCC, it is reasonable to assume that correcting these behaviors will potentially protect against the development of HCC.

However, there is insufficient direct evidence to indicate that changes in lifestyle reduce the risk of HCC in NAFLD/NASH patients. A meta-analysis of 30 RCTs in NAFLD patients (n = 3280), which evaluated the effect of diet, exercise, or their combination on the liver and metabolic markers, reported that a combination of diet and exercise resulted in a greater decrease of ALT [mean difference (MD): -13.27], AST (MD: -7.02) and Homeostatic Model Assessment for insulin resistance (MD: -2.07) compared to either of them[77]. However, no histological or imaging data were available. Moreover, an umbrella review of evidence from observational studies and RCTs looking at the association between lifestyle and NAFLD with regards to risk and treatment (41 meta-analysis from observational studies and 81 meta-analysis from RCTs) suggested that some interventions [*i.e.* green tea, omega 3 polyunsaturated fatty acids (PUFA), and exercise] were associated with some improvement in metabolic and hepatic markers, but more robust RCTs are needed to investigate the effect of lifestyle changes on liver outcomes[78]. In addition, the network meta-analysis by Buzzetti *et al*[79] (59 RCTs, 3631 participants; 2-24 mo of follow-up) could not draw a definite conclusion regarding the effect of the lifestyle interventions on any of the

Table 1 Summary of lifestyle and pharmacological intervention with potential preventive effects against developing nonalcoholic fatty liver disease/nonalcoholic steatohepatitis-associated hepatocellular carcinoma

Interventions with potential protective effects	
Lifestyle interventions	Weight loss
	Dietary changes
	Adherence to healthy eating patterns: Mediterranean diet, traditional Cantonese dietary pattern; Chinese Healthy Eating Index
	Reduced intake of: Saturated fats, sugar-sweetened beverages, alcohol
	Increased intake of: Vegetables, coffee; possibly fiber, white meat and fish, omega-3 polyunsaturated fatty acids, vitamin E
	Physical exercise
	Smoking cessation
Pharmacological interventions	Metformin
	Statins
	Aspirin
	Possibly also: Pioglitazone, GLP-1 RA, vitamin E (in nondiabetic individuals?), obeticholic acid

GLP-1 RA: Glucagon-like peptide-1 receptor agonists.

clinical liver outcomes (including HCC), as the number of events was too low (probably due to short duration of follow-up).

Diet: There is limited information regarding the impact of diet/dietary components on liver histology in patients with NAFLD/NASH and on the risk of progression to HCC. Most studies report data related to liver biomarkers/fat content or risk of liver cancer in overall population/patients with chronic liver diseases, regardless of etiology. The relationship between HCC and several nutrients, foods, and dietary patterns has been evaluated mostly in observational studies, but few data exist exclusively in patients with NAFLD/NASH[80]. There is a lack of high-quality data from large RCTs in this population, but it should be noted that it is quite challenging to evaluate dietary determinants of the disease in the context of multiple confounding effects of other lifestyle factors[81].

Dietary patterns represent a complex combination of foods/nutrients and beverages that are habitually consumed, and their evaluation may capture in a more integrated way the effect of diet on health outcomes[82]. A recent systematic review of 30 observational studies (5222534 participants from Asia, America, and Europe) investigated the association between diet and risk of HCC and found differences with regards to geographical regions and dietary patterns[83]. Specifically, the Mediterranean diet appeared to be protective for the European and American populations, while the Chinese Healthy Eating Index and the Cantonese Dietary Pattern seemed to be associated with lower risk of HCC in Asian countries[83].

Analysis of combined data from two case-control studies demonstrated that better adherence to the Mediterranean diet was associated with a lower risk of HCC (ORs: 0.66 and 0.51, $P < 0.001$ for trend)[84]. In addition, the Alternate Mediterranean diet score (an adaptation of the original Mediterranean diet score) was associated with a decreased risk of HCC [hazard ratio (HR): 0.68; $P = 0.02$], as indicated by a multiethnic cohort prospective study[85]. Another study has shown a suggestive but nonsignificant association with lower risk (HR: 0.75; $P_{trend} = 0.18$)[86]. The Singapore Chinese Health Study data also indicated that higher Alternate Mediterranean diet scores as well as higher scores of Alternative Healthy Eating Index-2010 (AHEI-2010) and Dietary Approaches to Stop Hypertension, representing a better dietary quality, were associated with a lower risk of HCC ($P_{trend} < 0.05$)[76]. The results were in agreement with the report of the National Institutes of Health-AARP Diet and Health study, indicating that higher HEI-2010 and Alternate Mediterranean diet score were significantly associated with lower HCC risk (HR: 0.72; $P_{trend} = 0.03$ and HR: 0.62; $P_{trend} = 0.0002$, respectively)[87].

Similarly, better adherence to the Chinese Dietary Guidelines, as assessed by the Chinese Healthy Eating Index, was shown to be associated with a lower risk of HCC (OR: 0.43; $P < 0.001$), even in the stratified analysis by risk factors[88]. The study by Lan *et al*[89] that enrolled 782 patients with primary liver cancer and evaluated their habitual dietary intake found that an urban prudent dietary pattern (consisting of higher intake of eggs, mushrooms, dairy products, soy foods and nuts, and lower intake of refined grains) and a traditional Cantonese dietary pattern (characterized by a high intake of fruit and vegetables, Cantonese soup, fish, and Chinese herb tea) have been associated with a lower risk of primary liver cancer (OR: 0.25 and 0.61, respectively), while a diet rich in meat and preserved foods

increased the risk (OR: 1.98). Moreover, a prospective study that enrolled 887 patients with newly diagnosed HCC suggested that a higher adherence to the 2016 Chinese Dietary Guidelines was associated with a lower risk of HCC-specific mortality (HR: 0.74) and all-cause mortality (HR: 0.75)[90].

Inflammation is a key pathogenetic mechanism that influences NASH progression and hepatocarcinogenesis. Two studies have evaluated the correlation between dietary inflammatory score/index (that reflect the overall inflammatory potential of a diet) and the risk of HCC/primary liver cancer mortality. The first one showed that higher dietary inflammatory index values (indicating a proinflammatory diet) were associated with an increased risk of liver cancer (HR: 2.05) and liver cancer-associated mortality (HR: 1.97)[91]. The second study reported that higher adherence to empirical dietary inflammatory pattern score (indicating a proinflammatory potential of the diet) was associated with an increased risk of HCC (HR: 2.03; P_{trend} = 0.001)[92]. The same study reported that several other scores (empirical dietary index/lifestyle pattern score for hyperinsulinemia and insulin resistance) indicating a higher insulin resistance potential of a diet were also correlated with a higher risk of HCC[92].

Thus, a healthy dietary pattern (generally characterized by an increased intake of vegetables, fruits, nuts, and whole grains and a decreased intake of red and processed meats) appears to be protective against HCC and HCC-related mortality. However, more specific evaluations are needed to confirm the data and assess the strength of these associations in patients with NAFLD/NASH.

There is little evidence with respect to the impact of dietary macro- and micronutrients upon liver histology in patients with NAFLD/NASH (*e.g.*, reversal of fibrosis) and the risk of progression to HCC.

Data coming from observational studies are heterogenous. Some studies showed that increased carbohydrate intake was associated with higher aminotransferases levels, liver fat, and NASH, while others indicated the opposite or were neutral[81,93-97]. A meta-analysis of ten RCTs concluded that low-carb diets significantly reduced the intrahepatic lipid content but did not change the serum liver enzyme concentrations in patients with NAFLD[98]. There is also sparse and inconsistent evidence regarding the association between food glycemic index and load with HCC[35]. Data from the European Prospective Investigation into Cancer and Nutrition cohort (EPIC) study (477206 subjects) did not find significant associations between dietary glycemic index or glycemic load or total carbohydrate intake with risk of liver cancer[99]. However, the risk of HCC in correlation with types of carbohydrates appeared to be divergent: 43% higher risk per 50 g total sugar intake/d and 30% lower per 50 g starch/d[99]. The analysis of the Shanghai Women's Health Study and the Shanghai Men's Health Study data also indicated no consistent association between dietary carbohydrates, glycemic index, and glycemic load and risk of liver cancer[100].

On the other hand, higher intake of dietary fructose/sugar-sweetened beverages (surrogate for free sugars) has been associated with NAFLD, independent of other risk factors in several studies, as well as with higher intrahepatic lipid content, mainly when consumed in the context of excessive caloric intake [81,101-105]. The meta-analysis by Li *et al*[106] (71 observational studies) reported that higher sugar-sweetened beverages intake was associated with higher overall cancer risk [relative risk (RR): 1.12; P = 0.000] and mortality risk (RR: 1.07; P = 0.029) as well as higher risk of HCC (RR: 2.00; P = 0.001) (but HCC results were based only on two studies). Interestingly, the EPIC study data suggested that consumption of > 6 servings of combined soft drinks per week was associated with higher HCC risk (HR: 1.83; P_{trend} = 0.01) but artificially-sweetened rather than sugar-sweetened soft drinks intake appeared to be deleterious[107]. A significant positive association between carbonated beverages consumption and HCC risk was also seen in a case-control study of 582 cirrhotic patients (181 with HCC) (OR: 2.44; P_{trend} = 0.021)[108].

The same study showed an inverse correlation of HCC risk with fiber intake (OR: 0.49; P_{trend} = 0.012), which is in accordance with the EPIC study results indicating a 30% HCC risk reduction per 10 g/d of total dietary fiber intake, even in viral hepatitis-free individuals[99,108]. However, the analysis of data from two United States cohort studies (125455 participants) did not find a significant association of HCC risk with cereal, fruit, or vegetable fiber intake[109]. On the other hand, lower daily (mainly soluble) fiber intake was observed in patients with NAFLD/NASH in several observational studies[94,96,110].

There is inconsistent evidence with regards to the role of dietary proteins (types, amount) in the progression of NAFLD/NASH and occurrence of HCC. The Rotterdam Study which included 3882 individuals (1337 with NAFLD) showed that animal protein intake was significantly associated with overweight NAFLD after adjustment for metabolic covariates (OR: 1.36)[111]. Indirect evidence, in line with these results, was provided by another cross-sectional study that showed that total and animal protein consumption was positively associated with estimates of liver steatosis (OR: 1.25 and 1.27, respectively), while vegetable proteins had an inverse association with these (OR: 0.81)[112]. On the other hand, a recent RCT that evaluated the effect of a low-carbohydrate high-protein diet on liver fat in 72 T2DM patients demonstrated that it reduced the liver fat content to a slightly greater extent compared to a conventional diet (64% *vs* 51%, P = 0.051) beyond the effects of (similar) weight loss[73]. Similarly, a small prospective study in NAFLD patients with T2DM showed that high protein diets (30% of total caloric daily intake), either animal or plant-based, significantly reduced liver fat independent of change in body weight, and decreased the serum levels of fibroblast growth factor 21[113]. The animal protein diet determined a greater increase in postprandial levels of methionine and branched chain amino acids but this was not accompanied by beneficial or deleterious effects[113]. Some experimental data suggested potential benefits of branched chain amino acids in terms of hepatocarcinogenesis

inhibition (changes in growth factors gene expression, inhibition of proliferation), increased apoptosis in liver cancer cell lines, and improvement of fibrosis[82,114,115]. In addition, few Japanese clinical studies suggested that branched chain amino acid supplementation in patients with HCC may reduce cancer recurrence after hepatic resection (mainly in patients with higher insulin resistance), but others showed no effect[116-119]. Certainly, additional good-quality evidence is needed regarding the role of dietary proteins/amino acids in the progression of NAFLD/NASH and associated HCC.

Most studies concerning dietary fats in NAFLD/NASH and HCC evaluated the role of type rather than amount of fats[120]. Observational studies seem to indicate that total dietary fat intake is not correlated with HCC risk[121-123]. Some but not all results suggested that vegetable fats might be associated with reduced HCC risk[121,123]. Higher consumption of saturated fatty acids however was shown to be associated with hepatic steatosis/NAFLD and NASH[81,95,124]. A large Chinese prospective cohort study also indicated that dietary saturated fats were associated with higher liver cancer risk [adjusted HR (aHR): 1.14; P = 0.005], but results from the EPIC study and a hospital-based case-control study from the United States did not support a direct association of saturated fats intake with HCC[125-127]. However, a meta-analysis of 14 studies by Zhao *et al*[128] demonstrated that higher dietary intake of saturated fats was associated with an increased risk of liver cancer (RR: 1.34 for highest *vs* lowest intake) in a dose-dependent manner.

On the other hand, the same United States case-control study showed that monounsaturated fatty acids (MUFA) but not total PUFA intake was inversely correlated with the risk of HCC (OR: 0.49 and 1.82)[122]. This was in contrast with the study by Yang *et al*[123] that showed that PUFA was inversely associated with HCC risk (aHR: 0.83; P = 0.03) (with MUFA being neutral). The study by Li *et al*[125] indicated that MUFA had a neutral effect (aHR: 1.26, 95%CI: 0.96-1.65; P = 0.034), while PUFA intake was associated with higher HCC risk (aHR: 1.41, 95%CI: 1.07-1.86 for highest *vs* lowest quartile; P = 0.005). Thus, data on MUFA and total PUFA seems quite heterogenous (maybe explained in part by dietary source, type of study, and study population).

In a NASH animal model, dietary intake of docosahexaenoic acid appeared to be superior to that of eicosapentaenoic acid in attenuating Western-diet induced liver injury, oxidative stress and fibrosis, and potentially in reducing the risk of HCC, thus suggesting differential effects of the two dietary omega-3 PUFAs[129]. Evidence is more convergent regarding the effect of omega-3 PUFA supplementation on liver fat content. Two meta-analyses actually showed that it decreased liver fat, and this was confirmed by a small histologic study in non-cirrhotic NASH patients that received 3000 mg/d omega-3 PUFA for 1 year[130-132]. However, the study did not reach the primary end-point (*i.e.* NAS reduction of ≥ 2 points without fibrosis progression)[132].

In addition, the evidence regarding the effect of omega-3 PUFA on HCC risk is not unequivocal. The case-control study by Moussa *et al*[127] demonstrated an inverse association between dietary omega-3 intake and HCC risk (OR: 0.50), but other epidemiological data seemed to indicate a neutral effect (HR: 0.63; P_{trend} = 0.14 and aHR: 0.89, 95%CI: 0.75–1.04; P = 0.14, respectively)[122,123,125]. Some observational studies showed a positive relationship between dietary intake of omega-6 PUFA and HCC risk (adjusted OR: 2.29 for highest *vs* lowest tertile, and OR: 4.36), indicating a potentially negative effect, although other data showed an inverse association (HR: 0.54; P_{trend} = 0.02)[123,127,133]. Linoleic acid intake might be inversely associated with HCC risk (OR: 0.35, P < 0.01)[122].

Even if the role of micronutrients in preventing the progression of NASH to HCC might be hypothetically explained from a pathogenetic perspective (*i.e.* antioxidant, anti-inflammatory, anti-fibrotic effects), and the experimental data is quite consistent. There is insufficient good quality clinical research regarding their dietary intake or supplementation effect in NAFLD patients[134].

Two United States prospective studies have shown an inverse relationship between (dietary and supplemental) magnesium intake and risk of liver cancer (HR: 0.65 and HR: 0.44; P_{trend} = 0.0065, respectively)[135,136]. Results from two large cohort studies in China demonstrated an inverse association between dietary manganese intake and liver cancer risk long-term (HR: 0.51; P_{trend} = 0.001), even after adjustment for HBV infection[137]. The case-control study by Rizk *et al*[108] also found that manganese intake was significantly lower in HCC patients *vs* controls (OR: 0.56; P_{trend} = 0.038) as well as potassium intake (OR: 0.44; P_{trend} = 0.004). On the other hand, the sodium intake was significantly higher [104]. Nevertheless, more studies are needed to clarify the role of minerals in NASH and HCC.

The same above-mentioned case-control study also indicated lower intake of vitamins E and B9 in HCC patients[108]. In the same sense, a report from two Chinese cohort studies showed that dietary vitamin E intake and supplement use were inversely associated with the risk of liver cancer (HR: 0.52) [138]. The effect of therapeutic intervention with vitamin E will be discussed below. An inverse association between HCC risk and (β) carotenes/vitamin A was noted in several studies (with OR: 0.48 for β carotene, 0.34 for vitamin A, and 0.35 for carotenes)[122,139].

Food items/groups have been evaluated with regards to their association with HCC risk. We will only briefly mention the main findings here. Several systematic reviews and meta-analyses have evaluated data regarding the association between meat consumption and risk of HCC. Even if not totally in agreement, they seem to indicate that read meat consumption is associated with increased HCC risk (RR: 1.22) or is neutral (RR: 1.04 and 1.10), and there is an increased HCC risk associated with processed meat consumption (RR: 1.20, and 1.01)[140-142]. Total meat intake had no significant effect [140-142]. On the contrary, white meat and fish intake were found to be inversely associated with the

risk of HCC (RR: 0.69, 0.76 for white meat and RR: 0.78, 0.91 for fish)[141,142]. Some epidemiological data suggested that increased dairy product intake (mainly milk and high-fat diary) was associated with a higher risk of HCC, although not all studies agreed (yogurt seemed to be associated with a decreased risk or had a neutral effect)[121,143-145]. Two meta-analyses indicated that increased vegetable consumption was associated with a lower risk of HCC[146,147]. For other food items, there is insufficiently consistent evidence so far.

There have been suggestions for the use of many herbal and dietary natural compounds, such as prebiotics/polyphenols, resveratrol, and curcumin in NAFLD/NASH therapy, including for HCC prevention, with some small studies suggesting anti-inflammatory effects of prebiotics, but until now there is very limited data from clinical trials, and no clear conclusion can be drawn[134,148-150]. Preclinical data demonstrated the anti-inflammatory effects of the catechin-rich green tea extract that may attenuate NASH-associated liver injury through a decrease of hepatic nuclear factor kappa-B activation but also indirectly through prebiotic and antimicrobial effects on gut microbiota, resulting in decreased translocation of the gut-derived endotoxins[151]. Green tea contains several bioactive compounds that may exert anticarcinogenic properties (*e.g.*, flavonoids, caffeine, polyphenols, *etc.*) through modulation of different signaling transduction and metabolic pathways (reduction of chronic inflammation, oxidative stress, insulin resistance, liver steatosis, *etc.*)[80,152]. The EPIC study data showed an inverse association between tea consumption and HCC risk (HR: 0.41, 95%CI: 0.22-0.78; P_{trend} = 0.003), while a meta-analysis of 15 RCTs demonstrated that green tea reduced the liver enzymes values in NAFLD patients[153,154].

The use of probiotics in NAFLD/NASH, cirrhosis, and HCC has been reported in several studies and indicated that they can improve aminotransferases and insulin resistance and have anti-inflammatory effects, but there is no evidence so far with regards to HCC prevention[150,155]. A meta-analysis of 21 RCTs (1252 participants) suggested that the use of probiotics/synbiotics was associated with a decrease of inflammation markers, liver stiffness, and steatosis in subjects with NAFLD[156]. However, well-designed RCTs are further needed to fully understand their protective effect in patients with NASH.

Coffee: Eight meta-analyses of prospective cohort and case-control studies provided consistent evidence regarding an inverse relationship between coffee drinking and risk of HCC (RR ranging between 0.54 and 0.78), with only one meta-analysis indicating a neutral effect (RR: 0.93)[157-164]. Caffeinated rather than decaffeinated coffee seemed to have a more consistent effect on HCC[158,161]. Also, the association appeared to be related to the amount of daily coffee intake. The beneficial effects generally started at two cups/d. An extra cup of coffee/d reduced the cancer risk by about 15%-25% (RRs between 0.75 and 0.85), and two extra cups/d by about 14%-44% (RRs between 0.56 and 0.86)[157-164].

Alcohol: Alcohol is a major risk factor for HCC, and it has synergistic effects with the metabolic risk factors (T2DM, obesity) in inducing carcinogenic mechanisms[80,165]. Evidence suggests that a modest alcohol intake is protective against fatty liver, NASH, and fibrosis, but it was not firmly established if it is also protective against HCC[82,166]. A meta-analysis of 19 cohorts (4445 incident cases of liver cancer) showed neutral effects of moderate drinking (< 3 drinks/d) on liver cancer risk (RR: 0.91, 95%CI: 0.81-1.02) compared to no drinking, while heavy drinking (defined as ≥ 3 drinks/d) significantly increased the risk (RR: 1.16, 95%CI: 1.01-1.34)[167]. In line with these results, a prospective evaluation of 8345 subjects with hepatic steatosis (mean follow-up duration of 11.1 years) demonstrated a dose-response relationship for advanced liver outcomes/liver cancer that became significant at ≥ 10 g of alcohol intake/d (for liver outcomes) and ≥ 30 g/d (for liver cancer) after multivariate adjustments[168]. The decrease in risk of HCC after alcohol drinking cessation is uncertain, but a meta-analysis of four studies suggested a decline of HCC risk with 6%-7%/year, although caution was advised in data interpretation [169].

Physical activity: The benefits of physical activity in reducing insulin resistance and hepatic liver content in NAFLD patients are well known[170,171]. The meta-analysis by Golabi *et al*[170] (eight studies with 8 to 48 wk duration) reported that aerobic and resistance exercises determined a liver fat reduction of 30.2%.

Physical exercise intervenes at multiple levels in the pathogenic pathways of NAFLD by reducing the free fatty acid (FFA) flux to the liver, FFA hepatic synthesis, the mitochondrial and cellular damage, oxidative stress, damage-associated molecular patterns release, and hepatic stellate cell activation and by increasing the FFA oxidation and activating the AMP-activated protein kinase-regulated pathways, *etc.*[172]. Physical exercise also improved mitochondrial function (*i.e.* autophagy, biogenesis) and modulated the carcinogenic signaling pathways[80,173,174]. Taken together, these might explain the potential protective effects of exercise in NAFLD/NASH and HCC. Indeed, a meta-analysis of 14 prospective studies indicated that physical activity was inversely correlated with the risk of liver cancer (HR for high *vs* low physical activity: 0.75, 95%CI: 0.63-0.89)[175]. These results were in accordance with the EPIC study that reported an aHR of 0.55, 95%CI: 0.38–0.80 for HCC in active *vs* inactive individuals [176]. The associations seemed to be at least in part mediated by obesity[176]. However, no prospective study evaluated the effect of physical exercise on HCC risk, as this might be rather difficult to perform [172].

Smoking cessation: Data from the Liver Cancer Pooling Project, a consortium of 14 prospective cohort studies comprising 1518741 individuals indicated that cigarette smoking significantly increased the risk of HCC (HR: 1.86, 95%CI: 1.57–2.20)[177]. It also demonstrated that in individuals who quit smoking for > 30 years, the risk of HCC decreased to values almost similar to non-smokers (HR: 1.09, 95%CI: 0.74–1.61)[177]. In addition, quitters seemed to have a lower risk of HCC-related mortality (HR, 0.62, 95%CI: 0.39-0.97), but this was seen in subjects without diabetes[178].

Pharmacological interventions

Several drugs have been suggested to bring benefits for NAFLD/NASH and to potentially reduce the risk of associated HCC, although there is limited evidence for HCC chemoprevention. It is assumed however that by improving histological features associated with NASH and the primary drivers of fibrogenesis ultimately leading to cirrhosis (and HCC), the disease progression will be attenuated, and HCC occurrence eventually prevented[179].

Metformin: Metformin does not seem to improve NASH/fibrosis, but several meta-analyses suggested that it might reduce the risk of HCC in patients with T2DM[180-182]. A recent meta-analysis of 24 studies including 1.4 million individuals reported that metformin was associated with a 41% lower risk of HCC in DM patients treated with metformin ($P < 0.001$) and a significant reduction of all-cause mortality (HR: 0.74, 95%CI: 0.66-0.83; $P = 0.037$)[180]. Moreover, a network meta-analysis that compared several antidiabetic medications has shown that in comparison with sulphonylureas and insulin, metformin significantly decreased the risk of HCC (RR: 0.45, 95%CI: 0.27–0.74 and RR: 0.28, 95%CI: 0.17–0.47)[181]. Postulated mechanisms of chemoprotective effects of metformin are the activation of AMP-activated protein kinase and inhibition of the mammalian target of rapamycin pathway, the inhibition of angiogenesis and induction of apoptosis[183]. It was also suggested that metformin may inhibit the progression of high fat diet-induced HCC by modulating the innate immune-mediated inflammation and restoring tumor surveillance[184]. However, these data should be interpreted with caution, and there is still need for further substantiation.

Thiazolidinediones: The meta-analysis by Musso *et al*[185] has delineated the effects of the two thiazolidinediones (TZDs), indicating that only pioglitazone (30/45 mg/d, for 6 to 24 mo) was associated with improvement in fibrosis (even advanced fibrosis) and NASH resolution in patients with or without diabetes. In addition to increasing adiponectin levels and decreasing excessive lipolysis and FFA flux into the liver, pioglitazone attenuated oxidative stress and inflammation and, the activation and proliferation of hepatic stellate cells, extracellular matrix deposition, fibrosis, *etc*[12,186-188]. The possible role of the TZDs in hepatic chemoprevention is further suggested by *in vitro* data, showing that they may inhibit hepatocarcinogenesis by the regulation of nucleophosmin, a ubiquitously expressed cellular phosphoprotein involved in both proliferation and growth-suppression pathways[189,190]. Animal data also showed that pioglitazone reduced the HCC development, possibly through the upregulation of the AMP-activated protein kinase pathway and the reduction of the mitogen-activated protein kinase activation[191]. However, data in humans are scarce, and no definite conclusions can be drawn yet. The results of the network meta-analysis by Zhou *et al*[181] suggested possible beneficial effects of TZDs in reducing HCC incidence, but these were seen only in comparison with sulphonylureas (RR: 0.47, 95%CI: 0.22–0.97) and with insulin (RR: 0.30, 95%CI: 0.14–0.61), but not *vs* observation alone.

GLP-1 receptor agonists: Two histological studies with GLP-1 receptor agonists (GLP-1 RAs) (liraglutide and semaglutide) in patients with or without T2DM have proven NASH resolution, but results regarding fibrosis were inconclusive[192,193]. Preclinical studies have suggested potential chemoprotective effects of the GLP-1 RAs through various mechanisms like enhancing natural killer cell-mediated cytotoxicity and, inducing autophagy and senescence of the HCC cells by the increase of transforming growth factor β1, promoting their apoptosis though activation of the JNK signaling pathway, *etc*[194-196]. There is very limited information regarding the long-term effect of GLP-1 RAs on HCC incidence in humans so far.

Statins: Two recent meta-analyses of observational and interventional studies have confirmed liver safety for statin use in patients with NAFLD and even a reduction of transaminases levels[197,198]. Moreover, the meta-analysis by Fatima *et al*[198], which also analyzed the liver histology outcomes, reported a significant reduction of steatosis grade, necro-inflammatory stage, and of significant fibrosis but not the fibrosis stage. Several meta-analyses (mostly of observational studies) have consistently reported reduced risk of HCC in statin users (RRs/ORs/HR between 0.52-0.75 for all statins), with some indication of differences between them[199-208]. In particular, it seemed that the lipophilic statins exert preventive effects (OR: 0.51, 95%CI: 0.46-0.57 and HR:0.49, 95%CI: 0.39–0.62) rather than the hydrophilic statins (OR: 0.77, 95%CI: 0.58-1.02 and HR: 0.73, 95%CI: 0.40–1.34)[205,208]. Higher doses appeared to be associated with better protective effects (HR: 0.38 *vs* 0.55)[195]. Moreover, a meta-analysis of nine retrospective cohort studies also reported a lower risk of HCC-related mortality (RR: 0.78, $P = 0.001$) and reduced HCC recurrence (RR: 0.55, $P < 0.001$) with statin use[208]. However, another meta-analysis

indicated that statin usage decreased the risk of all-cause mortality (HR: 0.80, 95%CI: 0.68-0.94; $P < 0.0001$) but not of HCC-specific mortality (HR: 0.80, 95%CI: 0.62-1.03; $P = 0.002$)[200].

Apart from exerting lipid-lowering effects, statins have anti-inflammatory, antioxidant, anti-proliferative, and anti-angiogenic properties[12,209,210]. Their potential anti-tumor effects might be mediated through the downregulation of the RAF/mitogen-activated protein kinase 1/extracellular signal regulated kinase and nuclear factor kappa-B pathways, limitation of the cyclin-dependent kinase inhibitors (p21 and p27) degradation, prevention of the c-Myc activation, reduction of proinflammatory cytokines, *etc.*, which determine apoptotic responses, tumor-suppressor effects, cell survival reduction, and cell growth inhibition[183,211]. Nevertheless, data from well-designed RCTs (that limit the effect of confounding factors) in support of the HCC chemopreventive effects of statins in NAFLD/NASH population is scant.

For the other anti-hyperglycemic and lipid-lowering drug classes there is no consistent evidence so far regarding potential HCC protective effects.

Resmetirom is a thyroid hormone receptor β-selective agonist, which was shown to significantly reduce the liver fat after 12 and 36 wk of treatment[212]. It is currently under evaluation for safety and efficacy in improving NASH and preventing progression to cirrhosis and/or advanced liver disease in a phase 3 RCT (MAESTRO-NASH; NCT03900429)[213].

Aspirin: Growing evidence coming from preclinical and clinical observational studies suggest that aspirin might play a role in HCC prevention[80,165]. The mechanisms are related to inhibition of selective cyclooxygenase-2 as well as of platelet-derived growth factor, P4HA2, nuclear factor kappa-B activation, and protein kinase 3 signaling, *etc.*, which may prevent proliferation of liver cancer cells and angiogenesis and promote fibrosis resolution[80,165]. Several meta-analyses have explored the potential HCC protective effect of aspirin and have shown that it is associated with reduced incidence/risk of HCC (RRs/HRs/ORs between 0.74 and 0.51)[214-219]. The meta-analysis by Memel *et al*[214] implied stronger association after adjustment for metformin/statin use and accounting for cirrhosis. Also, an inverse relationship seems to exist between aspirin dose/duration of use and HCC risk, but this should be further confirmed[215,219]. There was no evidence of higher risk of bleeding with aspirin use, including in patients with HCC in most meta-analyses except one[216-220]. Moreover, the systematic review and meta-analysis by Tan *et al*[216] showed that aspirin use was associated with improved liver-related mortality (OR: 0.32, 95%CI: 0.15-0.70) and reduced risk of HCC recurrence (HR: 0.80, 95%CI: 0.75-0.86). The same was observed in the meta-analysis by Li *et al*[220], which demonstrated a reduced risk of HCC recurrence (RR: 0.74, 95%CI: 0.59-0.93; $P = 0.01$) and all-cause mortality (RR: 0.59, 95%CI: 0.47-0.73; $P < 0.001$). Although the evidence points toward a potential benefit of aspirin use in prevention of HCC, further prospective data is still necessary in the NAFLD/NASH population.

Vitamin E has anti-oxidative properties, and it might modulate fibrogenesis through inhibition of transforming growth factor β1[12,221]. A recent meta-analysis of eight RCTs reported that vitamin E supplementation (400-800 IU/d, between 8-96 wk) was associated with reduction of fibrosis score (MD *vs* placebo: -0.26, 95%CI: -0.47 to -0.04; $P = 0.02$) as well as a decrease in steatosis, lobular inflammation, and hepatocellular ballooning compared with placebo[222]. However, only one study included patients with T2DM. In fact, the study by Bril *et al*[223] that randomized 105 T2DM patients with biopsy-proven NASH to vitamin E 400 IU b.i.d., vitamin E 400 IU b.i.d. plus pioglitazone 45 mg/d, or placebo did not reach the primary outcome (two-point reduction of NAS from two different parameters, without worsening of fibrosis) in patients receiving vitamin E *vs* placebo but resulted in improvement of steatosis. Thus, it is not clear if the benefit of vitamin E supplementation is restricted to individuals without diabetes, and more data is needed in the T2DM population.

Experimental studies have suggested that antifibrotic therapies may serve as HCC preventive interventions by addressing the underlying cause of carcinogenesis onset[224,225]. Unfortunately, no anti-fibrotic drug has been approved so far by the European Medicines Agency or by the United States Food and Drug Administration for NASH treatment.

Obethicolic acid (OCA) is a farnesoid X receptor agonist shown to improve fibrosis without worsening NASH in an interim analysis of a phase 3 RCT (REGENERATE; NCT02548351)[226,227]. In this analysis, a significantly higher proportion of patients treated with OCA 25 mg daily presented improvement in fibrosis by ≥ 1 stage with no worsening of NASH (23% *vs* 12% in placebo group, $P = 0.0002$)[227]. In the analysis that included patients receiving at least 15 mo of therapy, three times more patients in the OCA 25 mg/d group obtained ≥ 1 stage improvement in fibrosis (38%) *vs* progression of fibrosis (13%) compared with the placebo group, in which similar proportions of patients presented improvement (23%) *vs* worsening (21%) of fibrosis[227]. The FLINT (Farnesoid X Receptor Ligand Obeticholic Acid in NASH Treatment) trial had previously shown that treatment with OCA 25 mg/d for 72 wk improved liver histology (RR: 1.9, 95%CI: 1.3-2.8; $P = 0.0002$) in patients with biopsy-proven NASH[228]. However, in both trials, a higher proportion of patients treated with OCA presented pruritus (23% in FLINT study and 51% in REGENERATE study) in a dose-dependent manner[227,228]. In addition, OCA therapy was associated with increased low-density lipoprotein cholesterol levels, but this could be attenuated by concomitant statin therapy[80,229]. HCC animal model data suggested that the OCA might attenuate the development and progression of NASH-associated HCC by upregulating

sirtuin-1 and modulating the SOCS3/Jak2/STAT3 pathway[230].

Pentoxifylline: Although few small histological studies have suggested liver-related benefits with pentoxifylline 400 mg t.i.d. treatment [*i.e.* improvement of steatosis, lobular inflammation, and liver fibrosis score (mean change: -0.2 *vs* +0.4 on placebo, P = 0.038)] and regression of fibrosis when combined with vitamin E (P = 0.003), data is still scarce and not consistent[231,232]. Four meta-analyses have reported conflicting results with regards to the effect of pentoxifylline on liver fibrosis, with only two of them suggesting benefits[233-236]. Therefore, additional data is required.

Two other initially promising anti-fibrotic medications have failed to prove a significant impact on hepatic fibrosis in phase 3 clinical trials. Elafibranor, an agonist of PPAR-α/δ, reduced steatohepatitis without worsening fibrosis in a phase 2 trial in NASH patients, but the primary end-point was not met [237]. The phase 3 RCT (RESOLVE-IT-NCT02704403) was prematurely discontinued due to limited efficacy at the time of the interim analysis[238]. Cenicriviroc, a dual inhibitor of C–C motif chemokine receptor 2/5, reduced liver fibrosis in NASH patients but did not reach the primary outcome in a phase 2b study[239]. This was followed by a phase 3 trial (AURORA-NCT03028740), which was also terminated early due to lack of efficacy resulting from the planned interim analysis[240]. Several other drugs with potential anti-fibrotic effects are currently in development/evaluation, and the results are expected with interest[241].

CONCLUSION

Regardless of the risk status, all NAFLD/NASH patients should consider adopting lifestyle changes (healthy diet and physical exercise) and controlling their body weight, as these are the cornerstone interventions for NAFLD/NASH management and possibly through altering the natural course of the disease for HCC prevention.

Data suggest a possible role of comprehensive lifestyle changes in reducing the risk of HCC, but specific evidence in NAFLD/NASH patients is rather limited at this point and not sufficient to clearly indicate preventive effects on NAFLD/NASH-associated HCC. Moreover, there is no consensus regarding the composition of a protective diet but decreasing the intake of deleterious nutrients/foods and beverages (*i.e.* saturated fats, sugar-sweetened beverages, alcohol), increasing the beneficial nutrients/foods/beverages (vegetables, coffee, dietary fiber, omega-3 PUFA), and adherence to a healthy dietary pattern (such as the Mediterranean diet or traditional Cantonese dietary pattern) are reasonable and safe approaches. However, their role in HCC prevention still needs to be confirmed by further well-designed prospective studies and experimental research.

Several drug classes (metformin, statins, and aspirin and possibly TZDs, GLP-1 RA, vitamin E, and obeticholic acid) might exert chemopreventive effects by addressing the underlying mechanisms of the disease, but direct evidence regarding their role in NAFLD/NASH-associated HCC prevention is insufficient at the moment.

A timing combination of therapies/non-pharmacological interventions and perhaps adapting them to the stage of disease and/or patient particularities will be necessary to obtain disease resolution and prevention of cirrhosis/NASH-associated HCC.

FOOTNOTES

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REVIEW

Modern drug discovery for inflammatory bowel disease: The role of computational methods

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Abstract

Inflammatory bowel diseases (IBDs) comprising ulcerative colitis, Crohn's disease and microscopic colitis are characterized by chronic inflammation of the gastrointestinal tract. IBD has spread around the world and is becoming more prevalent at an alarming rate in developing countries whose societies have become more westernized. Cell therapy, intestinal microecology, apheresis therapy, exosome therapy and small molecules are emerging therapeutic options for IBD. Currently, it is thought that low-molecular-mass substances with good oral bio-availability and the ability to permeate the cell membrane to regulate the action of elements of the inflammatory signaling pathway are effective therapeutic options for the treatment of IBD. Several small molecule inhibitors are being developed as a promising alternative for IBD therapy. The use of highly efficient and time-saving techniques, such as computational methods, is still a viable option for the development of these small molecule drugs. The computer-aided (*in silico*) discovery approach is one drug development technique that has mostly proven efficacy. Computational approaches when combined with traditional drug development methodology dramatically boost the likelihood of drug discovery in a sustainable and cost-effective manner. This review focuses on the modern drug discovery approaches for the design of novel IBD drugs with an emphasis on the role of computational methods. Some computational approaches to IBD genomic studies, target identification, and virtual screening for the discovery of new drugs and in the repurposing of existing drugs are discussed.

Key Words: Inflammatory bowel disease; Computer-aided drug design; Janus Kinase; Molecular docking; Genome-wide association study; Molecular dynamics simulation

Core Tip: For the design of small molecule drugs to treat inflammatory bowel disease (IBD), highly effective and time-saving approaches, such as computational methods, are still a viable choice. By complementing experimental studies with computational approaches, the probability of successful drug discovery is increased while simultaneously reducing associated costs. This article provides a summary of the current drug discovery pipeline for IBD, with special emphasis on the part played by computational methods. The use of *in silico* genomic studies, target identification, and virtual screening to find new drugs and repurpose existing drugs for the treatment of IBD are discussed.

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INTRODUCTION

Inflammatory bowel disease (IBD) refers to a group of disorders characterized by chronic inflammation of the gastrointestinal tract that is oftentimes triggered by unknown causes. Crohn's disease (CD) and ulcerative colitis (UC) are the two most prevalent IBDs[1]. The clinical courses are characterized by consistent relapses and recoveries, linked to destructive idiopathic inflammation of the digestive tract. Despite the fact that the precise causes of IBD are still being investigated, it is believed to be the result of an intricate relationship between various parameters, including immune system dysfunction, microbial dysbiosis, a genetic susceptibility, and some environmental factors[2,3].

As the etiology of IBD continues to be a "crossword" to solve, its prevalence and significant global impact continue to rise[4-6]. Globally, the prevalence of IBD increased by 85.1% between 1990 and 2017, and the number of people diagnosed with the disease increased by 95%, as reported by the Global Burden of Disease 2017 Inflammatory Bowel Disease Collaborators. Since 1990, the incidence of IBD has risen in newly industrialized countries in Africa, Asia and South America, including Brazil, to the extent that at the turn of the 21st century this idiopathic disease[7] has become a global disease with accelerating incidence in countries whose societies have become more westernized[8]. Also of interest is the increasing prevalence of microscopic colitis, a subtype of IBD characterized by chronic watery diarrhea caused by colon inflammation. The disease has recently gained more attention due to the availability of more information about its pathogenesis, diagnosis and therapy. The incidence of microscopic colitis is progressively on the rise, approaching that of CD and UC in some populations[9].

Treatments options for IBDs, especially CD and UC, have made significant progress in the last few years[5]. A wide range of anti-inflammatory and symptom-relieving drugs is available for patients with UC. Recent treatments aim to improve the patient's quality of life by alleviating symptoms like abdominal pain and diarrhea and bringing the disease under control as a whole[10]. Conventional treatments include aminosalicylates, corticosteroids, immunomodulators and biologics as part of the pharmacotherapy to control symptoms. When necessary, they also include some general procedures, including surgery. The advent of biologics and other therapeutic advancements in recent years has altered not only the treatment modalities but also the perception of IBD therapy. While symptom remission, complication avoidance and complication treatment are all important, mucosal healing is also a major target. Healing of the mucosa occurs when inflammation in that area subsides and the mucosal lining is returned to its normal structure[11]. Emerging evidence suggests that mucosal healing is linked to improved long-term outcomes, including lower rates of clinical recurrence, hospitalization, surgery, and disability[12,13].

Many patients with IBD have seen a dramatic improvement in their long-term outcomes, both regarding disability and quality of life since the introduction of monoclonal antibodies targeting tumor necrosis factor (TNF) about 20 years ago. However, despite these developments, there are still many unfulfilled needs. For instance, less than a third of treated patients who begin a biologic therapy achieve and maintain drug remission at 1 year. Even in cases when clinical and endoscopic criteria for remission have been met, symptoms such as diarrhea, stomach discomfort, joint problems, and exhaustion may still be experienced[14]. Also, the lack of responsiveness to biologic therapies, which can be caused in part by the protein's immunogenicity upon administration, and the need to discontinue drugs because of intolerance or side effects show that a better generation of therapeutic alternatives is still needed. To achieve the desired results of immune homeostasis restoration and better symptom control, additional progress is still required. Emerging treatment options for IBD include cell therapy, intestinal microe-

cology, apheresis therapy, exosome therapy, and small molecules[15]. Low-molecular-mass compounds with excellent oral bioavailability and the capacity to cross the cell membrane to modulate the functions of parts of the inflammatory signaling pathway are now regarded to be promising therapeutic alternatives for the treatment of IBD. Several small molecule inhibitors are being explored as a possible treatment option for IBD, such as inhibitors of Janus kinase (JAK) enzymes[1]. For the development of these small molecule drugs, the use of highly effective and time-saving techniques, such as computational methods, remain a viable option.

Owing to the intricate nature of the molecular mechanisms involved in disease pathogenesis, the process of developing small molecule drugs is complicated[16,17]. The traditional methods of drug design and development require time-consuming, costly and laborious scientific procedures. On the other hand, computational tools hold great promise as a viable option for the design and development of new small molecules of biomedical relevance[18]. Computer-aided drug design (CADD) employs computer processing power, statistics, mathematics and three-dimensional (3D) graphics to elucidate the affinity and binding mode of small molecules against therapeutic targets.

As a whole, computational methods aid in the identification of candidate target proteins for drug screening and design by searching through large amounts of genomic data for the most important genes [19,20]. In combination with experimental data, protein structures can be predicted with some accuracy [21]. In order to find drug binding sites and understand how drugs work, it is necessary to conduct research into the structural and thermodynamic features of target proteins, both of which can be accomplished with biomolecular simulations using multiscale models[22]. Next, chemical libraries are explored using virtual screening to identify potential drug candidates based on the ligand binding sites on target proteins[23]. With a significantly reduced number of potential drug candidates, *in vitro* and/or *in vivo* experiments can be used to further assess the effectiveness of these molecules. Another CADD approach, besides virtual screening, is provided by *de novo* drug design techniques, which produce highly specific small molecules with good synthetic accessibility[24].

Medicinal chemists use CADD methods to help in rationalizing the selection of hit compounds and in hit-to-lead optimization. CADD has been used as an efficient method for identifying potential lead compounds toward the development of drugs for a wide range of diseases, including IBDs. A variety of computational approaches, such as molecular docking, molecular dynamic (MD) simulation, quantitative structure-activity relationship (QSAR) and pharmacophore modeling, are used to rationally design potent therapeutics with higher efficacies and fewer side effects[25]. The availability of more information about the molecular basis of IBD pathogenesis has further enhanced the use of computational methods in the design of small molecule drugs for IBD treatment. In the pathogenesis of IBD, there is an interplay between various factors such that any stimulus can lead to a cascade of events or even a vicious cycle. This gives a variety of therapeutic targets for which IBD drugs can be designed.

MOLECULAR ASPECTS OF IBD PATHOGENESIS

An interplay of genetic, epithelial and immune factors in the presence of intestinal microbiota are believed to be responsible for the development of the IBD[26].

Genetic factors

The genetic factors causing IBD were initially established through family and twin studies. The disease was observed to cluster in families, with a relative risk of 13-36 for siblings of CD patients and 7-17 for UC patients[27]. Hence, the prevalence of IBD is much higher among first-degree relatives of those with IBD than it is among the general population, suggesting a role for genetic factors in the pathogenesis of the condition. Monozygotic twins exhibit a much higher rate of disease concordance than dizygotic twins, and there are significant differences in the incidence and prevalence of IBD among various populations, according to the twin studies[27]. In CD, the concordance rate for monozygotic twins is approximately 50% compared to 15% for dizygotic twins. Meanwhile, the concordance of monozygotic twins for UC is only 16%, suggesting that genetic factors are less dominant in this form of IBD. In general, there is a growing number of genetic markers linked with the pathogenesis of IBD at various levels including innate immunity, epithelial integrity, antigen presentation, cell adhesion, and drug transporter. Along with environmental and immune system factors, the genetic markers at the different levels play a major role in genetic susceptibility to IBD[28].

Epithelial defects

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is a cytoplasmic protein that is induced in epithelial cells[29] and is constitutively expressed in neutrophils, macrophages, and dendritic cells[30]. Specifically, the leucine-rich repeat domain of NOD2 is necessary for recognition of muramyl dipeptide, a fragment of peptidoglycan present in bacterial cell walls. Ultimately, muramyl dipeptide causes nuclear factor kappa B activation and the induction of proinflammatory cytokines[31, 32]. Activation of the innate and adaptive mucosal immune responses as a result of NOD2 polymorphism causes the synthesis of cytokines, metalloproteinases and enzymes, which subsequently

results in tissue destruction and epithelial barrier defect.

Evidence from genome-wide association studies (GWAS)[33,34] also shows the involvement of hepatocyte nuclear factor 4α (HNF4α) in the pathogenesis of UC. HNF4α is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that is highly conserved and well expressed in liver and gastrointestinal organs. Several studies have linked HNF4α to intestinal epithelial differentiation, lipid metabolism and epithelial junctions[27,35], which are important components in colon development[36]. A zebrafish model suggests that the gut microbiota negatively regulates expression levels of HNF4α[37]. There is evidence linking alterations in HNF4 expression and activity, as well as germline variants, to IBD and colorectal cancer[38,39]. In UC, polymorphisms of HNF4α result in defects of epithelial tight junctions and intestinal permeability. Due to these epithelial barrier defects, there is transepithelial influx of bacteria, activation of the immune responses, release of cytokines, and further increase in epithelial junction permeability[40-42].

The development of UC has also been linked to a decrease in fatty acid oxidation in the colonic mucosal epithelium. Carnitine is a necessary cofactor in lipid metabolism and may be transported by the organic cation transporter (OCTN), a family of transporter proteins for organic cations. Carnitine helps shuttle long-chain fats into the mitochondria. Both OCTN1 and OCTN2 have been linked to CD-causing mutations: SLC22A4 1672C/T for OCTN1 and SLC22A5 +207G/C for OCTN2. The TC haplotype, formed by the presence of one or more of these mutations, is linked to the development of ileal, colonic and perineal symptoms and the requirement for surgical treatment of CD[28,43].

A number of tissues including the colon, small intestine, placenta, liver, heart and skeletal muscle contain high levels of Drosophila long disc homologue 5. The Drosophila long disc homologue 5 gene belongs to the membrane-associated guanylate kinase gene family, which encodes cell scaffolding proteins. It is thought to be involved in the maintenance of epithelial cell integrity and in signal transduction. Mutations in this gene have been linked to an increase in intestinal permeability[28]. The D haplotypes were found to be associated with UC and CD in a European cohort[44]. CD patients in Japan also showed another form of this gene (rs37462)[45]. Patients with IBD were found to have an excess transmission of haplotype D, which is defined by the haplotype-tagging single nucleotide polymorphisms (SNPs) G113A[28].

Mucosal immune responses

In normal intestinal mucosal immune response, the presence of gut microbiota has conditioned the dendritic cells present in the epithelium so that there is activation of T regulator cells that produce anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor-beta (TGF-β) as well as suppression of T effector cells. However, in IBD, the dendritic cells respond to gut microbiota by activating CD4+ T effector cells that differentiate into T helper (Th1, Th2 and Th17) cells depending on the IBD type[40,42]. Recently, the proinflammatory cytokine IL-12 family, which includes IL-22, IL-23, IL-25, and IL-27, has been linked to the pathophysiology of CD and other immune-mediated disorders [46]. The IL-12 family is responsible for the differentiation of Th cells into Th1 cells. IL-12 and IL-23 are heterodimeric proteins with a unique subunit linked to a shared p40 subunit. Patients with CD have elevated levels of these subunits[47,48]. Ustekinumab, a Food and Drug Administration (FDA)-approved drug for moderate-severe CD, inhibits IL-12 and IL-23 receptors on T cells, natural killer cells, and antigen-presenting cells[49]. In addition, several monoclonal antibodies have been discovered that target the Th1/Th17 pathway *via* IL-23. However, none of them have progressed to phase 3 trials[50,51].

The upregulation of IL-13, another crucial cytokine in the Th2 immune response, is considered to be a major trigger of mucosal inflammation in UC patients[52,53]. Research in mice revealed an increase in IL-13 in colitis, and this increase could be mitigated by inhibiting the ability of IL-13 to bind to its signaling receptor[54]. Tralokinumab is a human immunoglobulin G4 monoclonal antibody that binds to IL-13 and inhibits its activity.

Molecular studies have also identified NOD2 as a susceptibility gene in CD. The gene encodes a protein that transduces signals to activate nuclear factor kappa B in monocytes and functions as an intracellular receptor for bacterial products. Muramyl dipeptide activates NOD2, leading to autophagy in dendritic cells. Defective autophagy induction and decreased bacterial localisation in autophagolysosomes are observed in CD patients with susceptibility polymorphisms in the NOD2 gene. Genetic analyses have identified two other genes that are involved in autophagy and intracellular bacteria clearance, namely *IRGM* and *ATG16L1*, demonstrating the importance of autophagy in IBD immune responses. Other genes related with autoimmune disease, such as IL23R and PTPN2, reveal an additional feature of CD pathophysiology[38,40].

Another major player in IBD pathogenesis is TGF-β1, an essential factor in the healing of intestinal cells and the reduction of inflammation[55]. It is an immunosuppressive cytokine with the ability to inhibit pathogenic T cell activity and antigen-presenting cell responses. Increased levels of Mothers against decapentaplegic homolog 7 (SMAD7), an intracellular protein that binds TGF-β1 receptor and prevents TGF-β1- and SMAD-associated signaling, are believed to account for the deficiency in TGF-β1 activity observed in IBD patients. Mongersen, an oral medication that inhibits SMAD7 activity after administration to the terminal ileum and right colon is being investigated for efficacy in CD[56]. In its current form, the site of action is limited to the terminal ileum and right colon, hence it may not be beneficial for patients with more complicated CD or post-operative recurrence[57].

The involvement of the JAK family of intracellular non-receptor tyrosine protein kinases in the pathogenesis of a number of autoimmune diseases has also been demonstrated[58]. Many autoimmune diseases, including IBD, have been linked to specific SNPs in the genome, and the, JAK/signal transducer and activator of transcription proteins (JAK/STAT) signaling pathway has yielded several cytokines and receptors as potential treatment targets[59]. The JAK/STAT pathway interferes with a couple of inflammatory pathways and development of disease as seen in CD and UC patients is characterized by imbalanced effector T cells, leading to increased effector Th cells (Th1, Th2 and Th17 cells), thus mediating the inflammation[60]. Figure 1 shows a schematic description of the JAK/STAT pathway leading to the expression of genes encoding some inflammatory markers. Inhibition of JAK leads to the inhibition of signaling of a specific subset of cytokines, which are implicated in inflammation[61]. Some small molecule inhibitors of the JAK/STAT pathway with good oral bioavailability have been developed. A very good example is tofacitinib. When compared to monoclonal antibodies, JAK inhibitors have the therapeutic advantage of being able to target multiple downstream signaling pathways induced by inflammatory cytokines, whereas monoclonal antibodies can only block a single molecule[62].

The successful development of tofacitinib, as well as the promising results of other JAK inhibitors in both UC and CD (as shown in Figure 2), demonstrate that JAK inhibition has a role in the treatment of IBD. However, long-term safety studies in people with rheumatoid arthritis and UC who took tofacitinib showed a higher risk of reactivation of herpes zoster, especially at higher doses[63]. This increased risk is likely a class effect of JAK inhibitors and related to IFN and IL-15 inhibition. Also, there might be a possible thrombogenic risk, as shown by patients with rheumatoid arthritis[64]. More selective JAK-1, JAK-3 or tyrosine kinase 2 inhibitors are expected to improve safety while maintaining efficacy. However, these drugs are still systemic; a gut-selective JAK inhibitor with high intestinal exposure and target engagement but no systemic effects are still needed for treating people with IBD. Continued progress is being made in all of these areas[65].

IBD DRUG DISCOVERY AND DEVELOPMENT

The process of identifying a therapeutic agent for extensive study as a potential drug candidate is known as drug discovery[66]. In general, the modern drug discovery process involves identifying a condition to be treated and its unfulfilled medical necessity, selecting a druggable molecular target and validating it, developing *in vitro* assays followed by high-throughput screening of compound libraries against the target to identify hits and optimizing hits to create lead compounds with acceptable potency and selectivity towards the biological target and demonstrated efficacy in an animal model. Following that, the lead compounds are further refined to improve their effectiveness and pharmacokinetics before moving further with drug development (Figure 3). The discovery and development of innovative drugs is a complex process that takes around 12 years and an average of $1.8 billion to bring a new medication to market[66,67].

The process of IBD drug discovery begins with target discovery and selection followed by biological confirmation in cellular and animal models[68]; this is usually the pre-clinical phase. Several therapeutic targets of IBD have been identified, and some are listed in Table 1. Target identification in IBDs is based on qualitative relevance to the pathogenesis of the disease as well as increased magnitude of expression [68]. Biochemical pathways and specific proteins are targeted, and the drugs are developed based on the understanding of the mechanism of action of these targets. This is followed by pharmaceutical developments such as screening for safety, toxicity, pharmacokinetics and efficacy. At the preclinical stage, drugs are tested with targets to investigate levels of interactions and outcomes. Models and methods used differ between laboratories based on specific targets of interest.

For instance, in IBD, targets such as JAK/STAT and inflammatory mediator interleukins such as IL-12, IL-23, IL-6, IL-22Fc[69], tyrosine kinase and toll-like receptors[70] are explored using biologic agents at different stages of development. Following the *in vivo* studies using murine and human T cells, JAK/STAT inhibition by tofacitinib[71] has led to further application of the drug in several clinical trials. Integrins, a class of receptors that facilitate T lymphocyte trafficking into the gut[72] are targets as well. Integrins facilitate adhesive interactions between lymphocytes and endothelial cells, which leads to the trafficking[73] and can lead to T cell-dependent chronic intestinal inflammation in IBD[70]. Etrolizumab, an anti-adhesion agent approved by the FDA for treatment of IBD was subjected to *in vitro* testing, where it internalized β_7 integrin using cell culture models[74]. Etrolizumab selectively inhibits α 4β7 and αEβ7, which are involved in T lymphocyte homing in the gut[75]. Although there are challenges involved in the use of *in vivo* and *in vitro* methods in establishing IBD[68], conditions for the studies of potential drugs are optimized. The molecules with desired preclinical effect on the target go into clinical trial.

In clinical trials, drugs that scale through preclinical studies are expected to maintain remission in long-term mucosal healing. Clinical trial phases are regarded as long and extensive periods that take years from the point of research to the stage of approval of a candidate drug. Prior to these trials, detailed protocols that show the characteristics of the studies are provided[76]. The clinical phase comes

Table 1 Molecular targets for inflammatory bowel disease drug discovery

Target name	Abbreviation	Description	Disease implication	Modulatory effect of drug
Integrin alpha-4	ITGA4	A member of the family of integrins. Integrins alpha-4/beta-1 (VLA-4) and alpha-4/beta-7 are fibronectin and VCAM1 receptors. Integrin alpha-4/beta-7 is also a MADCAM1 receptor. On activated endothelial cells, VLA-4 integrin induces homotypic aggregation in the majority of VLA-4-positive leukocyte cell lines. ITGA4: ITGB1 binds fractalkine (CX3CL1) and may function as its coreceptor in fractalkine signaling dependent on CX3CR1[123]	ITGA4 upregulated in irritable bowel disease (IBD)	Inhibition
Interleukin 12B	IL12B	IL12B is also known as natural killer cell stimulatory factor 2 or p40, and it associates with IL23A to form IL23, a known stimulator of the JAK/signal transducer and activator of transcription (STAT) signaling pathway and a pathway with proven importance in IBD[124]	IL12B upregulated in IBD	Inhibition
Tumor necrosis factor	TNF	A type of cytokine, which binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is secreted by macrophages and is capable of triggering cell death of most tumor cell lines, although capable of promoting cell proliferation and induce cell differentiation under certain conditions[123]	TNF upregulated in IBD	Inhibition
Janus kinase 2	JAK2	A class of kinase, a non-receptor kinase that phosphorylates specific tyrosine residues on the cytoplasmic tails of the receptor. In the cytoplasm, JAK2 plays a pivotal role in signal transduction *via* its association with type I receptors such as growth hormone (GHR), prolactin (PRLR), leptin (LEPR), erythropoietin (EPOR), thrombopoietin (THPO) or type II receptors including IFN-alpha, IFN-beta, IFN-gamma, and multiple interleukins. It stimulates cell growth, development, differentiation or histone modification[123]	JAK2 upregulated in IBD	Inhibition
Prostaglandin-endoperoxide synthase 1 and 2	PTGS1/2	Also referred to as cyclooxygenase; are the primary enzymes involved in the synthesis of prostaglandin. They act both as a dioxygenase and as peroxidase, having two isozymes PTGS1 and PTGS2. This gene encodes the PTGS2 inducible isozyme. Its involvement in prostanoid-dependent inflammation and mitogenesis can be related to their regulation by specific stimulation[123]	PTGS1/2 upregulated in IBD	Inhibition
Peroxisome proliferator activated receptor gamma	PPARγ	A nuclear receptor. It consists of a group of approximately 50 transcription factors involved in many biological processes. It controls some regulatory genes involved in lipid metabolism and insulin sensitization as well as in inflammation and cell proliferation. It is highly expressed in the colon and majorly involved in bacterial-induced inflammation, also mediating the common aminosalicylate activities in IBD[125]. It acts as a critical regulator of gut homeostasis by suppressing nuclear factor-kappa B-mediated proinflammatory responses	PPARγ downregulated in IBD, mostly ulcerative colitis	Activation
Integrin subunit beta 7	ITGB7	Integrin alpha-4/beta-7 is an adhesion molecule that mediates lymphocyte migration and homing to gut-associated lymphoid tissue (GALT). The vascular endothelium of the gastrointestinal tract expresses MADCAM1, an adhesion molecule, which is the medium integrin alpha-4/beta-7 interacts with the gastrointestinal tract. VCAM1 and fibronectin found on the extracellular matrix of the cell also interacts with the integrin. Interaction with fibronectin is due to the CS-1 region[123]	ITGB7 upregulated in IBD	Inhibition
Nuclear receptor subfamily 3 group C member 1	NR3C1	This is a receptor recognized by glucocorticoids. It modulates the activities of cortisol and acts as a transcription factor that modulates the expression of its target genes[126]. It modulates inflammatory responses, cellular proliferation and differentiation in target tissues	NR3C1 downregulated in IBD	Activation
Janus kinase 3	JAK3	Non-receptor tyrosine kinase involved in signal transduction in the cytoplasm *via* its association with type I receptors sharing the common subunit gamma such as IL2R, IL4R, IL7R, IL9R, IL15R, and IL21R. It also plays a vital role in STAT5 activation. IBD pathology is associated with receptor-mediated signaling through the JAK and STAT DNA-binding families of proteins[127]	JAK3 upregulated in IBD	Inhibition
Arachidonate 5-lipoxygenase	ALOX5	ALOX5, an important member of the lipoxygenase gene family, exclusively involved in IBD development[128].	ALOX5 upregulated in IBD, especially	Inhibition

		Catalyzes the oxygenation of arachidonate (5Z,8Z,11Z,14Z)- eicosatetraenoate) to 5-hydroper-oxyeicosatetraenoate (5-HPETE) followed by the dehydration to 5,6- epoxyeicosatetraenoate (leukotriene A4/LTA4), the steps in the biosynthesis of leukotrienes, that mediates inflammation[123]	ulcerative colitis	
Tyrosine kinase 2	TYK2	A non-receptor kinase that carries out both structural and catalytic roles in numerous cytokines and interferons signaling. TYK2 plays a key role in mediating signaling and functional responses downstream of the IL-12, IL-23, and type I interferon (IFN) receptors and TYK2-mediated IL-12, IL-23 and type I IFN signaling activates STAT-dependent transcription, which promotes chronic inflam-mation[129]	TYK2 upregulated in IBD	Inhibition
Phosphoribosyl pyrophosphate aminotransferase	PPAT	PPAT is a key rate-limiting reaction in purine biosynthesis, transferring gamma-nitrogen from glutamine to 5-phosphoribosyl pyrophosphate (PRPP)[130]	PPAT upregulated in IBD	Inhibition
Vitamin D receptor	VDR	A nuclear, ligand-dependent transcription factor that regulates the expression of T cells and genes involved in different physiological functions when in complex with hormonally active vitamin D, 1,25(OH)2D3[131]. VDR plays a multifaceted role in the pathogenesis of IBD and is crucial in regulating autophagy, immune response, tight junctions, gut microbiome, and metabolites[132]	VDR downregulated in Crohn's disease	Activation
Matrix metallopeptidase 1	MMP1	An interstitial collagenase, that digests the spiral structure of collagen types I, II, III and X, subjecting them to hydrolysis by gelatinase and are major players in extracellular matrix degradation[123]	MMP1 upregulated in IBD	Inhibition
Matrix metallopeptidase 7	MMP7	A metallopeptidase member necessary for neutrophil migration into the airspaces by cleaving syndecan-1, a heparin sulfate proteoglycan necessary for the establishment of a neutrophil chemokine gradient[133]. Degrades casein, gelatins I, III, IV and V, and fibronectin and is responsible for the activation of procollagenase[123]	MMP7 upregulated in IBD	Inhibition
Dihydrofolate reductase	DHFR	An enzyme that converts dihydrofolate to tetrahydrofolate in folate metabolism and involved in purine and mitochondrial thymidylate biosynthesis[123]	DHFR upregulated in Crohn's disease	Inhibition
Matrix metallopeptidase 13	MMP13	A member of the family of collagenases. Matrix substrates of MMP13 include native collagen, gelatin and aggrecan. Lipopolysaccharide (LPS)-induced shock and dioctyl sodium sulfosuccinate (DSS)-induced colitis induce MMP13 upregulation in the gut, which results in MMP13-mediated shedding of transmembrane-bound TNF and release of bioactive, soluble TNF, thus triggering a cascade that leads to leakage of intestinal components, which increases systemic inflammation and subsequent organ damage[134]	MMP13 upregulated in IBD	Inhibition
Sphingosine-1-phosphate receptor 1	S1PR1	A type of G-protein-coupled receptor. S1P binds to the S1PR1, which triggers angiogenesis, endothelial barrier enhancement, blood vessel constriction, heart rate modulation and lymphocyte trafficking[135]	S1PR1 downregulated in IBD	Activation
ATPase H+/K+ transporting subunit alpha	ATP4A	A P-type cation-transporting ATPase. The gastric H+, K+-ATPase is a heterodimer made of high molecular, weight catalytic alpha subunit with a glycosylated beta subunit. It is a proton pump that catalyzes the hydrolysis of ATP coupled with the exchange of H (+) for K (+) ions across the plasma membrane and also responsible for gastric acid secretion due to its ability to generate proton gradient across the membrane[123]	ATP4A upregulated in IBD	Inhibition

IBD: Inflammatory Bowel Disease; ITGA4: Integrin alpha-4; IL: Interleukin; TNF: Tumor necrosis factor; JAK: Janus kinase; PTGS1/2: Prostaglandin-endoperoxide synthase 1 and 2; PPARγ: Peroxisome proliferator activated receptor gamma; ITGB7: Integrin subunit beta 7; NR3C1: Nuclear receptor subfamily 3 group C member; ALOX5: Arachidonate 5-lipoxygenase; TYK2: Tyrosine kinase 2; PPAT: Phosphoribosyl pyrophosphate aminotransferase; VDR: Vitamin D receptor; MMP: Matrix metallopeptidase; DHFR: Dihydrofolate reductase; S1PR1: Sphingosine-1-phosphate receptor 1; ATP4A: ATPase H+/K+ transporting subunit alpha; VLA-4: Very late antigen-4; VCAM 1: Vascular cell adhesion molecule 1; MADCAM1: Mucosal vascular addressin cell adhesion molecule 1; CX3CL1: C-X3-C Motif Chemokine Ligand 1; CX3CR1: C-X3-C Motif Chemokine Receptor 1; STAT: Signal transducer and activator of transcription; TNFRSF: Tumor necrosis factor receptor superfamily; GHR: Growth hormone receptor; PRLR: Prolactin receptor; LEPR: Leptin receptor, EPOR: Erythropoietin receptor; THPO: Thrombopoietin; IFN: Interferon; PTGS1&2: Prostaglandin G/H synthase 1 & 2; GALT: Gut-associated lymphoid tissue; 5-HPETE: 5-hydroperoxyeicosatetraenoate; LTA4: Leukotriene A4; prpp: Phosphoribosyl pyrophosphate.

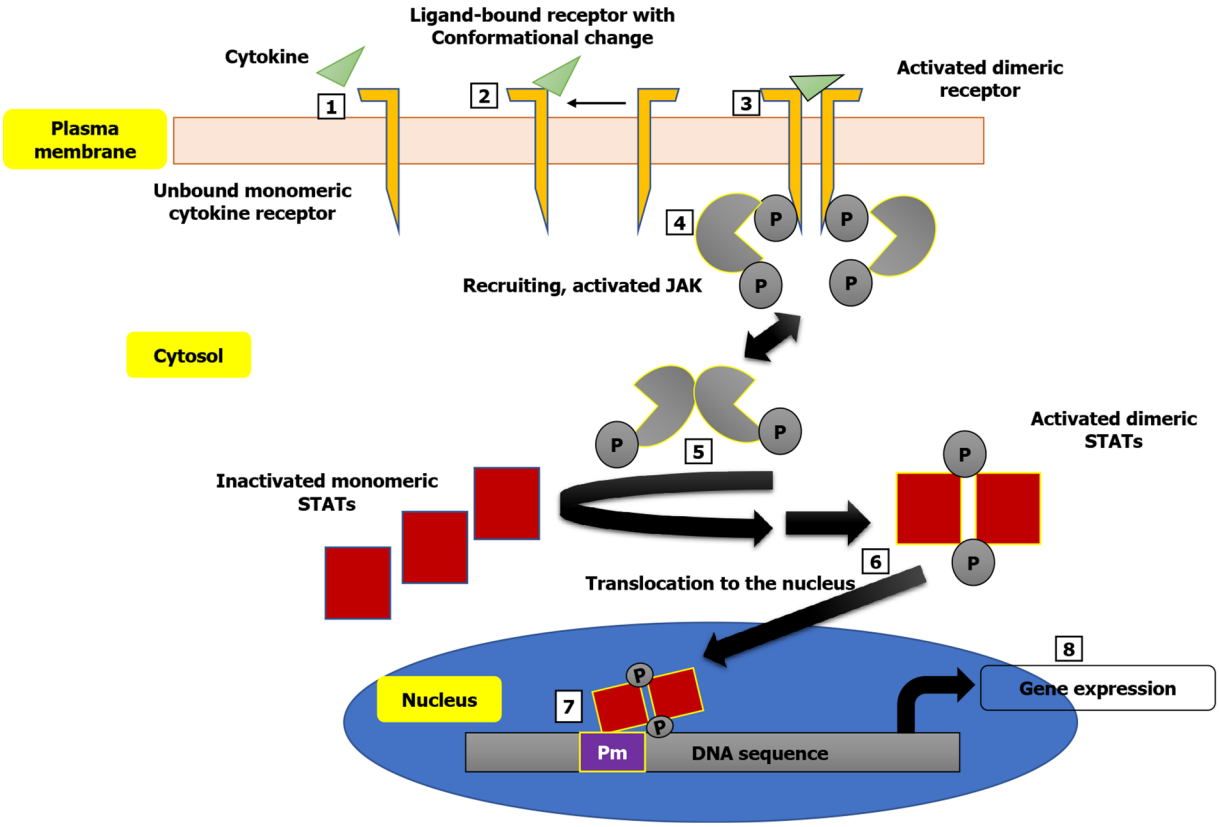

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Figure 1 A schematic description of the Janus kinase/STAT pathway. 1: Cytokine and cytokine receptor before interaction; 2: Ligand-reception interaction brings about conformational change that recruits the second cytokine receptor to dimerize with the first cytokine receptor; 3: The activated dimeric receptor recruits Janus kinase (JAK); 4: Thereby causing JAK to phosphorylate itself and the cytoplasmic tail of the cytokine receptor on the tyrosine residue; 5: Activated JAK phosphorylates monomeric STAT transcription factor, hence causing it to disengage from its endogenous inhibitor and dimerize; 6: The activated dimeric signal transducer and activator of transcription translocates to the nucleus; 7: Where it binds to a promoter; 8: That causes the gene expression of some inflammatory markers. JAK: Janus kinase; STAT: Signal transducer and activator of transcription.

after certainty has been established that the drugs/molecules are effective and non-toxic. Clinical trials involve three main phases before approval, known as phase I, phase II, and phase III. The first two phases are early phases.

The phase I is the stage in drug development when testing and pharmacokinetics studies are carried out using healthy or ill subjects[76] to ascertain safety level of the drugs. The pharmacokinetics and pharmacodynamics of the drugs are determined using volunteer patients or patients who are not responsive to previous treatments in order to determine drug safety alongside desired clinical activities and an Absorption, Digestion, Metabolism and Excretion (ADME) profile[68]. For instance, a phase I study of Japanese patients with UC treated with vedolizumab, an anti-integrin antibody, showed an appreciable level of tolerance in the desired clinical activity of the drug as well as a satisfactory level of pharmacodynamics and pharmacokinetics[77]. The outcome of this phase determines whether the drug scales through to the next phase. The phase I studies can last for up to many years and part of the process involves identification of adverse effects or events other than the occurrence(s) observed in the preclinical stage. Foreknowledge of a possible mechanism of action of a drug can also guide expectations in clinical trials.

The phase II is the stage that involves dose ranging. The efficacy of the drugs are determined alongside the optimum dose that would be considered safe[76]. At the point of dose determination, the outcome of the effect of different doses would guide the decision to proceed to the next phase. A phase II study of varied doses of ozanimod, an agent that targets sphingosine-1-phosphate receptors 1 and 5, was used to treat adult patients with moderate to severe CD based on an uncontrolled multicenter trial within 12 wk and the effects of different doses at different intervals were observed[78]. A drug that scales through is launched into the third phase.

Phase III is the stage whereby the efficacy of the drug is compared with that of an already established standard. Patients recruited for this phase are usually from the general population compared to phase I and II. The side effects of the drugs are studied as well as their effectiveness, comparing them with common treatments to help guide safety and proper use. Currently, etrolizumab, is undergoing a phase III clinical trial for treatment of IBD using six multicenter prospective randomized controlled trials and two open-label extension studies[75]. Upon conclusion of the studies, the drugs are considered for

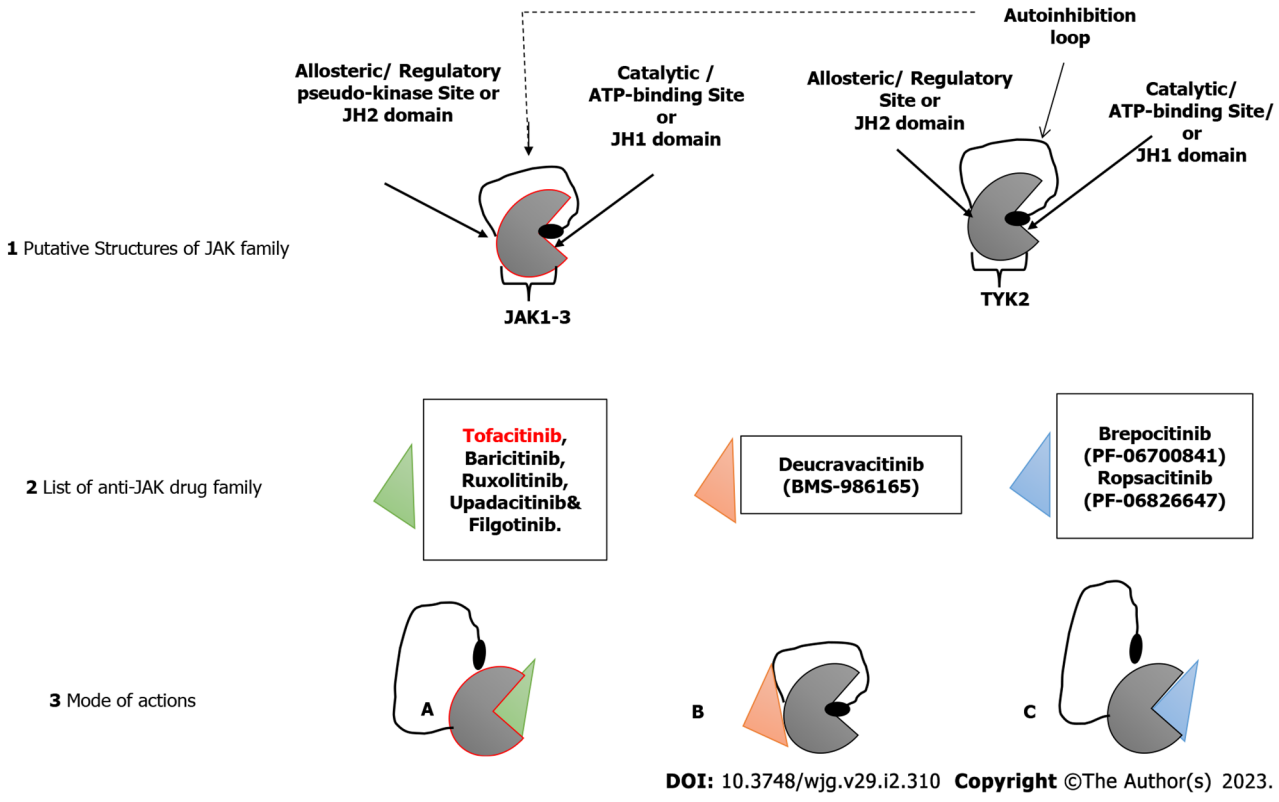

Figure 2 Putative representations. 1: Janus kinase (JAK) 1, 2 or 3, and tyrosine kinase 2 (TYK2) with their respective catalytic/ATP-binding/Janus homology (JH) 1 site, regulatory/allosteric/JH2 site and autoinhibition loop; 2: List of anti-JAK drug family differentiated with green, pink and blue shapes; 3: Different modes of action of the drugs differentiated with the colors of the shape: (A) Drugs in group A (*e.g.*, tofacitinib, baricitinib, ruxolitinib, upadacitinib and filgotinib) block the ATP-binding site of either/both JAK 1 or 2, while the inhibition loop is disengaged; (B) Drugs in group B (*e.g.*, deucravacitinib) bind to the allosteric site of TYK2, thereby stabilizing the autoinhibition loop from disengaging from the catalytic domain; (C) Drugs in group C (*e.g.*, brepocitinib and ropsacitinib) are dual inhibitors and have a similar mode of action as "A" by binding to the catalytic site of their targets but blocking the catalytic site of both JAK 1 and TYK2 or JAK2 and TYK2, respectively. JAK: Janus kinase; TYK: Tyrosine kinase; JH: Janus homology.

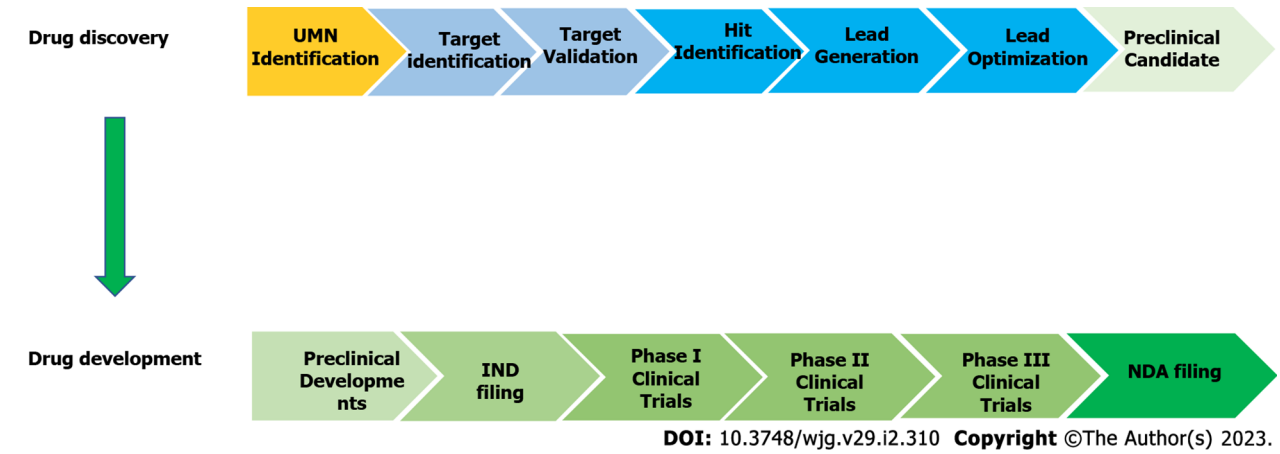

Figure 3 Overview of the drug discovery and development process. IND: Investigated new drug application; NDA: New drug application; UMN: Unmet medical needs.

approval based on FDA standards. Drugs are reviewed by the FDA based on the evidence of preclinical and clinical outcomes and approved for marketing based on the terms of the FDA.

ROLE OF COMPUTATIONAL METHODS IN IBD DRUG DISCOVERY

The use of computational methods has become a crucial part of the drug discovery process[18]. A

number of commercially available medications as well as several clinical candidates have been discovered or improved with the help of molecular modeling techniques. CADD combines multiple chemical-molecular and quantum approaches to the discovery, design and development of drugs. Structure-activity relationships form the basis of many CADD methods. The objectives of CADD are multidisciplinary in nature, with the end goal being the modification of bioactive molecules, the formation of therapeutic alternatives and the knowledge of biological events at the molecular level[79].

From drug identification to pharmacological role discovery and from preclinical testing to drug marketing, computers have played a fundamental role in the entire drug discovery and development process[15]. In addition, CADD opens the door to the possibility of drug repurposing, which is the systematic identification of new potential uses from drugs that have already been approved for other indications[79]. According to the results of a computational drug repurposing study conducted by Bai *et al*[80], atorvastatin shows promise as a novel treatment approach for improving symptoms in patients with UC. They developed a framework for systematically integrating publicly accessible heterogeneous molecular data with clinical data on a large scale in order to repurpose FDA-approved drugs for a wide variety of human diseases.

Computational methods are useful in various aspects of modern drug discovery for IBD. These include but are not limited to genetic studies for the identification of pathways linked to IBD pathogenesis, target identification, and virtual screening.

IBD genetic studies

Computational methods have made tremendous contributions to the field of IBD genetic studies. As mentioned earlier, genetic factors contribute to the pathogenesis of IBD and the understanding of the molecular events are important for target identification. The advent of GWAS is a turning point in the history of genetic research into complex human disease. GWAS is a technique for identifying genetic markers that are linked to an increased probability of developing a disease or exhibiting a certain trait. The strategy entails analyzing the genomes of a large number of people to identify genetic variations that are more common in people who have a disease or a particular trait than in those who do not. Following the discovery of such genomic variants, researchers often use GWAS to search for other potentially causal variants located in close proximity[81]. IBD is a prime example of the usefulness of GWAS and related analyses. Using GWAS based on SNPs, researchers were able to identify 163 genetic loci and numerous signaling pathways that are linked to IBD[82]. Many of these loci have pleiotropic effects, and risk prediction models were developed using a wide variety of genetic variations. Key contributions of the gut microbiome and important interactions between genes and the environment are emerging as a result of these studies[83].

The development of computational methods has contributed significantly to the identification of the vast quantity of genetic determinants. There are a number of different software packages available for use in GWAS research[84], and a common example is PLINK. In addition to standard GWAS features like quality control filtering and SNP association testing, PLINK also includes more advanced tools like gene-based analysis, annotation and epistasis testing in a compact, user-friendly package. PLINK initially discovered the actin-related protein 2/3 complex subunit 2[85], IL10, HNF4a, cadherin-3, cadherin-1 and laminin subunit beta-1[33] loci and recently discovered 163 IBD loci[82]. In the majority of GWAS-based genetic studies of IBD, PLINK has served as the primary analytical tool. A linear mixed model, which accounts for both fixed and random effects, has been used in GWAS in recent years. Factored Spectrally Transformed Linear Mixed Models[86], Efficient Mixed-Model Association expedited[87] and Genome-wide efficient mixed-model association (GEMMA)[88] are all examples of linear mixed model-based software. They are able to adjust for both overt and covert connections within a population simultaneously. Multiple correlated phenotypes can be analyzed with GEMMA as well.

There is a plethora of pathway analysis software packages available, many of which are based on GWAS[89]. The development and implementation of GWAS-based pathway software has frequently been illustrated with CD as an example. To examine the CD dataset from the Wellcome Trust Case Control Consortium, Wang *et al*[90] used the GenGen software to find significant associations of the IL12/IL23 pathway with CD status in multiple cohorts genotyped with different SNP chips and from different ethnic backgrounds[91]. This was among the first analyses of IBD pathways based on GWAS data. IL3 activation and signaling pathway were also linked to CD, based on the research by Torkamani *et al*[92]. They used the MetaCore program, which is a for-profit product created by GeneGo Inc. (St. Joseph, MI, United States).

Using the statistical technique of Simes/false discovery rate, Peng *et al*[93] discovered significant enrichment of the JAK/STAT signaling pathway, as well as the cytokine-cytokine receptor interaction. With a model-based strategy, Carbonetto *et al*[94] found a similar result for a number of cytokine signaling pathways. Holmans *et al*[95] used the software ALIGATOR to discover the involvement of MHC genes in addition to IL/cytokine signaling. Similarly, Jostins *et al*[82] examined the largest IBD GWAS to date for enrichment of canonical pathways or Gene Ontology terms and discovered significant enrichment of Gene Ontology terms pertaining to the regulation of cytokine production, lymphocyte activation, and the JAK/STAT signaling pathway.

It is possible that the differences between the studies can be attributed to the different statistical methods and/or the different pathway databases used in their studies. However, all of these pathways were related to interleukins and the immune system. Torkamani *et al*[92] found a significant association between calcium signaling and the carbohydrate response element binding protein regulation pathway; Peng *et al*[93] found a significant association between ABC transporters and the extracellular matrix receptor interaction. Numerous user-friendly web-based software programs, such as improved Gene Set Enrichment Analysis for (i-GSEA4) GWAS[96] and GSEA-SNP[97], have been developed for pathway analysis using GWAS data.

IBD target identification

Following the discovery of the biological basis of a disease, the next step in drug discovery is the identification of potential drug targets. The most promising targets for drug discovery are hypothesized to be highly prevalent in the disease-affected population, to have a well-established function in the underlying pathology and to be directly linked to the disease of interest. Potential drug targets are defined as disease-modifying rather than disease-causing. In the present day, a wide range of methods, both experimental and computational, are used to identify drug targets. The experimental methods rely heavily on comparative genomics, with phenotype and gene association analysis serving as complementary tools. All experimental methods yield credible findings, but they have significant drawbacks, including the high cost and extensive scientific labor needed to experimentally probe the entire space of chemical compounds to identify viable drug targets[98]. In light of these drawbacks, researchers and pharmaceutical companies increasingly rely on computational methods for initial investigations before turning to experimental approaches for validation and other purposes.

Various bioinformatics resources are available for the identification of drug targets as illustrated in Table 2. These programs efficiently process a large volume of data from genomic, transcriptomic and proteomic databases and ultimately provide potential drug targets in a short period of time and at a low cost. Several computational methods are currently accessible, each of which makes use of a unique type of molecular information, such as a gene or genomic sequence, molecular interaction data or the 3D structure of a protein[98]. There are strong connections between most of these methods.

Mohan *et al*[99] reported using genetic databases to find new molecular targets for IBD. They used four different genetic databases to categorize the protein-coding genes associated with UC (3783 genes), CD (3980 genes), uveitis (1043 genes), arthritis (5583 genes), primary sclerosing cholangitis (1313 genes), and pyoderma gangrenosum (119 genes). The databases used were Genecards: The Human Gene Database, DisGeNET, the Comparative Toxicogenomics Database, and the Universal Protein Resource. Then, they used Network Data Exchange to map a distinct signal pathway based on the identified common genes underlying the aforementioned diseases. Across UC, CD, uveitis, arthritis, pyoderma gangrenosum and primary sclerosing cholangitis, they identified a distinct set of 20 genes with the highest probability of overlap. Different disease processes were linked to some unique immune modulators. IL-25 and monensin-resistant homolog 2 were observed in UC, CD, pyoderma gangrenosum, and arthritis. Arachidonate 5-lipoxygenase was found to contribute to the development of UC, CD, and arthritis. The involvement of solute carrier organic anion transporter family member 1B3 is unique to pyoderma gangrenosum, UC, and CD. TNF was found to be involved in the pathogenesis of UC, CD, psoriatic spondylitis, and arthritis.

Virtual screening

Drug discovery relies heavily on the physical screening of large chemical libraries for biological targets in order to find new lead compounds. High-throughput screening is a method for finding active molecules in experiments by analyzing more than a million compounds biochemically. However, developing and deploying this technology takes a long time and a sizable investment. Therefore, virtual high-throughput screening was developed as a more affordable and effective calculation method. This technique has seen extensive use in the earliest stages of drug discovery. The goal is to search through huge compound libraries to find the structure of a novel, active small molecule. To some extent, this supports the goals of high-throughput screening. Virtual screening saves money by reducing the number of compounds used to measure pharmacological activity, while high-throughput screening uses all compounds in the database[18,24,100]. In this sub-section, we will go over the various virtual screening techniques that are commonly used in IBD drug discovery.

Molecular docking: Molecular docking is commonly used in the drug discovery and development process because of its ability to predict interaction patterns between proteins and small molecules as well as proteins[18,101,102]. The principle behind this phenomenon proposes that ligand and receptor recognition is predicated on a similarity in spatial shape and energy. Understanding the action mechanism of a drug requires first establishing its binding conformation to a specific protein receptor [24]. The goal of docking is to accurately assess the strength of binding by fitting the structure of a ligand within the requirements of a receptor binding site[103]. The most frequently cited molecular docking software packages for use in drug discovery are summarized in Table 3.

Table 2 Bioinformatics resources for the identification of drug targets

Tool/Database	Description
Open Targets Platform	To facilitate systematic target identification and prioritization for drug discovery based on underlying evidence, the Open Targets Platform offers users a searchable knowledgebase and user interface[123]
SELF-BLM	A self-training support vector machine-based bipartite local model that predicts drug-target interactions[136]
iDTIESBoost	A model for detecting drug-target interactions based on evolutionary and structural features[137]
GEO	Database that stores array- and sequence-based transcriptomics data that can be applied to functional genomics[138]
DASPfind	Predicts drug-target protein interactions that stem from shared structural features[139]
NetCBP	Network methods for predicting drug-target interactions. Furthermore, it suggests new drugs even when no data on their interactions with their targets are available[140]
DbMDR	Offers a database of multidrug resistance (MDR) genes and their orthologs, which could be used to develop new treatments [141]
TDR targets	Drug development molecular target identification and prioritization[142]
DrugBank	An extensive drug database with annotations covering drug targets and mechanisms of action[143]
PDTD	Database of potential proteins for *in silico* drug target identification[144]
DEG	Contains all known essential genes from different organisms[145]
TTD	Publicly accessible cross-links database that provides inclusive information about known therapeutic targets with related information, *i.e.* pathway information and the corresponding drugs/ligands[146]
KEGG	Offers information about the pathway, gene and ligands in three different databases, *i.e.* Pathway, Gene and Ligand[147]
Genecards	Officially known as Genecards: The Human Gene Database, it is an all-inclusive, authoritative compilation of annotative information about human genes[148]
DisGeNET	A public resource that houses a massive database of genetic variants and their links to human disease[149]
CTD	The Comparative Toxicogenomics Database is a vast, freely accessible database with the objective of increasing understanding of the effects of environmental exposures on human health. It includes information on chemical-gene/protein interactions, chemical-disease relationships and gene-disease links that has been curated by humans[150]
UniProt	The Universal Protein Resource is the world's most comprehensive, high-quality and freely accessible database of protein sequence and functional information[151]

SELF-BLM: Self-training bipartite local model; GEO: Gene Expression Omnibus; UniProt: Universal Protein Resource; CTD: Comparative Toxicogenomics Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; TTD: Therapeutic Target Database; DEG: Database of Essential Genes; PDTD: Potential Drug Target Database; DbMDR: Database of multidrug resistance: NetCBP: Network-Consistency-based Prediction.

Keretsu *et al*[104] used computational methods to design new JAK1 inhibitors from a series of pyrrolopyridine derivatives. Autodock 4.2 was utilized to predict the protein-ligand binding. Tofacitinib, an established ligand, which was co-crystallized with the structure of JAK1 (protein data bank ID: 3EYG) obtained from the protein data bank (www.rcsb.org), was used as a reference compound to validate the docking procedure. The docked conformation of tofacitinib closely matched that of one obtained in the crystal structure. The binding affinity of compound 42 for JAK1 was found to be -10.2 kcal/mol. The F958 and L959 residues of the protein formed H-bond interactions with the pyrrolopyridine moiety. As a result, the methyl group of the methyl piperidine moiety protruded beyond the binding pocket and into the bulk solvent. The chlorobenzyl group slid into the hydrophobic pocket made by the residues of the activation loop, the α-helix and the P-loop. The selected binding conformation was prepared for further MD simulation study. The finding of the study was presented as a possible guide in the design of more effective JAK1 inhibitors[104].

In order to identify the bioactive compounds of the *Phyllanthus nivosus* leaf responsible for its activity against UC for further drug design, Johnson *et al*[101] combined molecular docking with an *in vivo* study. Levels of TNF-α, IL-6, nitric oxide, malondialdehyde, reduced glutathione, superoxide dismutase and catalase in the serum of rats experimentally induced with UC and treated with *Phyllanthus nivosus* were monitored. The bioactive ingredients of the most active fraction were identified using gas chromatography-mass spectrometry followed by molecular docking against IL-1b converting enzyme (caspase-1), beta-2 adrenergic receptor, cyclooxygenase-2 and TNF-α. The study led to the identification of ethyl iso-allocholate cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate and alpha-cadinol as promising compounds for further development into drugs for the treatment of UC[101].

In another study, Halder *et al*[105] used molecular docking in combination with other *in silico* techniques for the repurposing of FDA-approved medications and provided a framework for drug exploration and computational methods in the discovery of drugs for the treatment of IBD. After being imported and processed using Protein Preparation Wizard and LigPrep, respectively, the molecular

Table 3 Molecular docking programs most frequently employed in drug discovery

Program	Description	Website
AutoDock [152]	A docking toolkit. It is meant to foretell the binding mode of small molecules to a receptor with a known 3D structure, such as a substrate or a drug candidate. There have been multiple engines developed, and it has undergone constant evolution and refinement over the years to incorporate new features	https://autodock.scripps.edu/
AutoDock Vina[153]	One of the AutoDock Suite's docking engines. It is a free and open-source molecular docking software. Dr. Oleg Trott of The Scripps Research Institute's Molecular Graphics Lab (now CCSB) created and initially implemented the system. The most recent version of AutoDock Vina is v.1.2.0	https://vina.scripps.edu/
Hex[154]	Invented by Dave Ritchie and is a program for molecular superposition and protein docking. Hex can read protein and DNA structures in the Protein Data Bank format as well as small-molecule SDF files. It has been downloaded over 40000 times as of December 2015	http://hex.loria.fr/
MOE[155]	Integrated computer-aided molecular design platform for small molecule and biological therapeutics. Common platform for chemists, biologists and crystallographers. Small Molecules - Peptides – Biologics	https://www.chemcomp.com/
Glide Schrodinger [156]	Provides a full range of speed *vs* accuracy options, ranging from the high-throughput virtual screening mode that efficiently enriches million compound libraries to the standard precision) mode that reliably docks tens to hundreds of thousands of ligands with high accuracy to the extra precision mode that eliminates false positives by sampling more extensively and using more advanced scoring, resulting in even higher enrichment	https://www.schrodinger.com/

CCSB: Center for Computational Structural Biology; SDF: Structure data files; DNA: Deoxyribonucleic Acid; MOE: Molecular operating environment.

target TNF (protein data bank ID: 2AZ5) with a small molecule inhibitor and the FDA-approved drugs (from the Zinc database) were subjected to molecular docking, ADMET analysis and binding free-energy calculation [Molecular mechanics with generalised Born and surface area solvation (MMGBSA)]. Following that, the medications were ranked based on docking score, ADMET parameters and MMGBSA dG binding score. The selected drugs were then subjected to an induced-fit docking study. The MD simulation study was conducted on the two most promising compounds, iopromide (ZINC000003830957) and deferoxamine (ZINC000003830635). Finally, the bioisosteric substitution was applied to enhance the ADMET properties of these compounds[105].

Pharmacophore modelling: Virtual screening of databases with the pharmacophore model has become one of the most important ways to find new lead compounds as compound databases and computing power have advanced. A pharmacophore is a conceptual description of the molecular features required for molecular recognition of a ligand by a biological macromolecule, which describes how structurally distinct ligands can bind to the same receptor site. To achieve therapeutic efficacy, drug molecules adopt an active conformation that is both geometrically and energetically complementary to that of the target macromolecule.

Medicinal chemists have discovered that modifications to specific chemical groups in drug molecules greatly affect the interaction between drugs and targets, while modifications to other groups have little to no effect[106]. Additionally, it was discovered that molecules exhibiting the same activity share common properties. Accordingly, Ehrlich proposed the idea of pharmacophores in 1909[107], which referred to the molecular framework of atoms with active essential characteristics. In 1977, Gund[108] provided further elaboration on the concept of pharmacophores as a class of molecules that recognize receptors and form structural features of molecular biological activity.

Pharmacophores can be discovered using one of two common approaches. If the structure of the target molecule is known, then the structure of the pharmacophore can be inferred using techniques like conformational analysis and molecular folding[109]. The pharmacophore recognition procedure will then choose an active compound that can be used to create the model. Conversely, pharmacophore studies are conducted on a number of compounds when either the structure of the target or its action mechanism is still unknown; this allows for a summary of data on certain groups that are crucial to the activity of the compound[24].

Babu *et al*[110] combined ligand-based pharmacophore modeling with virtual screening and molecular docking to find JAK1 inhibitors with high potency and selectivity. In the first step, they developed ligand-based pharmacophore models and checked them for accuracy with potency and selectivity validation techniques. A pharmacophore-based virtual screening was carried out on eight selected pharmacophore models using six different databases. ADME prediction and molecular docking were used to narrow down the hits found during screening. Docking results were verified using the binding free-energy calculation and induced fit docking techniques. A cross docking analysis was then performed to determine which lead compounds are selective for JAK1. In the end, five promising compounds were chosen and subjected to further investigation using MD and density functional theory. T5923555 and T5923531 were identified as the most promising leads among the five compounds and will be pursued for additional validation *via in vitro* and *in vivo* techniques.

QSAR: QSAR is frequently used in the drug discovery process to find compounds with desirable inhibitory effects on target proteins, with minimal side effects (nonspecific activity). The QSAR model is a quantitative investigation into the relationships that exist between small organic molecules and large biological macromolecules. The calculated properties of molecules (such as the absorption, distribution and metabolism of small organic molecules in living organisms) are correlated with their biological activity, as determined experimentally[111]. QSAR is the most precise and efficient approach to drug design when the structure of the receptor being targeted is unknown.

In the 1980s, 3D structural information was incorporated into the quantitative structure-activity relationship to form the 3D-QSAR method. Since the 1990s, structure-based drug design has increasingly replaced QSAR in the area of drug design due to the increase in computational power and the availability of the 3D structure of many biomolecules. However, QSAR with its advantages of small amount of calculation and good predictive ability[112], continues to play an important role in pharmaceutical studies.

3D-QSAR enables the investigation of the three-dimensional structure of bioactive compounds and the correct representation of energy changes and interaction patterns between bioactive molecules and receptors. Different drugs are evaluated by fitting their physicochemical and 3D structural parameters to the quantitative relationship. The structures of the newly created compounds are subsequently predicted and improved upon. 3D-QSAR analysis is a research method that integrates QSAR with computational chemistry and molecular graphics. It is an effective method for determining the nature of drug-target interactions, creating hypothetical images of simulated targets, determining the correlation between drug structure and activity and developing new medications. In addition, predictive 2D-QSAR models, 3D-QSAR models and 3D target and ligand-based approaches have all been developed for the purpose of finding IBD drugs[24].

Yang *et al*[113] incorporated molecular docking and QSAR study in the design of a series of TNF-α converting enzyme (TACE) inhibitors with the ability to bind in the S1′ pocket of the enzyme. A total of 12 analogues were synthesized by altering the chain length and alkylation pattern on the aromatic ring of the side chain. Most compounds inhibited cellular TNF-α production and TACE *in vitro*. The most promising compound from *in vitro* and *in vivo* pharmacokinetic studies had a moderate systemic clearance and a good oral bioavailability of 42%. It was also tested in a rat model of carrageenan-induced paw edema and found to be effective at reducing edema in the animal's paws. The series of α-alkoxyaryl alkyl substituted chromen-based analogues was then validated by means of a QSAR study and docking. Coumarin core TACE inhibitors with long, bulky α-substituent groups are able to enter the S1′ and S3′ pockets, where they form van der Waals interactions, with increased inhibitory activity. The docking study of the compound demonstrated its dual-function inhibitory activity toward TACE and matrix metallopeptidase-3. Based on the resulting QSAR descriptors, new α-substituted chromen-based TACE inhibitors with enhanced TACE inhibitory activity can be developed.

MD simulation in IBD drug discovery: MD simulation[102,114-116] is another popular approach to studying biomolecules; it is based on Newtonian mechanics and applies empirical molecular mechanics force fields. The drug discovery process can be aided by using explicit/implicit solvent models, which allow for simulations of time and space, and all-atom, united-atom and coarse-grained MD simulations [117,118]. MD simulations have typically been used to identify potential drug binding sites on target proteins, calculate the binding free energy between proteins and ligands, determine the mechanism of action of drug molecules, and more[119,120].

Taldaev *et al*[121] used MD to create structural representations of the arrangement of binding sites for the JAK family enzymes in order to elucidate the selectivity of upadacitinib for JAK1 among other isoforms. They found that the high affinity of upadacitinib was due to its ability to form four hydrogen bonds with amino acid residues in the hinge region of JAK1 as opposed to just two with other JAK isoforms. Structural features of the JAK1 binding site, including the unique residues S963 and E966, are responsible for stabilizing the molecule at the hinge region, as proposed by the authors. Hydrogen bonding with the JAK1 (E883) and JAK2 (N859) amino acid residues in the glycine loops was reported to increase the affinity. The research findings were presented as having the potential to direct the creation of more selective and effective next-generation JAK inhibitors, thereby enhancing the treatment of a wide range of cytokine-mediated diseases.

In another MD simulation research conducted by Du *et al*[122], the interaction mechanism between oncostatin M (OSM) and its receptor (OSMR) at the atomic level was predicted. Binding of OSM to OSMR is said to be implicated in the pathogenesis of IBD. The OSMR interaction domain was built using the homology modeling approach. Docking was used to establish the near-native structure of the OSM-OSMR complex, and long-time scale MD simulation in an explicit solvent was used to sample the conformations when OSM binds to OSMR. Following the equilibration of the simulated system, the per-residue energy contribution was determined to describe the key residues for the formation of the OSM-OSMR complex. Premised on these key residues, eight residues (OSM: Arg100, Leu103, Phe160, and Gln161; OSMR: Tyr214, Ser223, Asp262 and Trp267) were identified as "hot spots" by computational alanine mutagenesis analysis and confirmed by further MD simulation of the R100A (one of the discovered "hotspots") mutant. Furthermore, the FTMap analysis revealed six cavities at the OSM-

OSMR interface, which were proposed as key binding sites. The predicted 3D structure of the OSM-OSMR complex and the discovered "hotspots" provide useful information in understanding the OSM-OSMR interactions, and the identified locations serve as potential targets in designing small molecules to inhibit the interactions[122].

CONCLUSION

The global prevalence of IBD is increasing, with developing countries experiencing an increase due to modernization. There is still a need for a new generation of alternative therapies due to the loss of response to biologic drugs, which can be caused in part by the immunogenicity of the administered protein as well as the need to discontinue drugs due to intolerance or side effects. The development of small molecule drugs is difficult because of the complexity of the molecular pathways involved in the progression of disease. The conventional drug design and development processes are lengthy, expensive and filled with arduous scientific procedures. However, computational tools show considerable promise as a practical means of creating new small molecules with biomedical application. There have been a number of groundbreaking successes with drugs for several diseases developed using CADD. Many of these drugs are either FDA-approved or under clinical trials. However, computational methods appear to be underutilized for IBD drug research, as observed during this literature search. To hasten efforts in finding treatments for IBD, more scientists need to turn to computational methods. The modern drug discovery process for IBD makes use of a wide range of computational tools, some of which are geared toward specific tasks such as genomic studies, target identification, and virtual screening.

FOOTNOTES

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MINIREVIEWS

Current opinion on the regulation of small intestinal magnesium absorption

Siriporn Chamniansawat, Nasisorn Suksridechacin, Narongrit Thongon

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Abstract

Magnesium (Mg^{2+}) has an important role in numerous biological functions, and Mg^{2+} deficiency is associated with several diseases. Therefore, adequate intestinal absorption of Mg^{2+} is vital for health. The small intestine was previously thought to absorb digested Mg^{2+} exclusively through an unregulated paracellular mechanism, which is responsible for approximately 90% of total Mg^{2+} absorption. Recent studies, however, have revealed that the duodenum, jejunum, and ileum absorb Mg^{2+} through both transcellular and paracellular routes. Several regulatory factors of small intestinal Mg^{2+} uptake also have been explored, *e.g.*, parathyroid hormone, fibroblast growth factor-23, apical acidity, proton pump inhibitor, and pH-sensing channel and receptors. The mechanistic factors underlying proton pump inhibitor suppression of small intestinal Mg^{2+}, such as magnesiotropic protein dysfunction, higher mucosal bicarbonate secretion, Paneth cell dysfunction, and intestinal inflammation, are currently being explored. The potential role of small intestinal microbiomes in Mg^{2+} absorption has also been proposed. In this article, we reviewed the current knowledge on the mechanisms and regulatory factors of small intestinal Mg^{2+} absorption.

Key Words: Hormone; Magnesium absorption; Paneth cells; Proton pump inhibitor; Regulation; Small intestine

Core Tip: Small intestinal epithelium absorbs digested magnesium (Mg^{2+}) through both transcellular active and paracellular passive mechanisms. Several regulatory factors of small intestinal Mg^{2+} uptake have been reported. Parathyroid hormone and fibroblast growth factor-23 directly inhibit transcellular Mg^{2+} absorption in the duodenum, jejunum, and ileum. The apical proton triggers acid-sensing ion-channel 1a and purinergic $P2Y_2$ receptor activities, which stimulates mucosal bicarbonate secretion and induces $MgCO_3$ precipitation to suppress absorption. Omeprazole suppresses Mg^{2+} absorption in the duodenum, jejunum, and ileum.

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INTRODUCTION

Magnesium (Mg^{2+}) has an essential role in numerous cellular biochemical functions ranging from DNA structure stability and repairing, cell proliferation, neuronal excitability, bronchodilatation, vasodilatation, muscle contraction, myocardial excitability, bone hydroxyapatite formation, and anti-inflammatory function to exocrine and endocrine function of the pancreas[1]. Mg^{2+} deficiency has been implicated in several diseases, such as Alzheimer's disease[2], osteoporosis[3], hypertension[4], diabetes mellitus[5], and cancer[6]. Therefore, its plasma level is tightly regulated within a narrow range (0.7–1.1 mmol/L) by the collaborative actions of intestinally digested Mg^{2+} absorption, bone and muscle Mg^{2+} storage, and excess renal Mg^{2+} excretion[1]. The mechanism underlying regulation of transepithelial Mg^{2+} transport has been extensively explored in the renal tubular epithelium[1]. However, few research articles on the mechanism and regulatory factors of intestinal Mg^{2+} absorption have been published.

Since dietary intake is the sole source of Mg^{2+} in humans, adequate intestinal absorption of Mg^{2+} is vital for normal Mg^{2+} balance. It was previously hypothesized that bulk Mg^{2+} uptake occurs in the small intestine through an unregulated paracellular pathway, whereas fine-tuning of colonic Mg^{2+} absorption occurs through a regulated transcellular mechanism[1,7,8]. Colonic Mg^{2+} absorption can be modulated by dietary Mg^{2+} content and inulin fibers[7,9] but not by hormones[1,7]. In contrast, recent studies have provided new insights into the mechanisms and modulatory factors of small intestinal Mg^{2+} uptake. The aim of this article was to review the current knowledge of the mechanisms and regulatory factors of small intestinal Mg^{2+} absorption.

MECHANISM OF SMALL INTESTINAL MG^{2+} ABSORPTION

The mechanism of small intestinal Mg^{2+} absorption is currently under debate. One research group has proposed that transient receptor potential melastatin 6 homodimer channel (*TRPM6*) mRNA expression and transcellular Mg^{2+} absorption were not present in the small intestine[1,7,8]. However, a study from the same group showed positive immunofluorescence staining of TRPM6 protein in the absorptive cells along the brush border membrane of the villi in the duodenum[10]. Another group has proposed that the small intestinal epithelium absorbs Mg^{2+} through transcellular active and paracellular passive transport mechanisms[11-13]. In an Ussing chamber study, transport of transcellular and paracellular Mg^{2+} was detected in the duodenum, jejunum, and ileum[11-13]. The proposed mechanism of small intestinal Mg^{2+} absorption is shown in Figure 1.

Transcellular Mg^{2+} absorption

Transcellular Mg^{2+} absorption occurs through mucosal Mg^{2+} uptake by TRPM6 and TRPM7 homodimer channel, both of which were markedly detected in the small intestinal epithelium of human and murine cells[10-14]. In addition, recent mass spectrometric peptide sequence analysis confirmed the expression of TRPM6 and TRPM7 in the duodenum and jejunum[15]. The channel activities of both homodimers of TRPM6 and of TRPM7 are negatively regulated by physiological Mg·ATP and Mg^{2+} levels[10,16-19]. A recent study reported the expression of a heterodimer TRPM6/7 channel in the plasma membrane of duodenal and jejunal epithelium[15]; therefore, Mg^{2+} enters the small intestinal epithelial cells through TRPM6/7, TRMP6, and TRPM7. However, the heterodimer TRPM6/7 channels do not respond to physiological intracellular Mg^{2+} and Mg·ATP[17,19]; thus, continuous epithelial Mg^{2+} absorption can occur through the TRPM6/7 channel, regardless of intracellular Mg^{2+} and concentrations. Basolateral Mg^{2+} extrusion from the small intestinal epithelium occurs through cystathionine β-synthase domain divalent metal cation transport mediator 4[11-13,20] by means of a sodium (Na^+) gradient-dependent

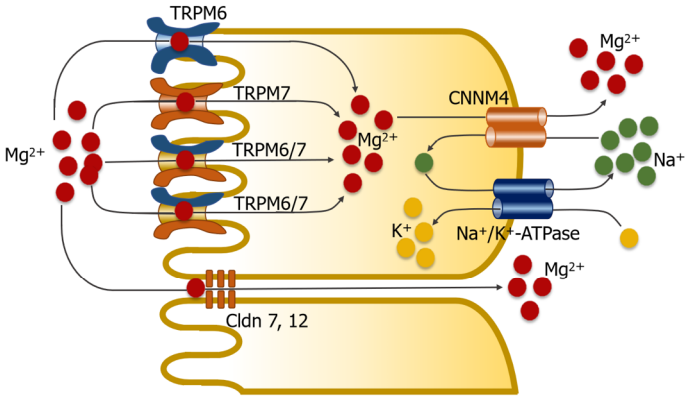

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Figure 1 Magnesium absorption in the small intestine through two absorption pathways. The transcellular transport mechanism involves magnesium (Mg^{2+}) influx into enterocytes through the transient receptor potential melastatin 6 homodimer channel (TRPM6), transient receptor potential melastatin 7 homodimer channel (TRPM7), and transient receptor potential melastatin 6/7 heterodimer channel (TRPM6/7). Cystathionine β-synthase domain divalent metal cation transport mediator 4 (CNNM4) mediates basolateral Mg^{2+} extrusion by means of secondary active transport. In the paracellular mechanism, Mg^{2+} moves through tight-associated paracellular pores of Claudin 7 (Cldn 7) and Claudin 12 (Cldn 12).

secondary active transport[20]. However, mutation of cystathionine β-synthase domain divalent metal cation transport mediator 4 does not affect the plasma concentration in humans[21,22], suggesting that other Mg^{2+} extrusion mechanisms probably occur.

Paracellular Mg^{2+} absorption

It has been suggested that paracellular Mg^{2+} absorption is responsible for 90% of total intestinal Mg^{2+} uptake[23]. Paracellular permeability is regulated by the paracellular claudin (Cldn) channel of the tight junction[24]. In 1999, the first discovery of a paracellular channel at the tight junction was Cldn-19 or paracellin-1, which form a paracellular Mg^{2+} channel[25]. It is thought that paracellular Mg^{2+} channels in epithelial tissues are formed by Cldn-16 and -19[25-27]; mutations in these genes lead to severe hypomagnesemia. The small intestinal epithelium expresses Cldn-1–5, -7, -8, -12, and -15 but not -16 and -19[28,29]. A previous study proposed that Cldn-7 and -12 modulated intestinal paracellular Mg^{2+} absorption[30]. However, the processes involving Cldn-regulated paracellular Mg^{2+} absorption in the small intestine still must be elucidated.

REGULATORY FACTORS OF SMALL INTESTINAL MG^{2+} ABSORPTION

Hormones

In general, hormones mainly modulate the transcellular electrolyte transport to regulate epithelial electrolyte absorption or secretion. Hormonal regulation of small intestinal Mg^{2+} absorption also modulates transcellular Mg^{2+} absorption. A recent study reported that parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF-23) systemically and directly inhibited transcellular, but not paracellular, Mg^{2+} absorption in the duodenum, jejunum, and ileum[13]. There was no additional effect of PTH and FGF-23, suggesting that they acted through the same intracellular signaling molecule. Both PTH and FGF-23 activate their corresponding receptors that further stimulate the same protein kinase C pathway to suppress plasma membrane-associated TRPM6 expression (Figure 2). Since native TRPM6 primarily functions as a subunit of heteromeric TRPM6/7 channels[31], the suppression of plasma membrane TRPM6 probably suppresses plasma TRPM6/7 heterodimer expression. The suppression of plasma TRPM6 and TRPM6/7 activity leads to diminution of transcellular Mg^{2+} absorption[13]. The inhibitory effect of PTH and FGF-23 could be nullified by Gö 6850[13], which inhibits the conventional (α, β1, β2, and γ) and novel (δ and ε) protein kinase C isoforms. However, the exact signaling pathway of PTH and FGF-23 inhibition of small intestinal transcellular Mg^{2+} absorption requires further study.

The proposed physiologically relevant magnesiotropic actions of PTH and FGF-23 are shown in Figure 3. During hypocalcemia, the parathyroid gland actively secretes PTH into the blood stream. PTH stimulates the bone resorption process, which increases plasma calcium (Ca^{2+}), inorganic phosphate (Pi), and Mg^{2+} levels[32,33]. PTH stimulates renal 1,25-dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] production, which subsequently induces small intestinal Ca^{2+} absorption[34]. PTH also activates renal tubular Ca^{2+} and Mg^{2+} reabsorption[32]. Plasma Pi and PTH trigger bone-derived FGF-23 release, which acts as a negative feedback regulator to abolish $1,25(OH)_2D_3$-induced intestinal Ca^{2+} absorption[33]. PTH and FGF-23 synergistically suppress the small intestinal absorption of dietary Mg^{2+}[13] to prevent hypermagnesemia. PTH and FGF-23 downregulate the Na^{2+}-dependent Pi cotransporters, (NaPi)-IIa and NaPi-IIc,

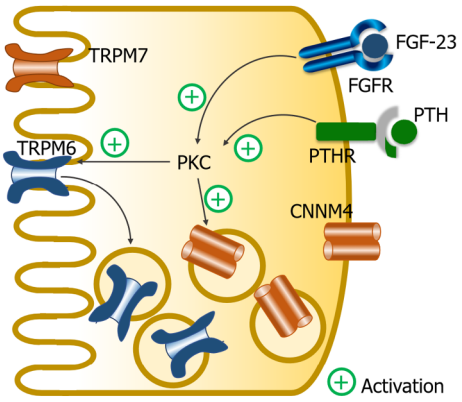

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Figure 2 Fibroblast growth factor-23 and parathyroid hormone regulate magnesium absorption in the small intestine. Fibroblast growth factor-23 (FGF-23) and parathyroid hormone (PTH) act through their corresponding receptors to suppress magnesium absorption in the protein kinase C (PKC)-dependent pathway; they suppressed membrane transient receptor potential melastatin 6 homodimer channel (TRPM6) expression, which leads to the suppression of membrane transient receptor potential melastatin 6/7 (TRPM6/7) expression. FGF-23 and PTH also increase cytosolic cystathionine β-synthase domain divalent metal cation transport mediator 4 (CNNM4) expression. TRPM7: Transient receptor potential melastatin 7 homodimer channel; FGFR: Fibroblast growth factor receptor; PTHR: Parathyroid hormone receptor.

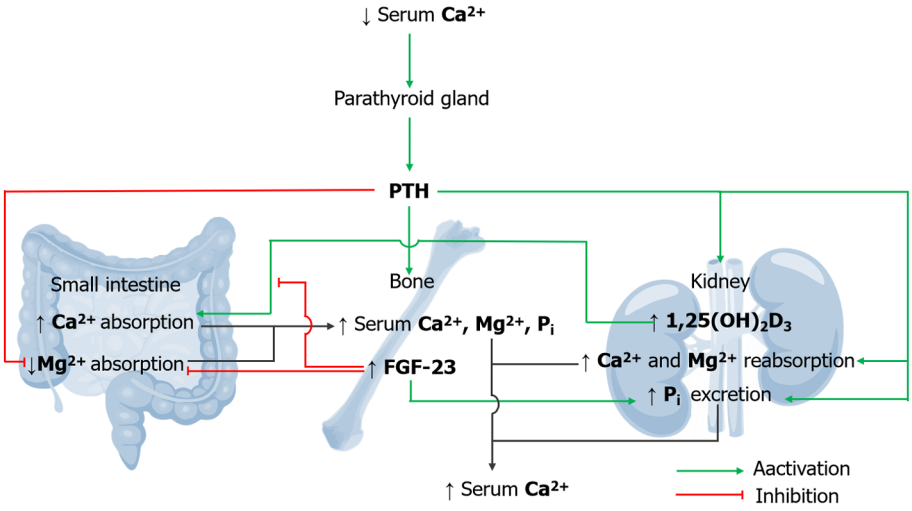

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Figure 3 Integrated magnesiotropic action of fibroblast growth factor-23 and parathyroid hormone. Parathyroid hormone (PTH) stimulates the bone resorption process, which increases plasma calcium (Ca^{2+}), inorganic phosphate (P_i), and magnesium (Mg^{2+}) levels. PTH stimulates the release of 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) to subsequently induce small-intestinal Ca^{2+} absorption. PTH also activates renal tubular Ca^{2+} and Mg^{2+} reabsorption. Plasma Pi and PTH trigger the release of fibroblast growth factor-23 (FGF-23) to abolish $1,25(OH)_2D_3$-induced intestinal Ca^{2+} absorption. PTH and FGF-23 synergistically suppress small intestinal absorption of dietary Mg^{2+}. PTH and FGF-23 induce urinary P_i excretion.

and increase urinary Pi excretion[32] to prevent hyperphosphatemia. Therefore, PTH and FGF-23 exert their calcemic effect by preventing hyperphosphatemia and hypermagnesemia.

Luminal acidity

The hypothesis that apical acidity and mucosal bicarbonate secretion (MBS) affect luminal Mg^{2+} solubility and intestinal Mg^{2+} absorption was previously proposed in 2014[11,35], which was confirmed in a recent review article[36]. The luminal acidity along the entire human and rodent small bowel varies from pH 5.0–7.3[12,37]. The luminal protons provide an appropriate environment for mineral absorption by stabilizing their ionized forms[38]. The elevation of luminal pH led to a lower soluble Mg^{2+}, which decreased from 79.61% of total luminal Mg content at pH 5.15% to 8.71% of total luminal Mg at pH 7.8[39]. Therefore, luminal acidity enhances Mg^{2+} absorption in the human small intestine[40] and epithelial-like Caco-2 monolayers[30,35]. The MBS and luminal pH elevation diminished duodenal, jejunal, and ileal Mg^{2+} absorption[11,12].

pH-sensing channel and receptor

Small intestinal enterocytes are regularly exposed to strong gastric acid. When luminal protons are present in the duodenal lumen, the intestinal epithelium cells can directly detect and modulate their cellular response through the proton-sensing channels, *e.g.*, the acid-sensing ion-channel 1a (ASIC1a) or proton-sensing receptors, such as ovarian cancer G protein-coupled receptor 1 (OGR1) and $P2Y_2$ purinoceptor[41-44].

OGR1, also known as GPR68, is expressed in the human small intestine, spleen, testes, brain, lungs, placenta, heart, and kidneys but not in the colon[44]. OGR1 is a proton-sensitive receptor with pH values at half activation ($pH_{0.5}$) and full activation of 7.2 and 6.8, respectively[45-47]. When the luminal pH decreases to 6.5, OGR1 activity is inactivated[45]. Activation of OGR1 triggers the phospholipase C–protein kinase C signaling pathway to activate intestinal Mg^{2+} absorption[35] (Figure 4).

ASIC1a is a proton-sensitive Ca^{2+} channel with a $pH_{0.5}$ of 6.2[41,43]. Activation of ASIC1a activates intracellular Ca^{2+} signaling and subsequently induces MBS. In the intestinal epithelium, luminal proton stimulates ASIC1a activity that further activates MBS in a Ca^{2+} signaling-cystic fibrosis transmembrane conductance regulator-dependent mechanism[35] (Figure 4). Secreted bicarbonate has previously been found to reduce luminal protons[48] and induce precipitation of luminal free Mg^{2+}[49], thus reducing free soluble Mg^{2+} and suppressing intestinal Mg^{2+} absorption.

Purinergic regulation of luminal pH and electrolyte transport in the small intestine have been described[50-52]. Duodenocytes regularly secrete ATP into its lumen. If luminal pH is low, luminal alkaline phosphatase activity is diminished and luminal ATP increases, which subsequently activates $P2Y_2$ purinoceptor. Simultaneously, $P2Y_2$ is a proton-sensitive receptor that is activated by luminal protons[42]. Active $P2Y_2$ purinoceptors further activate MBS to increase luminal pH. A previous study showed that $P2Y_2$ activation induced MBS through a cystic fibrosis transmembrane conductance regulator- and Na^+-HCO_3^- cotransporter-1-dependent mechanism, which subsequently suppressed intestinal Mg^{2+} absorption[53] (Figure 5).

Proton pump inhibitor

Proton pump inhibitor (PPI)-induced hypomagnesemia (PPIH) and hypomagnesuria in humans have been reported since 2006[54-57]. Intravenous Mg^{2+} supplementation or withdrawal of the PPI was able to rapidly normalize plasma and urinary Mg^{2+} levels in PPIH patients, though oral Mg^{2+} supplementation could not. Clinical assessments have reported that PPIH patients had normal renal Mg^{2+} handling[54,56,57]. These findings suggest that PPIs could suppress intestinal Mg^{2+} absorption. Our group has extensively studied the underlying mechanisms of PPI-suppressed intestinal Mg^{2+} absorption for a decade[11,12,15,30,35,53,58,59]. Our results suggest that PPIs mainly suppressed small intestinal Mg^{2+} absorption.

Omeprazole, the first introduced PPI, significantly suppressed total, transcellular, and paracellular Mg^{2+} absorption in the duodenum, jejunum, ilium, and colon of PPIH rats[11,12]. Regarding the percent suppression of total Mg^{2+} absorption in the duodenum (81.86%), jejunum (70.59%), ileum (69.45%), and colon (39.25%), the small intestine is the segment most adversely affected by prolonged PPI administration. However, previous articles have proposed that PPIs mainly inhibit colonic Mg^{2+} absorption[36,60,61], but those study results remain controversial[60,61]. They also proposed that colonic fermentation of dietary fibers probably increased serum Mg^{2+} and cured patients with PPIH[36]. A previous study clearly showed that dietary inulin fibers significantly induced cecal and colonic fermentation, but not plasma Mg^{2+} levels, in control and PPIH mice[61]. In contrast, dietary inulin fibers significantly induced renal Mg^{2+} excretion in PPIH mice[61], which should aggravate hypomagnesemia in PPIH. Therefore, the large intestine may not be a suitable intestinal segment that should be modulated to counteract PPIH.

The proposed mechanism of PPI-suppression of small intestinal Mg^{2+} absorption is shown in Figure 6. PPIs markedly suppress membranous TRPM7 and TRPM6/7[15]. Membranous TRPM6-channel activity is suppressed by hyperphosphorylation at the T1851 residue and hyperoxidation at the M1755 residue [15]. Phosphorylation of the T1851 residue of the TRPM6 protein induces TRPM6-channel suppression by intracellular free Mg^{2+} and activated 5 C-kinase 1[62]. Oxidation of the M1755 residue in the TRPM6 protein also suppresses its channel permeability[63]. Suppression of membranous TRPM6, TRPM7, and TRPM6/7 disrupts mucosal Mg^{2+} entry into the small intestinal epithelium and then inhibits transcellular Mg^{2+} absorption[11,12]. Plasma FGF-23 was markedly increased in PPIH rats[12]. The mechanism by which FGF-23 inhibits transcellular small intestinal Mg^{2+} absorption is described in the above section[13]. Therefore, PPI-suppressed transcellular Mg^{2+} absorption is due, at least in part, to FGF-23.

PPIs suppress paracellular Mg^{2+} absorption (Figure 6). The small intestinal epithelium only expresses Cldn-1, -2, -3, -4, -5, -7, -8, -12, and -15[28,29]. Overexpression of Cldn proteins and higher paracellular resistance have been demonstrated in the small intestines of PPIH rats[11,12]. Paracellular tight junction width was significantly decreased in the small intestine of PPIH rats[58]. PPIs also suppress epithelial paracellular Mg^{2+} permeability and cation selectivity[30,59]. These results shed light on the mechanism of PPI-suppressed paracellular Mg^{2+} absorption in the small intestine.

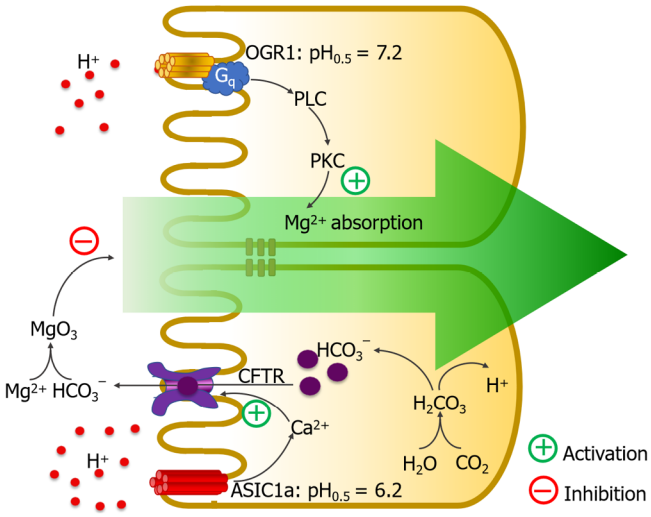

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Figure 4 Ovarian cancer G protein-coupled receptor 1 and acid-sensing ion-channel 1a modulate intestinal magnesium absorption. Activation of ovarian cancer G protein-coupled receptor 1 (OGR1) triggers the phospholipase C (PLC)–protein kinase C (PKC) signaling pathway to activate intestinal magnesium (Mg^{2+}) absorption. Activation of acid-sensing ion-channel 1a (ASIC1a) activates intracellular calcium (Ca^{2+}) signaling to induce mucosal bicarbonate secretion in a cystic fibrosis transmembrane conductance regulator (CFTR)-dependent mechanism. Secreted bicarbonate (HCO_3^-) reduces free soluble Mg^{2+} and suppresses intestinal Mg^{2+} absorption. H: Hydrogen.

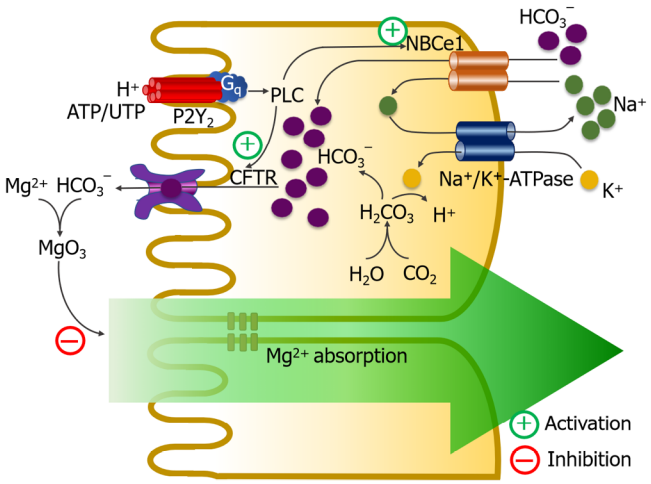

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Figure 5 $P2Y_2$ purinoceptors modulate intestinal magnesium absorption. Activation of $P2Y_2$ purinoceptor stimulates luminal cystic fibrosis transmembrane conductance regulator and basolateral Na^+-HCO_3^- cotransporter-1 (NBCe1) activities through a phospholipase C (PLC)-dependent mechanism. Active cystic fibrosis transmembrane conductance regulator (CFTR) and Na^+-HCO_3^- cotransporter-1 induce mucosal bicarbonate (HCO_3^-) secretion, which reduces luminal free magnesium (Mg^{2+}) and suppresses Mg^{2+} absorption. Ca^{2+}: Calcium; H: Hydrogen; K: Potassium; Na^+: Sodium.

PPI-induced small intestinal MBS (Figure 6) has been reported in humans[64], PPIH rats[11], and PPI-treated Caco-2 monolayers[35,53]. PPIs have also been shown to significantly increase ASIC1a and $P2Y_2$ expression in PPI-treated epithelium[35,53]. Active ASIC1a and $P2Y_2$ trigger MBS. Higher secreted bicarbonate in PPIH small intestines reduces free soluble Mg^{2+}, which disrupts Mg^{2+} absorption (Figure 6). Inhibition of MBS significantly increases duodenal Mg^{2+} absorption in PPIH rats[11].

In addition to the change in magnesiotropic protein expression and function and MBS, PPIs have been shown to induce structural change in the absorptive epithelium of the small intestine[58]. Prolonged PPI administration markedly decreased the villous length and absorptive area in the duodenal, jejunal, and ilial epithelium of PPIH rats. The underlying mechanism involves Paneth cell dysfunction in the small intestine[58]. Paneth cells have an important role in host-microorganism homeostasis in the small intestine by providing antimicrobial α-defensin peptides[65,66]. Disruption of the secretory function of Paneth cells leads to infection and chronic inflammation of the small intestine [65,66]. In PPIH rats, a reduction in secretory granules and metaplasia of Paneth cells occurs in the duodenum, jejunum, and ileum, suggesting Paneth cell secretory dysfunction[58]. Chronic inflam-

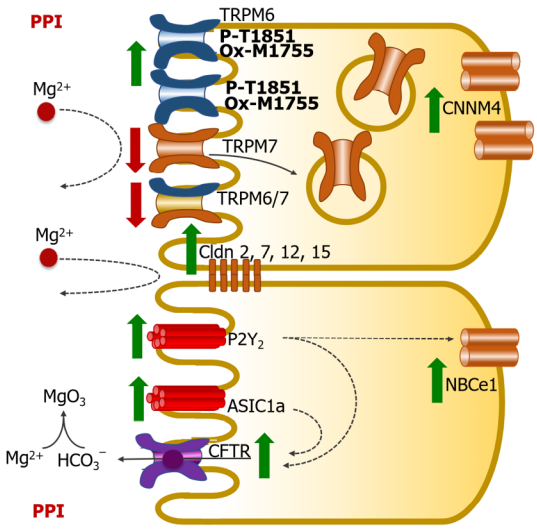

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Figure 6 Mechanism of proton pump inhibitor-suppressed small intestinal magnesium absorption in proton pump inhibitor-induced hypomagnesemia rats. Proton pump inhibitor (PPI) suppresses membrane transient receptor potential melastatin 7 homodimer channel (TRPM7) and transient receptor potential melastatin 6/7 homodimer channel (TRPM6/7) expression but increases membrane transient receptor potential melastatin 6 homodimer channel (TRPM6) and cystathionine β-synthase domain divalent metal cation transport mediator 4 (CNNM4) expression. PPI induces phosphorylation of the T1851 residue and oxidation of the M1755 residue of the membrane TRPM6 channel, which reduces their channel permeability. These PPI effects reduce transcellular magnesium (Mg^{2+}) absorption. Overexpression of claudin 2 (Cldn2), claudin 7 (Cldn7), claudin 12 (Cldn12), and claudin 15 (Cldn15) reduces paracellular permeability, which suppresses paracellular Mg^{2+} absorption. PPI also enhances $P2Y_2$- and acid-sensing ion-channel 1a (ASIC1a)-suppressed intestinal Mg^{2+} absorption. CFTR: Cystic fibrosis transmembrane conductance regulator; HCO_3^-: Bicarbonate; NBCe1: Na^+-HCO_3^- cotransporter-1.

mation in the small intestinal epithelium leads to villous atrophy and reduction of the absorptive area in the small intestine of PPIH rats[58].

Gut microbiota

The potential role of gut microbiota in colonic Mg^{2+} absorption has previously been proposed[36]. However, it is currently unknown how the small intestinal microbiome affects small intestinal Mg^{2+} absorption. Previous studies have shown that the small intestine is colonized by a complex gut microbiota community and is less numerous and diverse (approximately 10^3–10^7 microbial cells/gram) than in the colon (approximately 10^{12} microbial cells/gram)[67]. The dominant bacterial phyla in the small intestine are *Streptococcus sp*., *Lactobacillaceae*, and *Enterobacteriaceae*, whereas in the colon, the dominant phyla are *Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae*, and *Ruminococcaceae*[68,69]. Prolonged PPI treatment can lead to gut microbiota dysbiosis, such as the reduction of *Actinobacteria* and *Bifidobacteria spp*., which are responsible for maintaining the mucosal barrier function[68].

Furthermore, long-term treatment with PPIs causes small intestinal bacterial overgrowth because of the loss of the gastric acid defensive barrier[70]. The jejunal samples of small intestinal bacterial overgrowth patients regularly showed increased production of toxic agents, such as serum endotoxin and bacterial compounds that stimulate the secretion of proinflammatory cytokines[71]. Apart from these findings, our previous study showed Paneth dysfunction and chronic inflammation in the small intestine of PPIH rats[58]. From the perspective of relevant gut microbiota, Paneth cell defects have been found to be associated with increased *Bacteroidetes* and *Enterococcus* and decreased *Bifidobacterium*[72], whereas *Bifidobacterium longum* has been found to promote cell proliferation and expression of Lgr5 and Wnt3a in intestinal organoids and alleviate microbiota dysbiosis by regulating the functions of Paneth cells[73]. It is also possible that the synthesis of gut microbiota metabolites could lead to changes in the absorptive surface in the gut and/or stimulate gene expression[74].

In the colon, bifidobacterial fermentation leads to acidification of the colon, which shows beneficial absorption of Mg^{2+}[9,61,75]. In humans, small intestinal microbiota can also ferment the available carbohydrates and induce intestinal acidification[76]. In the human small intestine, a dominant bacterial phylum is *Streptococcus sp*.[77,78], which is an anaerobe that can ferment relatively simple carbohydrates at a high rate[79]. According to the above, luminal acidity markedly induces small intestinal Mg^{2+} absorption. Therefore, small intestinal fermentation should induce small intestinal Mg^{2+} absorption.

CONCLUSION

Bulk absorption of digested Mg^{2+} occurs in the small intestine through transcellular active and

paracellular passive mechanisms. PTH, FGF-23, luminal protons, ASIC1a, OGR1, $P2Y_2$, PPIs, and the microbiome have recently been proposed as regulatory factors of small intestinal Mg^{2+} uptake. However, the regulatory mechanism of small intestinal Mg^{2+} requires additional extensive studies.

FOOTNOTES

Author contributions: Chamniansawat S wrote the manuscript; Suksridechacin N wrote the manuscript; Thongon N wrote and edited the manuscript.

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MINIREVIEWS

Hepatocellular carcinoma in non-alcoholic steatohepatitis without cirrhosis

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Abstract

Cirrhosis is an emerging major cause of the development of hepatocellular carcinoma (HCC), but in non-alcoholic fatty liver disease (NAFLD), up to 50% of patients with HCC had no clinical or histological evidence of cirrhosis. It is currently challenging to propose general recommendations for screening patients with NAFLD without cirrhosis, and each patient should be evaluated on a case-by-case basis based on the profile of specific risk factors identified. For HCC screening in NAFLD, a valid precision-based screening is needed. Currently, when evaluating this population of patients, the use of non-invasive methods can guide the selection of those who should undergo a screening and surveillance program. Hence, the objective of the present study is to review the epidemiology, the pathophysiology, the histopathological aspects, the current recommendations, and novel perspectives in the surveillance of non-cirrhotic NAFLD-related HCC.

Key Words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Hepatocellular carcinoma; Genetic variants; Microbiota; Obesity

Core Tip: Cirrhosis is an emerging major cause of the development of hepatocellular carcinoma (HCC), but in non-alcoholic fatty liver disease (NAFLD), up to 50% of patients with HCC had no clinical or histological evidence of cirrhosis. In the present study, we evaluated data regarding the epidemiology, the pathophysiology, the histopathological aspects, the current recommendations, and novel perspectives in the surveillance of non-cirrhotic NAFLD-related HCC. We believe that using non-invasive methods can guide the selection of patients who need to undergo screening and a surveillance program.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has been traditionally defined by the evidence of hepatic steatosis by imaging or histology and by the lack of secondary causes of hepatic fat accumulation such as significant alcohol consumption, long-term use of steatogenic medications, hereditary disorders and other causes of chronic liver diseases[1]. Recently, there has been a proposal to rename NAFLD to metabolic associated fatty liver disease (MAFLD), thus eliminating the need to exclude other causes of liver diseases and adopting inclusive criteria according to coexistence with other liver diseases[2]. The diagnosis of MAFLD is based on histological, imaging or blood biomarker evidence of fat accumulation in the liver (steatosis) in addition to one of the following criteria, namely overweight/obesity, type 2 diabetes mellitus (DM) or evidence of metabolic dysregulation[2].

NAFLD is a well-known cause of chronic liver disease, compromising more than 25% of the global population, and up to 25% may have nonalcoholic steatohepatitis (NASH) with or without fibrosis. NASH with fibrosis is the most active form of disease which is associated with significant morbidity and mortality due to complications of liver cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC)[3].

Cirrhosis is an emerging major cause of the development of HCC, but in NAFLD, up to 50% of patients with HCC had no clinical or histological evidence of cirrhosis[4-6].

NAFLD and the components of metabolic syndrome, especially obesity and DM, are independently associated with HCC development and contribute to the risk of HCC in a non-cirrhotic liver[7,8]. Non-cirrhotic NAFLD patients have a 2.5-fold higher risk of developing HCC than other etiologies of chronic liver disease without cirrhosis[9].

The objective of the present study is to review the epidemiology, the pathophysiology, the histopathological aspects, the current recommendations and novel perspectives in the surveillance of non-cirrhotic NAFLD-related HCC.

EPIDEMIOLOGY OF NAFLD-RELATED HCC

Liver cancer, most of which corresponds to HCC[10,11], ranks sixth among the most common malignancies and second among the leading causes of cancer-related death worldwide. HCC affected 11.6/100000 individuals in 2020, leading to a mortality rate of 10.7/100000[12]. Remarkably, despite major advances in the treatment of viral hepatitis, it is estimated that the incidence rate of liver cancer will keep increasing until 2030, which can be partly explained by a striking increase in the incidence of NAFLD-related HCC[13].

NAFLD affects approximately one-fourth of individuals in the world[14], reinforcing its importance in the etiology of HCC[15]. In 2019, 36300 new cases of HCC and 34700 HCC-related deaths were attributed to NAFLD[16]. The increasing burden of NAFLD will probably lead to a growth in the age-standardized incidence rate of NAFLD-related liver cancer, with an estimated average percentage change of 2.12 between 2018 and 2030[13]. The growing importance of NAFLD as a cause of HCC becomes apparent when two cohorts from South America, a continent with a high prevalence of NAFLD, are compared. While from 2005 to 2015, 9% of HCC cases were attributed to NAFLD[17], 34% of cases were associated with NAFLD from 2019 to 2020[18].

Most cases of HCC develop in cirrhotic livers. Nevertheless, it is noteworthy that HCC may also occur in NAFLD without cirrhosis[15,19-21]. Aside from cirrhosis, diabetes and other metabolic traits, older age, male sex, alcohol consumption and tobacco smoking also seem to be risk factors for developing HCC in patients with NAFLD[10,22-24]. In cirrhosis associated with NAFLD, the annual incidence of HCC is reported as 0.5% and 2.6%[25,26].

In a large retrospective cohort study of European primary care databases, including 136703 patients with NAFLD and matched controls, the incidence rate of HCC was 0.3 per 1000 person-years among individuals with NAFLD, which was significantly higher than among controls, with a hazard ratio of 3.51. The risk of developing HCC was higher according to the Fibrosis-4 (FIB-4) score, which might reflect the odds of having cirrhosis[22].

In another retrospective cohort study performed using a large American administrative database, including 296707 individuals with NAFLD and an equal number of matched controls, HCC was diagnosed in 490 patients with NAFLD and 55 controls. This translated into an annual incidence rate of HCC of 0.21 cases per 1000 person-years among individuals with NAFLD, which was significantly higher than among controls (0.02 cases per 1000 person-years). In a subgroup analysis, the annual incidence rate of HCC was 10.6 per 1000 person-years among individuals with cirrhosis, 0.08 per 1000 person-years among those with NAFLD without cirrhosis and 0.02 per 1000 person-years among controls[27]. Nonetheless, the study had substantial methodological limitations, especially regarding misclassification risks and lack of database granularity. Therefore, its results should be interpreted with caution.

Regarding non-cirrhotic HCC, a meta-analysis has demonstrated that around 38% of NAFLD-related HCCs are diagnosed in individuals without cirrhosis[9]. However, it should be emphasized that the risk of liver cancer is substantially higher in patients with NAFLD and cirrhosis when compared to those without cirrhosis. A recent meta-analysis found an incidence of 3.78 *vs* 0.03/100 person-years in patients with non-cirrhotic NAFLD[28].

Table 1 shows the studies that evaluated the incidence/prevalence of HCC and risk factors in patients with NAFLD without cirrhosis.

PATHOPHYSIOLOGY OF NAFLD-RELATED HCC

The pathophysiology and etiology of NASH progression to HCC are not entirely known, and many mechanisms have been proposed. Neoplastic transformation of NAFLD is driven by metabolic imbalance, lipotoxicity consequent to hepatocyte lipid overload, oxidative stress and immunological aspects, whereas many other factors such as genetic markers, gut dysbiosis and alcohol or tobacco abuse may interact as risk modifiers[29].

Genetic factors

Three main single-nucleotide polymorphisms (SNPs) have been described as associated with a higher risk of steatosis, fibrosis and even HCC, Patatin-like phospholipase domain-containing 3 (*PNPLA3*), membrane-bound o-acyltransferase domain-containing 7 (*MBOAT7*), and transmembrane 6 superfamily member 2 (*TM6SF2*) genes[30].

The variant in the *PNPLA3* gene is the strongest genetic variant predisposing from fatty liver to HCC, and its frequency ranges from 17% to 49% according to ethnicity and the geographic distribution of NAFLD[31]. This variant codifies adiponutrin, a protein responsible for the export of lipids from the liver. The substitution of a single nucleotide (from isoleucine to methionine – I148M) modifies the function of adiponutrin, leading to the accumulation of triglycerides, retinyl esters in lipid droplets in both hepatocytes and hepatic stellate cells, leading to fibrogenesis and tumorigenesis. Patients with at least one G allele, primarily those with GG homozygosis, have a higher risk of developing steatosis, fibrosis and HCC[32]. A subgroup analysis from a systematic review involving 9915 patients showed an association between the *PNPLA3* rs738409 SNP and HCC among patients with NASH or alcohol-related cirrhosis with an odds ratio of 1.67 and a 95% confidence interval of 1.27-2.21, but not among patients with cirrhosis of other etiologies[33].

Studies investigating *MBOAT7* association with HCC are scarce. In a cohort of 765 Italian patients with NAFLD, especially those without advanced fibrosis, the *MBOAT7* rs641738 variant was strongly associated with HCC. On the other hand, it showed no association with HCC in a validation cohort of 358 patients with NAFLD without cirrhosis in the United Kingdom[34].

TM6SF2 polymorphism is also associated with increased liver fat content in NASH, advanced hepatic fibrosis and cirrhosis. *TM6SF2* variants have a moderate to significant effect on the risk of NAFLD. Additionally, the *E167K* allele has an allelic odds ratio of 1.82 for steatosis[30]. Whether or not the variant is associated with an increased risk of NAFLD-related fibrosis and HCC remains to be determined.

Recently, the odd-skipped related transcription factor 1 (*Osr1*) has been reported as a novel tumor suppressor gene, as well as a potential prognostic biomarker in gastric cancer. Some authors suggest that *Osr1* plays an essential role in regulating cell survival, cell inflammation, and macrophage migration in the liver. Accordingly, *Osr1* was identified as a novel repressor gene in the progression of NAFLD/NASH[35]. So far, the role of *Osr1* in the progression of NAFLD towards HCC development is not established.

Human telomerase reverse transcriptase (*hTERT*) mutations are associated with familial liver diseases. Telomere length and germline *hTERT* mutations were evaluated to determine their association

Table 1 Studies that included the incidence/prevalence and risk factors for hepatocellular carcinoma in non-alcoholic fatty liver disease without cirrhosis

Ref.	Study design	Aim	Number of patients	Results and conclusion
Mohamad *et al*[5], 2016	Retrospective	To characterize patients with NAFLD and HCC comparing cirrhotic *vs* non-cirrhotic patients	All patients with NAFLD and HCC between 2003-2012 (n = 83)	36 (43.4%) NAFLD HCC non-cirrhotic *vs* 47 (56.6%) NAFLD HCC cirrhotic patients. HCC patients without cirrhosis are more likely to present at an older age with larger tumor and higher rates of tumor recurrence
Piscaglia *et al*[6], 2016	Multicenter observational prospective	To assess the clinical features of patients with NAFLD-related HCC and to compare to those with HCV related HCC	N = 756 (145 NAFLD *vs* 611 HCV)	Cirrhosis was present in about 50% of NAFLD-HCC patients, in contrast to the near totality of HCV-HCC. Survival was significantly shorter in patients with NAFLD-HCC than in those with HCV-HCC (25.5 mo *vs* 33.7 mo)
Stine *et al*[9], 2018	Systematic review with meta-analysis	To compare the prevalence of NAFLD-related HCC to other chronic liver diseases	19 studies (n = 168571)	The prevalence of NAFLD-related HCC in patients with NASH without cirrhosis is approximately 38% compared with 14% for other liver diseases
Tobari *et al*[24], 2020	Prospective	To evaluate the characteristics of HCC in non-cirrhotic NAFLD	48 non-cirrhotic HCC *vs* 71 cirrhotic HCC patients	In patients with non-cirrhotic NAFLD, important risk factors for HCC were male gender, alcohol consumption, and the FIB-4 index. HCC recurrence and survival were only influenced by the tumor stage
Kanwal *et al*[27], 2018	Retrospective	To estimate the risk of incident HCC among patients with NAFLD	296707 NAFLD *vs* 296707 matched controls	NAFLD individuals with cirrhosis had the highest annual incidence of HCC. 20% of NAFLD patients with HCC had no evidence of cirrhosis. The absolute risk of HCC in patients without cirrhosis is too low to recommend HCC surveillance
Orci *et al*[28], 2022	Systematic review with meta-analysis	Evaluate the pooled HCC incidence in patients with NAFLD at distinct severity stages	18 studies (470404 individuals)	Evidence documenting the risk in patients with NASH or simple steatosis is limited, but the incidence of HCC in these populations may lie below thresholds used to recommend a screening (0.03 per 100 person-years)
Donati *et al*[34], 2017	Sectional	To evaluate whether the *MBOAT7* rs641738 risk T allele predisposes to HCC in NAFLD patients stratified by the presence of severe fibrosis	765 Italian NAFLD patients	The *MBOAT7* rs641738 T allele is associated with reduced *MBOAT7* expression and may predispose to HCC in patients without cirrhosis
Demirtaş *et al*[71], 2021	Retrospective	To investigate the characteristics and survival course of non-cirrhotic individuals with HCC	N = 384 HCC; 43 (11.2%) without cirrhosis; 10 (23%) with NAFLD	HCC in non-cirrhotic liver is diagnosed at more advanced stage and with larger tumor size. The overall survival is shorter in HCC without cirrhosis, due to late recognition

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; *MBOAT7*: Membrane-bound o-acyltransferase domain-containing 7.

with NAFLD-HCC. The authors observed an association between shorter peripheral blood telomeres and NAFLD-HCC development and found that rare germline mutations in *hTERT* predispose NAFLD progression to HCC, potentially assisting the identification of high-risk individuals[36].

Metabolic imbalance

Insulin resistance (IR) is the key pathogenic event associated with the development of hepatic steatosis and is also related to the development of HCC[37].

Hyperinsulinemia can promote the synthesis and activity of insulin-like growth factor-1, inhibiting cell proliferation and apoptosis[38], which increases the risk of hepatocellular carcinogenesis. Hyperglycemia provides a substrate for energy metabolism in tumor cells and leads to a glycosylation reaction activating the inflammatory signaling cascades and generating reactive oxygen species (ROS) to induce HCC development[39]. IR may also directly accelerate hepatocarcinogenesis by stimulating hepatic neovascularization[40].

These events affect cell growth by inducing the transcription of the protooncogenes, so fibrosis and carcinogenesis are promoted in the liver. Additionally, hyperinsulinemia increases hepatic lipid accumulation and leads to oxidative stress due to the increased beta-oxidation of free fatty acids and the formation of ROS. There is positive feedback between oxidative stress in mitochondria and endoplasmic reticulum (ER) through ER stress, further contributing to cell injury and carcinogenesis in NASH. In contrast to insulin-mediated apoptosis inhibition, hepatic lipotoxicity activates proapoptotic cell signals. Another recently discovered mechanism involves the association between lipolysis and autophagy, with conflicting evidence due to its double-natured, divergent role in NASH-associated HCC[41].

Lipotoxicity

Lipotoxicity is the dysregulation of intracellular lipid components resulting in the accumulation of

harmful lipids, which are associated with cellular damage and death[42]. Lipotoxicity causes cellular damage as lipids alter the biology and function of intracellular organelles, such as the ER and mitochondria. Also, a direct modification of intracellular signaling pathways may occur, deregulating the metabolic and inflammatory pathways[43].

The ER is an intracellular organelle that engages in many critical cellular processes, including folding membranes and secreted proteins, synthesizing lipids and sterols, and storing free calcium. Disturbance of any of these processes results in stress on the ER and interrupts the protein folding process. When ER stress cannot be restored, the apoptotic pathway is stimulated, leading to cell death to eliminate the stressed cells[44].

ER stress is linked to the development and progression of liver inflammation. Because it is a crucial mediator of liver inflammation, the immunoglobulin protein promotes the inflammatory response associated with NASH[45]. ER stress has been identified as a mediator of NAFLD-promoted HCC *in vitro*. Also, enhanced ER stress increases tumor necrosis factor production by macrophages, leading to tumor formation[46].

Oxidative stress

Oxidative stress results from an imbalance between the excessive formation of prooxidants (ROS and/or reactive nitrogen species) and limited antioxidant defenses, leading to cell death and tissue damage[47].

In NAFLD, there are some mechanisms for producing mitochondrial ROS. Thus, mitochondrial dysfunction and ROS production are exacerbated. In this context, some hepatocytes may develop adaptive cell survival and proliferation mechanisms that promote precancerous transformation and/or tumor growth[48].

Immunological aspects

During the progression of NAFLD from steatosis to NASH and more advanced stages of NASH with liver fibrosis, the immune system plays an important role. There are inflammation triggers within hepatic (lipid overload, lipotoxicity, oxidative stress) and extra-hepatic systems (gut-liver axis, adipose tissue, skeletal muscle), resulting in unique immune-mediated pathomechanisms in NAFLD[49].

Immune cells play a role in hepatocarcinogenesis through processes that are independent of fibrosis. Hepatocyte damage promotes neutrophil infiltration in the liver, resulting in DNA damage to other hepatocytes and promoting HCC development without fibrosis. Furthermore, lymphoid aggregates are often present in the setting of chronic inflammation. Additionally, the selective loss of CD4+ T lymphocytes occurs, which was shown to be critical for the progression of HCC[41].

Although immunological response can promote HCC, the immune system also plays an important role in suppressing tumor growth through immunosurveillance. Furthermore, HCC actively promotes tumor tolerance by inducing immunosuppression, and the fibrotic microenvironment leads to the overproduction of transforming growth factor beta, a potent immunosuppressant, thereby promoting disease progression[41].

Microbiota

Increased gut permeability and altered microbiome composition are associated with NAFLD and its disease severity, contributing to hepatocarcinogenesis[50].

The gut microbiota has been described as a cofactor in liver disease progression and in the development of HCC through the interaction with immune compartments *via* the gut–liver axis. Dysbiosis characterizes the microbiota of patients with NAFLD-cirrhosis, with compositional and functional shifts occurring with HCC development. It has been suggested that the gut microbiota in NAFLD-HCC is characterized by a distinctive microbiome/metabolomic profile and can modulate the peripheral immune response[50]. Human metagenomic data support an emerging core microbiome signature that characterizes NAFLD-cirrhosis, with increased *Ruminococcus gnavus*, *Clostridium bolteae*, *Streptococcus parasanguinis*, and *Klebsiella pneumoniae*, and a reduced number of beneficial species, including *Faecalibacterium prausnitzii*, *Alistipes putredinis*, and *Eubacterium eligens*. Furthermore, *Veillonella parvula* and *Bacteroides caecimuris* are also identified to distinguish NAFLD-HCC from NAFLD-cirrhosis. In agreement with these findings, rRNA analyses of patients with NAFLD-HCC have detected enrichment in *Bacteroides* and *Ruminococcaceae*, which correlated with several systemic inflammatory and immune markers[51]. Ren *et al*[52] also observed a decrease in butyrate-producing bacterial families, namely *Ruminococcus*, *Oscillibacter*, *Faecalibacterium*, *Clostridium IV*, and *Coprococcus* in patients with HCC.

Increased intestinal permeability, intestinal bacterial overgrowth and elevated serum endotoxin have been reported in NAFLD and NAFLD-HCC[53]. Endotoxemia-induced toll-like receptor 2 induction leads to cyclooxygenase-2 (COX2) mediated prostaglandin E (PGE) production, which suppresses antitumor immunity by inhibiting antitumor cytokine production from liver immune cells leading to HCC progression in a mouse model. In human non-cirrhotic NAFLD-related HCC, COX2 overexpression and excess PGE production are detected. Although these findings suggest that hepatocellular inflammation may be secondary to altered intestinal permeability and translocation of either intact bacteria or microbial cell components into the circulation, the causal link between them is not entirely

clarified[53,54].

Other factors

Many factors have been associated with the potential to increase the risk of HCC in NAFLD, such as male gender, older age, ethnicity, presence of type 2 DM, obesity, any degree of alcohol consumption and smoking[27,55].

Among these, risk factors for NAFLD-related HCC, which have long been recognized, are male sex, older age and Latino ethnicity[56]. Kanwal *et al*[27] described in a large cohort study involving 296707 patients with NAFLD that age above 65 years was an independent risk factor for HCC. It was more often identified in men and was higher in Hispanic individuals compared to white (0.21 per 1000 patient-years) and African American individuals (0.12 per 1000 patient-years)[27].

Clinical variables such as diagnosis of type 2 DM and obesity are also significant risk factors among patients with NAFLD. They can act independently or jointly with NAFLD to increase the risk of HCC development[56]. Type 2 DM doubled the risk of developing this outcome[19]. DM is a recognized risk factor for HCC regardless of the etiology of liver disease, and some authors suggest that DM has the strongest association with HCC[57], being related to the duration of DM and adequate glycemic control [58]. On the other hand, it is unclear if the correlation between DM and HCC in patients without cirrhosis applies, as a recent study evaluating the differences between cirrhotic and non-cirrhotic HCC in NAFLD found an inverse association between DM and HCC in the non-cirrhotic group, emphasizing that non-cirrhotic HCC tended to occur in older patients and those with a lower body mass index[59].

Obese patients with cirrhosis were 47 times more likely to have HCC than persons without liver disease, and there is strong evidence that obesity impacts HCC development and promotes an increase in mortality, especially in those with early age onset and the presence of visceral fat[58].

Obesity is a well-known risk factor for many cancers but is significantly linked to liver cancer[60]. A study from the Mayo Clinic has shown that the diagnosis of type 2 DM increased the risk of HCC by fourfold. Therefore, it is recommended that type 2 DM in every individual with NAFLD should be investigated due to its association with more advanced disease and increased risk of HCC[61].

Alcohol consumption is independently associated with a higher risk of HCC in individuals with NAFLD[62]. Some studies suggest that the increased risk would apply only to those with heavy alcohol use (*e.g.*, > 50 g/d or ≥ 3 drinks/d or ≥ 7 drinks/d), better supporting the recent definition of MAFLD instead of NAFLD. The additive effect of alcohol in those with NAFLD might explain the increase of HCC in this specific group[63].

The study by Ascha *et al*[64] suggested that any degree of alcohol consumption may increase the risk of HCC occurrence in patients who, by the classic definition, do not have a significant intake. The deleterious effects of continuous and excessive ethanol intake on the liver are well established; however, there is uncertainty regarding the impact of mild to moderate ethanol consumption[65].

In the same way, elevated alanine aminotransferase has been proposed as an independent factor associated with an increased HCC risk[65].

Environmental factors such as tobacco smoking are associated with insulin resistance, the development of NAFLD and liver cancer. Current and former smoking is associated with a 70% and 40% increased risk of liver cancer, respectively[66]. Similarly, in a meta-analysis of 81 studies, the pooled odds ratios for HCC development were 1.55 in current smokers *vs* 1.39 in former smokers[67]. Currently, there is no specific data on the risk of smoking in NAFLD-related HCC.

Many studies[22,27,54,57,64,65,68] have assessed the risk of HCC or other liver complications in patients with non-cirrhotic NAFLD, but they have many limitations. Most of them were retrospective and heterogeneous in terms of the inclusion criteria; did not have data on liver fibrosis stages; or had a short follow-up to assess complex outcomes such as HCC or complications of cirrhosis. In addition, most of them had relatively few cases of HCC diagnosed.

HISTOPATHOLOGICAL ASPECTS OF NAFLD-RELATED HCC

Patients with NAFLD and HCC without cirrhosis have larger tumors, but more often, they have well-differentiated tumors and a single nodule compared to those with cirrhosis[59,69,70]. On the other hand, due to late diagnosis, some cases have a higher rate of vascular invasion and extra-hepatic metastasis [71]. Frequently, the nontumor liver has significant steatosis and histological findings of steatohepatitis [72] (Figure 1).

Paradis *et al*[69], studying patients with HCC and metabolic risk factors, demonstrated that the neoplasia in 5 of 31 patients with NASH without cirrhosis developed on a preexisting liver cell adenoma.

Approximately 90% of HCCs are the conventional subtype, but patients with NAFLD with or without cirrhosis or patients with metabolic risks can present a histological subtype of HCC identified as a steatohepatitis-related variant[73]. Macroscopically, the nodule is golden-yellow in color and slightly firm because of steatosis and fibrosis[74].

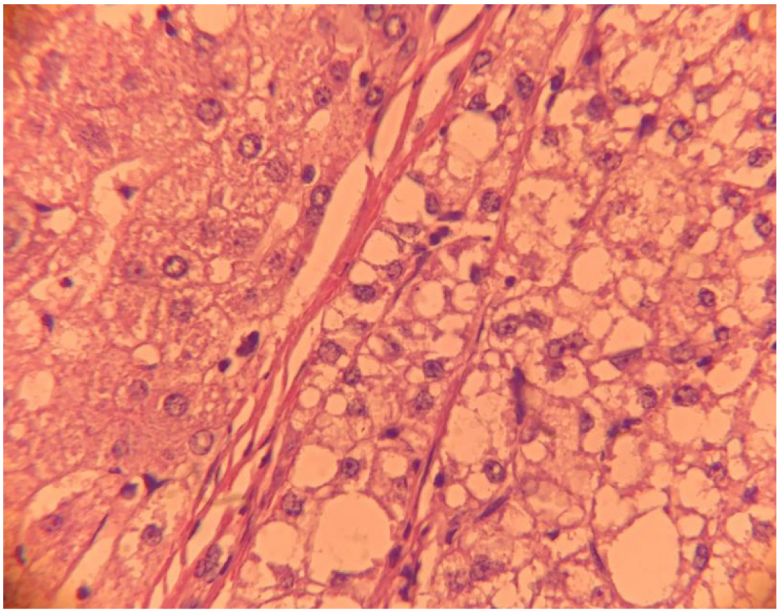

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Figure 1 Histopathological aspect of a non-alcoholic fatty liver disease-related hepatocellular carcinoma in a non-cirrhotic liver. On the left side: Non-cirrhotic liver with steatosis. On the right side: Hepatocellular carcinoma, steatohepatitis variant with steatosis, hepatocellular ballooning and macrotrabecular arrangement.

The histologic features of this subtype are macrovesicular steatosis, ballooning malignant hepatocytes, lymphocytic inflammation, Mallory Denk bodies and pericellular fibrosis, often with a "chicken-wire pattern"[75]. More frequently, neoplasia has a trabecular arrangement and minimal mitotic activity[74,75].

Regarding immunohistochemistry, a study evaluating 62 cases demonstrated a similarity between steatohepatitis HCC in non-cirrhotic livers and inflammatory liver cell adenomas, demonstrating a higher expression of C-reactive protein and serum amyloid A[70].

These tumors also have distinct molecular features: They frequently showed IL-6/JAK/STAT activation and less often Wnt/β catenin/CTNNB1 and P53 pathway alterations[76].

CURRENT RECOMMENDATIONS ON SURVEILLANCE FOR HCC IN NAFLD

HCC in the setting of NASH is known to occur even in the absence of liver cirrhosis, an event previously mainly related to hepatitis B virus infection. Thus, knowing whom to screen for HCC and which patient population deserves surveillance is essential.

The objective of screening and surveillance in patients with cirrhosis is to reduce mortality, as this patient population will develop HCC. Cost-effectiveness studies suggest that an incidence of 1.5% per year or more would warrant HCC surveillance in cirrhotic patients, regardless of etiology[23,77]. Recent analysis has confirmed the importance of surveillance in patients with cirrhosis, resulting in longer survival[78]. In Brazil, when we performed screening in a population of more than 500 patients with cirrhosis, the prevalence of HCC was around 5%[79]. Likewise, when we followed a cohort of 450 patients with cirrhosis, the estimated cumulative incidence of HCC was 2.6% in the first year, 15.4% in the fifth year and 28.8% in the tenth year, demonstrating the relevance of carrying out a surveillance program[80].

NAFLD, with or without NASH, is a hepatic manifestation of metabolic syndrome and predisposes to HCC in cirrhotic and non-cirrhotic patients. Despite the high prevalence of NAFLD in the general population, as previously mentioned, it is believed that the incidence of HCC in these patients with non-advanced disease is not sufficiently high for a universal surveillance program to be proposed. In a systematic review, considering only studies that include patients with or without cirrhosis, the incidence of HCC in NAFLD patients with cirrhosis was 15% at 10 years, while the incidence in NAFLD patients without cirrhosis was 2.7% and 23 per 100000 person-years[81]. Given the lowest risk of HCC in non-cirrhotic livers (approximately 0.1 to 0.8 per 1000 patient-years), the development of cost-effective HCC surveillance strategies to identify high-risk NAFLD patients without cirrhosis are needed[58].

Although type 2 DM and obesity have been implicated as independent risk factors for HCC, studies establishing a clear link with HCC in non-cirrhotic livers are scarce[82]. Therefore, it becomes essential to assess the benefits of predictive models based on clinical data to identify patients with HCC in the

population of NAFLD patients without cirrhosis.

Some authors use different tools to stratify patients according to the risk of developing HCC. Thus, FIB-4 was evaluated in European databases, including more than 18 million individuals. When the NAFLD group was classified according to the FIB-4 score, it was possible to identify which patients were at greater risk. When compared to individuals with a FIB-4 score < 1.30, those with a score between 1.30 and 2.67 had a risk ratio for HCC of 3.74, and those with a score > 2.67 had a risk ratio for HCC of 25.2[22]. Although not accepted by all[27], it is possible that the FIB-4 score can be used in selected patients for surveillance.

In a European longitudinal study, Younes *et al*[83] applied various scores (NAFLD fibrosis score - NFS, FIB-4, BARD, APRI) and the Hepamet fibrosis score to predict HCC in 1173 patients with NAFLD (75% non-cirrhotic). These patients were followed for a mean period of 81 mo, with 17 patients (1.5%) developing HCC. The NFS performed significantly better than the other non-invasive scores (C-index: 0.901 ± 0.0302; AUROC = 0.889 ± 0.048)[83].

The latest European Association for the Study of the Liver guideline recommends surveillance in patients with metabolic syndrome or NASH in the presence of significant fibrosis on histology or elastography. However, it is noted that the role of surveillance for NAFLD patients without cirrhosis is unclear[84].

Recently, at a meeting of experts, an evidence-based review was performed addressing the risk of HCC in patients with NAFLD. This review concluded that NAFLD patients with evidence of advanced fibrosis, even when suggested by non-invasive markers, should be considered for HCC screening. Thus, the need for surveillance would be indicated when there is an agreement between two non-invasive tests with different methodologies (FIB-4 and elastography, for example). These results were endorsed by the American Gastroenterological Association[85].

NOVEL PERSPECTIVES IN SURVEILLANCE FOR HCC IN NAFLD

The most validated predictive factor for HCC development in NAFLD is the presence of advanced fibrosis. However, many other factors may be considered to identify those at high risk for liver cancer, even though we still do not have enough evidence to change HCC surveillance strategies in NAFLD[86].

In addition to surveillance based on imaging and serological methods, mainly ultrasound and alpha-fetoprotein, there are no scores or predictive models with enough strength to use in the daily surveillance of NAFLD-related HCC. The development of novel tools might help risk stratification and accurately identify high-risk patients, even those without cirrhosis, leading to individualized surveillance strategies.

In future studies, some of these clinical scores should be combined with genetic risk factors for risk stratification of patients with NAFLD, since the genetic markers currently available still have limitations. As noted, different genetic polymorphisms have varying effects on HCC risks; the 17-β hydroxysteroid dehydrogenase 13-HSD17B13, for example, has protective effects, while others such as the *PNPLA3* (variant I148M) increase HCC risk[58].

The combination of genetic polymorphisms to determine a genetic risk score has shown a low accuracy with a sensitivity of 43% and specificity of 79% in the prediction of HCC with an AUROC of only 0.65[85]. Moreover, the genetic polymorphisms are not ready to be used in clinical routine due to high cost and low availability. Another large study by Bianco *et al*[87] investigated the polygenic risk score (PRS) in a German and an Italian cohort with NAFLD compared to the general population regarding the development of HCC. The polygenic risk score (PRS) was composed of TM6SF2-GCKR-MBOAT7 combined in hepatic fat PRS (PRSHFC), further adjusted for HSD17B13 (PRS-5). This study showed a strong association between hepatic fat and HCC. The PRS improved the accuracy of HCC detection and may help stratify HCC risk in individuals with dysmetabolism, including those without severe liver fibrosis[87].

Also, multiple new panels, including biomarkers such as multiprotein-based and circulating tumor-derived DNA-based ("liquid biopsy") panels[88], as well as abbreviated magnetic resonance imaging protocols and other imaging-based protocols, are currently under investigation as potential screening tests. Studies investigating the accuracy of liquid biopsies are ongoing. Liquid biopsy strategies for sampling tumor products in the bloodstream include substances such as circulating tumor cells (CTCs), circulating tumor DNA (ct-DNA) and extracellular vesicles (EVs)[88]. CTCs include cells released from primary or metastatic tumor sites, CT-DNA consists of DNA from cellular necrosis or apoptosis, and EVs are cell membrane-derived particles such as apoptotic bodies, micro-vesicles and exosomes, containing molecular cargoes specific to the origin cell with an essential role in cell-to-cell communication[88]. Data from a systematic review with 67 studies evaluated liquid biopsy techniques for early-stage HCC detection, including studies evaluating CTCs, ct-DNA and EVs. They have shown good accuracy for HCC detection, with higher accuracy than alpha-fetoprotein (AFP) for distinguishing patients with HCC from controls and the capacity to identify AFP-negative HCC patients. In this study, combinations with AFP were superior to AFP alone[62]. When included in a panel, a liquid biopsy was also associated with poorer survival (EVs and ct-DNA)[89] and with tumor progression.

Some blood-based biomarkers, such as lectin-bound AFP (AFP-L3) and des-gamma carboxyprothrombin (DCP), have been proposed for detecting HCC in some regions like Japan and are under investigation in other countries. Moreover, there is an increased interest in early detection panels using multiple combined biomarkers. The best example is GALAD, which combines demographic and clinical variables with blood-based biomarkers such as gender, age, AFP, AFP-L3, and DCP[90]. In a multinational case-control study, its sensitivity was 60%–80% for detecting early-stage HCC[90]. The GALAD panel was recently evaluated in a case-control study of 125 patients with NAFLD. It showed a similar diagnostic performance at a cut-off of -0.63, with a sensitivity and specificity of 68% and 95%, respectively, for early-stage HCC[91]. Interestingly, in the prospective study arm, the GALAD score identified patients who developed HCC as early as 1.5 years before their diagnosis[91]. However, although it is a promising tool, it is not yet available for clinical use since it still needs to be validated in phase III and IV studies.

After basic serological tests, elastographic techniques are the cornerstone for NAFLD's non-invasive staging of liver fibrosis. Vibration-controlled transient elastography (VCTE) can also assess steatosis through the controlled attenuation parameter and is considered the point of care method among elastography-related techniques[40]. 2D-Shear wave elastography and point-shear wave elastography have the additional capacity to evaluate the macroscopic aspect of the liver and identify nodular lesions as patients with NAFLD-related cirrhosis should have an ultrasound every six months to screen for HCC. Thus, the elastography evaluation and the evaluation of liver lesions have been studied as additional methods for HCC surveillance[92]. A recent study in type 2 DM individuals with NAFLD who had VCTE at baseline and were followed for 50 mo has shown that those with liver stiffness > 13 kPa had a higher incidence of liver decompensation and HCC[93].

Boursier *et al*[94] evaluated the prognostic significance of liver stiffness in NAFLD. They proposed defining a new fibrosis classification stage based on liver stiffness by VCTE categorized in seven different classes of liver fibrosis: LSM1 (2.0 to 4.6 kPa), LSM2 (4.6 to 6.1 kPa), LSM3 (6.1 to 8.8 kPa), LSM4 (8.8 to 12.0 kPa), LSM5 (12.0 to 18.0 kPa), LSM6 (18.0 to 38.6 kPa) and LSM7 (greater than 38.6 kPa to 75 kPa). In this study, overall survival decreased as liver stiffness increased. For instance, overall survival for LSM1 in ten years was close to 100%, whereas, for LSM7, it was near 30%. The authors evaluated liver-related deaths in this study, not specifically HCC[94]. As a reflection, based on the data presented, we could suggest performing elastography in patients with NAFLD, and, when a greater liver stiffness is evidenced, they would be selected to join a screening and surveillance program.

CONCLUSION

It is currently challenging to propose general recommendations for screening patients with NAFLD without cirrhosis, and each patient should be evaluated on a case-by-case basis based on the profile of specific risk factors identified. For HCC screening in NAFLD, a valid precision-based screening is needed.

Currently, when evaluating this population of patients, we believe that the use of non-invasive methods can guide the selection of patients who will undergo a screening and surveillance program. So far, ultrasound with or without AFP is still the screening method of choice and should be used for all NAFLD patients with advanced fibrosis. In the future, it is possible that new technologies and liquid biopsy methods might add precision in screening these large populations, including those without cirrhosis.

FOOTNOTES

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MINIREVIEWS

Secondary bile acids and the biliary epithelia: The good and the bad

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Abstract

The biliary tract has been considered for several decades a passive system just leading the hepatic bile to the intestine. Nowadays several researches demonstrated an important role of biliary epithelia (*i.e.* cholangiocytes) in bile formation. The study of biliary processes therefore maintains a continuous interest since the possible important implications regarding chronic cholestatic human diseases, such as primary biliary cholangitis or primary sclerosing cholangitis. Bile acids (BAs), produced by the liver, are the most represented organic molecules in bile. The physiologic importance of BAs was initially attributed to their behavior as natural detergents but several studies now demonstrate they are also important signaling molecules. In this minireview the effect of BAs on the biliary epithelia are reported focusing in particular on secondary (deriving by bacterial manipulation of primary molecules) ones. This class of BAs is demonstrated to have relevant biological effects, ranging from toxic to therapeutic ones. In this family ursodeoxycholic and lithocholic acid present the most interesting features. The molecular mechanisms linking ursodeoxycholic acid to its beneficial effects on the biliary tract are discussed in details as well as data on the processes leading to lithocholic damage. These findings suggest that expansion of research in the field of BAs/cholangiocytes interaction may increase our understanding of cholestatic diseases and should be helpful in designing more effective therapies for biliary disorders.

Key Words: Cholangiocytes; Biliary secretion; Cholestasis; Bile acids; Secondary bile acids; Ursodeoxycholic acid; Lithocholic acid

Core Tip: The biliary epithelia present important physiologic activities that are of interest with regard to chronic cholestatic liver diseases. Secondary bile acids (BAs) are derived by bacterial manipulation of the primary BAs produced by the liver. This review summarizes the most important recent findings with regard to secondary BAs interaction with biliary epithelia.

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INTRODUCTION

The biliary system is composed of a delicate structure of anastomosing ducts, leading the bile from the liver toward the intestine[1]. While for several years this anatomical apparatus was considered just as an inert route for bile transport, recently several studies have demonstrated that important qualitative/quantitative bile changes occur within the biliary tract. The isolation and characterization of the biliary epithelia (composed of bile duct cells or cholangiocytes) has deepened our understanding of several important molecular process involving the biliary tree, also shedding some light on the mechanisms leading to chronic cholestatic liver diseases.

Bile acids (BAs) are the main organic molecules secreted in bile[2]. Their physiological importance, which in the beginning was identified only with regard to the physicochemical processes leading to micelle formation[3], nowadays has been expanded by the evidence that BAs are also essential signaling molecules[4]. In this minireview, the most important findings involving secondary BAs and biliary epithelia will be reported together with the possible implications of these mechanisms in human liver diseases.

SECONDARY BAS

BAs are synthesized by the liver starting from cholesterol and are the most represented lipidic component in bile[5]. Taurine or glycine conjugation, occurring after synthesis, confers increased water solubility to these molecules in bile. BAs are traditionally classified as primary (produced by the liver) or secondary (derived by primary BAs after bacterial dehydroxylation in the intestine)[6]. In humans, the primary BAs are cholic (CA) and chenodeoxycholic (CDCA) acid, while the most represented secondary ones are deoxycholic (DCA) and lithocholic (LCA) acid. The removal of a hydroxyl group (C-7 position) in general determines reduced water solubility and increased detergency in comparison with primary precursors. The hydrophilic or hydrophobic character of a specific BA has been put in relation with its potential cytotoxicity and damaging effects[7]. In this perspective, secondary BAs are generally regarded as possibly damaging molecules when they reach adequate concentrations since their detergent/destabilizing effect on cell membranes. Being the bile a mixture of different (primary and secondary) BAs, the concept of hydrophilic/hydrophobic balance of the bile (and so the net concentration of secondary BAs) has been related with possible liver injury in some conditions[8]. The most hydrophobic human BA is the monohydrate LCA. Sulfation of this molecule by the liver greatly reduces its intestinal absorption (also enhancing its hydrophilicity and urine elimination) and the consequent damage induced by LCA enterohepatic recirculation[9]. It in fact represents less than 5% of total BAs in human bile[6]. The number of hydroxyl groups, however, is not the only determinant of the specific hydrophilic/hydrophobic character of a specific BA. In fact, another secondary BA, ursodeoxycholic acid (UDCA), despite having an equal number of OH groups (two) in comparison with CDCA and one less than CA, is more hydrophilic in comparison with the latter molecules. This physico-chemical characteristic is related to the fact that, differently from CDCA (3α, 7α), in UDCA, the two hydroxyl groups are not on the same plane (3α, 7β). See Table 1 for a quick reference on hydroxyl group number and position, together with some other features, of the principal BAs found in human bile. On Figure 1 the approximate amount of each individual BA in human bile is reported.

BILIARY EPITHELIA

Together with hepatocytes, cholangiocytes constitute the liver epithelial compartment. These latter cells, lining the intrahepatic and extrahepatic biliary ducts, despite representing less than 10% of liver mass, are able to support nearly 50% of bile volume under stimulation[10]. They in fact contribute almost

Table 1 Some physico-chemical features of the most relevant primary and secondary bile acids in human bile

	Hydroxil groups number and position	Solubility in water (protonated form, μM)[1]	Critical micellar concentration (sodium salt, mM)[1]	Hydrophobicity index (taurine conjugated)[2]
Primary bile acids				
Cholic acid	3 (3, 7, 12)	273	13	0
Chenodeoxycholic acid	2 (3, 7)	27	9	0.46
Secondary bile acids				
Deoxycholic acid	2 (3, 12)	28	10	0.59
Lithocholic acid	1 (3)	0.05	0.9	1
Ursodeoxycolic acid	2 (3, 7)	0.9	19	-0.47

[1]Values assessed in water as reported by Hofmann *et al*[44].
[2]Cholic acid and Lithocholic acid were assumed to have (by definition) a value of 0 and 1 respectively[8].

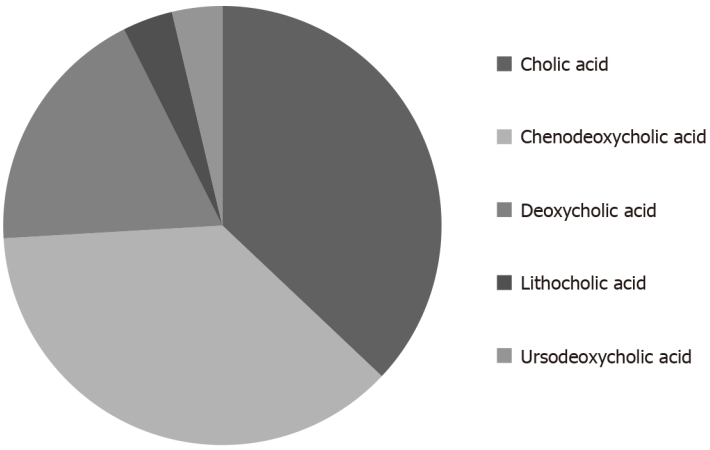

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Figure 1 The relative amount of individual bile acids in human bile is depicted. For each bile acid the extent of conjugation with glicine *vs* taurine is approximately 3 to 1.

exclusively to the so-called BA-independent bile flow. Cholangiocytes are heterogeneous in size and function; the larger ones represent the physiologically functional compartment, while smaller cells (harboring small branches) may replace large ones when the latter are injured[11]. The most studied mechanism of bile duct secretion concerns the interaction between secretin (Sec) and a specific Sec receptor (SR) expressed by cholangiocytes only within the liver. The subsequent downstream molecular mechanisms are characterized by increased intracellular cAMP in bile duct cells, followed by PKC phosphorylation, extrusion of Cl^- by the cystic fibrosis transmembrane regulator and finally its reabsorption and exchange with bicarbonate operated by the Cl^-/HCO_3^- exchanger (AE2)[12]. With this process, a bicarbonate-enriched choleresis is obtained. See Figure 2 for a schematic representation of this mechanism. However, several hormones and neuropeptides (such as somatostatin, histamine, melatonin, gastrin and others) may regulate bile duct cell activity, as these cells have been demonstrated to express the corresponding receptors[13]. BA receptors and transporters are also present on cholangiocytes. They are responsible for important physiological mechanisms.

BAS/BILIARY EPITHELIA INTERACTIONS

The biliary epithelium is constantly exposed to significant concentrations (mM) of BAs. This strict connection is at the basis of important processes under both normal and pathological conditions. As previously stated, BAs are mainly present in bile as glycine or taurine conjugates; however, more than 30 years ago, the possibility that unconjugated BAs may cross the biliary epithelium and recirculate in the liver (the so-called chole-hepatic shunt) was hypothesized, thereby inducing increased choleresis with multiple passages[14]. Later, uptake of BAs by the biliary epithelium was demonstrated by the

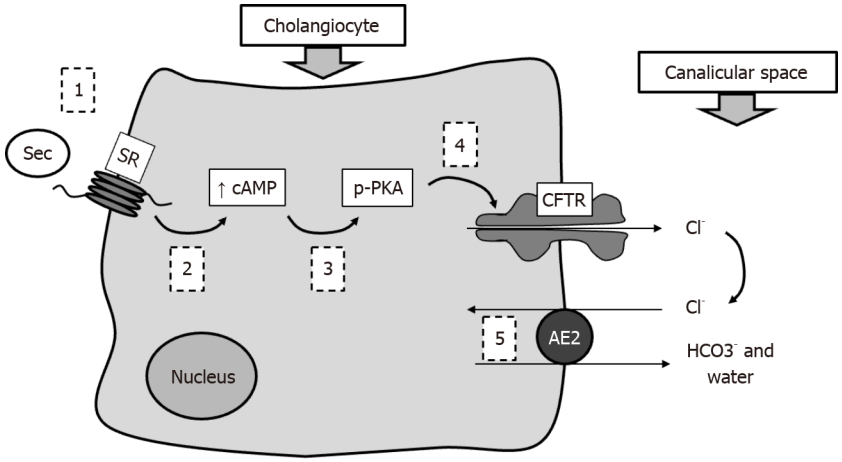

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Figure 2 A step by step representation of secretin-induced biliary secretion is depicted. 1: Secretin (Sec) bind to specific Sec receptor on cholangiocytes; 2 and 3: Increased intracellular levels of cAMP stimulate formation of p-PKA; 4: Cystic fibrosis transmembrane regulator is opened determining Cl- efflux; 5: chloride-bicarbonate exchanger (AE2) favors reuptake of Cl- and releases HCO_3- (osmotically recalling water) in the canalicular space. CFTR: Cystic fibrosis transmembrane regulator; Sec: Secretin; SR: Secretin receptor.

identification of the apical sodium-dependent BA transporter (ASBT) on cholangiocytes[15]. ASBT in the same study was demonstrated to be expressed only by cholangiocytes within the liver and to prompt unidirectional BA transport from the apical to the basolateral cellular domain. ASBT is also expressed in the small intestine, actively reabsorbing BAs and having a major role in maintaining the appropriate entero-hepatic recirculation of these molecules. Gene disruption of this transporter, in fact, nearly completely abolished intestinal recovery of BAs[16], even if a reduced proportion of unconjugated protonated BAs is absorbed by passive uptake in the colon[17]. ASBT function in cholangiocytes, however, remains less clear. In one study, it was demonstrated that Sec stimulation of cholangiocytes was able to increase choleresis, also promoting the transfer of ASBT from the plasma membrane to the apical domain and supporting the original concept of the BA cholehepatic shunt[18]. In another study, a relationship between biliary BAs concentration and ASBT expression was found, suggesting a possible regulatory mechanism of this transporter in maintaining an appropriate biliary BAs concentration[19]. At present, ASBT inhibitors are under study to reduce the BA pool in diseases possibly related to its pathological increment, such as primary biliary cholangitis (PBC)[20].

Further information regarding BAs and cholangiocyte molecular interactions came after the identification of the TGR5, specific for BAs[20]. While in the liver the FXR is mainly expressed in the hepatocyte nucleus where it regulates the transcripts for the synthesis of these molecules[21], on the other hand, TGR5 is prevalently found on the cholangiocyte apical domain[22]. Studies on TGR5(-/-) mice showed an effect on body weight, the immune system and glucose homeostasis[23]. With regard to the biliary tree, TGR5 seems to be an important regulator of cell proliferation with the opposite effect when it is activated in ciliated *vs* non-ciliated cells[24]. In fact, activation of TGR5 on cholangiocyte cilia depresses cAMP formation and proliferation while the same signal in non-ciliated cholangiocytes enhances intracellular cAMP and cell growth. The important role of TGR5 as a possible regulator of biliary mass suggests that this receptor is a possible target in human diseases characterized by uncontrolled cholangiocyte growth, such as cholangiocarcinoma[25] or polycystic liver disease[26]. More recently, other BA receptors, such as the S1PR2, have been identified on cholangiocytes[27]. These signals enhance biliary growth upon stimulation with taurocholic acid (TCA), employing an ERK1/2 dependent mechanism. In conclusion, accumulating evidence demonstrates that the role of BAs in bile is not restricted to lipid dissolution. In fact, BAs are also important molecular signaling molecules.

SECONDARY BAS AND THE BILIARY EPITHELIA

As previously reported, secondary BAs originate from manipulation of the original molecules synthesized by the hepatocytes, by intestinal bacteria. However, within this family, molecules with opposite physicochemical and biological characteristics cohabit. The extremities of this class of organic compounds, in terms of heterogeneity, are represented by UDCA and LCA. At the same time, these two BAs seem particularly interesting and relevant with regard to human biliary diseases, as evidenced by several studies.

UDCA (the good one)

UDCA was first detected as primary BA in Chinese black bear bile, and later also identified in human bile as a secondary BA, in small amounts (≤ 3%)[28]. Interest in UDCA was first focused on its therapeutic potential for cholesterol gallstone dissolution[29,30]. However, its clinical efficacy for gallstone treatment is: (1) Limited to small (≤ 1 cm) non-calcified stones; and (2) affected by frequent recurrent disease when UDCA is withdrawn. On the other hand, early studies on gallstone dissolution, conducted in patients with concurrent chronic hepatitis, also demonstrated the capabilities of UDCA in improving liver function[31].

UDCA beneficial effects on biliary epithelia (general)

Some clinical studies specifically underscored the UDCA beneficial effects in diseases targeting biliary cells and causing an impaired biliary secretion (*i.e.* cholestasis), such as PBC[32]. UDCA (oral dose 13 to 15 mg/kg/day) is in fact, nowadays, a first line treatment for this disease[33,34]. Several mechanisms seem responsible for the improved clinical picture when UDCA is employed in biliary cholestasis[35]. First, due to its intrinsic hydrophilicity, UDCA seems able to reduce the cytotoxicity/hydrophobicity of the total BA pool against bile duct cells. Second, increased biliary secretion is observed if UDCA enrichment occurs in bile. Finally, immune-modulatory and antiapoptotic effects have been demonstrated[32]. Moreover, also regarding PBC, impairment of AE2 and consequent inadequate formation of a delicate bicarbonate film in the canalicular biliary space (the so-called bicarbonate umbrella) has been suggested to facilitate biliary damage by protonated BAs. In this setting, UDCA seems to be able to reconstitute adequate bicarbonate secretion, thus mitigating PBC injury[36] and also reducing the endoplasmic reticulum stress and autophagy acting as a chaperone[37]. With regard to the biliary epithelium, experimental studies have elucidated some important mechanisms.

Molecular basis of UDCA beneficial effects

In early research, conducted in the cholestatic model of the bile duct ligated (BDL) rat (a condition inducing a hyperplastic growth of the biliary tree), UDCA feeding was able to attenuate biliary mass proliferation[38]. A subsequent study using the same model (BDL) clarified that both pathologically enhanced proliferative and secretive processes of cholangiocytes were mitigated by UDCA, as demonstrated by reduced H3 histone, protein cellular nuclear antigen (PCNA) and SR gene expression, and decreased Sec-induced choleresis[39]. Decreased proliferation was not related to cholangiocyte apoptosis and was dependent (as was decreased secretion) on PKCα activation. Another molecular aspect characterizing the effects of UDCA was the decreased ASBT cholangiocyte expression leading to reduced intracellular BA influx. These findings were extended in a more complex model combining rat BDL and vagotomy. In fact, when vagotomy was performed in the BDL rat, the consequent lack of cholinergic stimuli impaired the hyperplastic cholangiocyte response to cholestasis and led to apoptosis in bile duct cells[40]. When UDCA was administered in this model, it was able to counterbalance bile duct cell loss and apoptosis by a PKCα/Ca^{2+} dependent mechanism[41].

UDCA effects in animal model of human biliary disease

Further information regarding UDCA and biliary epithelia came from the Mdr2(-/-) mice model. This mouse is not able to transport phospholipids in bile and develops a chronic cholestasis, resembling the human primary sclerosing cholangitis (PSC), with similar scars and strictures within the biliary tree[42]. In Mdr2(-/-) mice UDCA attenuated reactive cholangiocyte proliferation as well as inflammatory and fibrotic processes. These effects were in part related to the inhibition of mast cells, which are activated during experimental and human PSC[43].

LCA (the bad one)

LCA is a monohydrate secondary BA that is known for its particular hydrophobicity, remaining water insoluble in its free form while it presents a very low critical micellar concentration (concentration at which micelles are spontaneously formed) in saline[44]. According to its physico-chemical properties, LCA has longer been known as a cholestatic and injurious agent in animal experiments[45,46] and, in parallel with this, increased levels of this BA have been found in human with chronic liver disease[47].

General mechanisms of LCA-induced cholestasis

Several mechanisms were identified at the basis of LCA-induced cholestasis such as: (1) Impairment of bile secretion (both BAs dependent and independent)[48]; (2) bile salt export pump translocation from apical membrane to cytosol with its consequent reduced activity[49]; (3) changes in apical membrane fluidity and tight junction permeability[50]; and (4) impairment of canalicular contraction[51].

LCA effects on biliary epithelia

With regard to biliary epithelia, a study on LCA feeding in Swiss albino mice evidenced interesting features[52]. After 4 d of a 1% LCA diet, destructive cholangitis characterized by stenosis of biliary ducts, solid crystal precipitation and bile infarcts was observed. Neutrophil infiltration surrounded the

Table 2 Main findings regarding secondary bile acids and biliary epithelia

Model	Administration route	Main results	Main molecular, immunologic findings	Ref.
Ursodeoxycholic acid				
BDL rat	Feeding (both the unconjugated and taurine-conjugated form)	Decreased biliary proliferation and secretion. No apoptosis	Decreased H3-histone. PCNA, SR and ASBT expression. No apoptosis. Increased PKC α expression	[38, 39]
BDL + vagotomy rat	Feeding (both unconjugated and taurine-conjugated form)	Reversal of duct loss and apoptosis induced by vagotomy	PKCα/Ca^{2+} dependent mechanism	[41]
Mdr2(-/-) mice	Feeding	Decreased proliferation, inflammation and fibrosis	Inhibition of mast cells activity	[43]
Lithocolic acid				
Mouse	Feeding	Destructive cholangitis, bile duct stenosis, bilary infarcts	Damage related to direct toxic effect and not to neutrophil infiltration	[52, 53]
In vivo rat and isolated cholagiocytes	Feeding (cholic acid or Lithocholic acid both taurine conjugated)	Similar effect in increasing proliferation and secretion	Effect restricted to large cholangiocytes	[54, 55]
In vivo rat and isolated cholagiocytes	Feeding (cholic acid or Lithocholic acid both taurine conjugated)	Cholangiocytes proliferation	Dependent by PKA-mediated ASBT expression	[56]
Deoxycholic acid				
Human gallbladder cancer (specimens and cell lines)	*In vitro* exposure	Increased concentration associated with inhibition of tumor growth	Reduced miR-92b-3p inhibits PI3K/AKT activity	[60]

ASBT: Apical sodium bile acids transporter; BDL: Bile duct ligated; PCNA: Protein cellular nuclear antigen; SR: Secretin receptor.

small biliary branches and periductal fibroblast activation with collagen deposition was reported. A subsequent study, conducted in the same experimental system, helped to clarify that LCA-related biliary damage was dependent on direct toxicity of this BA and not to the immune response since neutrophil inhibition did not significantly change the pathological picture[53]. With regard to secretive and proliferative cholangiocyte activities, *in vitro* experiments demonstrated that LCA and CA (taurine conjugated) had similar effects in promoting biliary growth and Sec-stimulated bile output[54]. These results were observed with the large cholangiocyte population, which is well-known as the main functional pool in the biliary tree. Similar results were later confirmed in *in vivo* experiments[55]. In fact, TCA or TLCA rat feeding (1% diet, 1-4 wk) both similarly increased biliary mass and enhanced cholangiocyte biliary secretion. Further experiments suggested that the TLCA stimulation of cholangiocytes function (similarly to TCA) was associated with increased ASBT activity and consequently enhanced intracellular (PKCα/Ca^{2+} dependent) BA trafficking[56]. This process, with regard to LCA, due to the changes in Ca^{2+} flux, was also related to impaired gap junction permeability and consequent cholestasis [57].

Other secondary BAs

With regard to other secondary BAs that may play a role in human biliary physio-pathology, DCA is the only one possibly reaching significant concentrations (10%-35% of total BAs pool) in human bile[58]. DCA liver toxicity has been well-established since the early 1990s and, in a study on rat feeding, this was enhanced in comparison to LCA due to its increased intestinal reabsorption and bile enrichment [59]. Despite this and concerning the biliary epithelia, one study has raised interest by showing the suppression of gallbladder cancer growth by DCA, possibly due to interference with miR-92b-3p[60]. This miR in fact would be responsible of the activation PI3K/AKT pathway that is enhanced in several tumors and also represents a target for anticancer treatment[61]. Several other secondary BAs may be found in different species[62]. For instance in rodents the main represented primary BA is β-muricholic acid (β-MCA; 3α, 6β, 7β)[63]. Bacterial manipulation of β-MCA may give origin to different secondary BAs including HDCA (3α, 6α)[64]. HDCA is reported as the strongest regulator of BA-sensitive ion channel (BASIC) that is normally expressed in brain, intestine and cholangiocytes only, within the liver [65]. While the exact physiologic function of cholangiocyte BASIC has not been well established, evidences demonstrate enhanced activity of this channel, with increased trans-epithelial ion transport, after exposure to HDCA[66]. This suggests BASIC as a further possible regulator of biliary secretion. Table 2 summarizes the main findings regarding secondary human BAs and biliary epithelia.

CONCLUSION

BAs are important organic molecules. For several decades, researchers have focused on their physico-chemical characteristics, due to their reported detergent properties. From this perspective, the hydrophilic or hydrophobic character of a BA has been considered in the past as the main determinants of physiologic effect. This preliminary view is clearly challenged nowadays, with many studies demonstrating the important molecular signaling systems activated by BAs, not only in the hepatocytes but also in the biliary epithelium. Artificial manipulation of native BA molecules, moreover, has led to the discovery of new agents, such as obeticholic acid, that may be helpful for human therapy[67]. Given all the above, it is clear that the original classification of BAs as primary and secondary compounds only expresses aspects of their synthesis and not necessarily beneficial or negative physiologic effects. Similarly, the division of secondary BAs as good or bad ones (as reported in this review) is questionable, since this does not adequately recapitulate the multitude of effects (probably discovered just in part at the present stage) these molecules may have. In fact, UDCA (generally supposed as beneficial) has been demonstrated to be detrimental in experimental obstructive cholestasis as it can lead to bile infarcts and should not be administered in this clinical condition[68]. On the other hand, LCA has shown interesting curative properties and anti-tumoral and anti-inflammatory effects on intestinal environment, in some studies[69]. In conclusion, UDCA and LCA clearly represent the extremities of a field in which research may growth and a revision in our present beliefs regarding these secondary BAs remains therefore possible in the near future. With regard to normal human physiology and in practice, however, LCA accumulation is prevented by a detoxification system while UDCA is formed only in trace amounts. However, bile enrichment is possible when BAs are exogenously administered to manipulate the BAs pool for therapeutic purposes.

FOOTNOTES

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MINIREVIEWS

Non-alcoholic fatty liver disease and COVID-19: Harmless companions or disease intensifier?

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Abstract

The pandemics of coronavirus disease 2019 (COVID-19) and non-alcoholic fatty liver disease (NAFLD) coexist. Elevated liver function tests are frequent in COVID-19 and may influence liver damage in NAFLD, while preexisting liver damage from NAFLD may influence the course of COVID-19. However, the prognostic relevance of this interaction, though, is unclear. Obesity is a risk factor for the presence of NAFLD as well as a severe course of COVID-19. Cohort studies reveal conflicting results regarding the influence of NAFLD presence on COVID-19 illness severity. Striking molecular similarities of cytokine pathways in both diseases, including postacute sequelae of COVID-19, suggest common pathways for chronic low-activity inflammation. This review will summarize existing data regarding the interaction of both diseases and discuss possible mechanisms of the influence of one disease on the other.

Key Words: COVID-19; Postacute sequelae of COVID-19; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Inflammation; Fatty liver

Core Tip: The "colliding" pandemics of coronavirus disease 2019 (COVID-19) and non-alcoholic fatty liver disease (NAFLD) influence each other in several ways. Molecular similarities of cytokine pathways in both diseases including postacute sequelae of COVID-19 (PASC) may be responsible for amplification of chronic low-active inflammation. While there are conflicting data regarding the clinical influence of NAFLD on acute COVID-19 and vice versa, further research is necessary to study the long-term influence of COVID-19 hygienic measures and PASC on NAFLD.

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INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD) has increased rapidly over the past 30 years, particularly in Western countries. This is due to a lifestyle with hypercaloric diets and obesity leading to a concomitant increase in metabolic syndrome[1]. It is estimated that approximately 30% of people in Western countries have NAFLD, and approximately 5% have non-alcoholic steatohepatitis (NASH), the inflammatory variant of fatty liver[2]. NAFLD and NASH represent chronic liver diseases with high morbidity and potential mortality.

The coronavirus disease 2019 (COVID-19) pandemic began in Wuhan, China, in late 2019. From there, the disease spread rapidly throughout the world. To date, over 500 million people have contracted COVID-19 and over 6 million people have died from it[3]. Despite effective vaccination, it is foreseeable that the coronavirus cannot be eradicated. While vaccination protects against a severe course, it cannot completely prevent infection and minor disease. To that extent, COVID-19 is likely to persist in the world as a disease, its severity depending on the prevailing variants, and to impact the population and preexisting concomitant diseases in an individual. After acute COVID-19 resolves, a proportion of COVID-19 patients suffer from postacute sequelae of COVID-19 (PASC) also named "long COVID" - as a range of new, returning, or ongoing health problems people can experience four or more weeks following initial severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection[4,5]. Therefore, in addition to acute COVID-19, postacute sequelae from COVID-19 are an emerging global health crisis, and there are hints that metabolic factors and a chronic inflammatory state (both characteristics of NAFLD) predispose patients to PASC[6].

While NAFLD is a noninfectious disease whose pandemic spread depends on people's lifestyle, especially dietary habits, COVID-19 has an acute course due to its nature as an infectious disease. This leads to waves of infection that have prompted epidemic hygiene countermeasures to contain the infection, especially in the past 2 years (2020 and 2021). Lockdowns have occurred in numerous countries to restrict the mobility of people and thus prevent the spread of the coronavirus. The harsh isolation and lockdown measures in the past 2 years under the more pathogenic previous variants had significant sociological and psychological effects. Thus, in addition to the direct viral effects of COVID-19 on the liver, there are also indirect effects on the liver or liver disease, which may play an important role in the further development of these diseases. As COVID-19 can still cause renewed lockdowns and isolation measures in the future, for example, when more lethal mutations arise again or due to newly emerging infectious diseases, such effects should also be modeled and taken into account in the future.

NAFLD and COVID-19 can be referred to as "syndemic"[7]. They represent, in a sense, "colliding pandemics"[8] due to the various possible interactions, which have different dynamics but some molecular and pathogenetic commonalities. These effects are summarized in this review and the current state of the evidence is evaluated. Between August and September 2022, we searched PubMed using the terms coronavirus, COVID-19, SARS-CoV-2, NAFLD, fatty liver, NASH, MAFLD. We analyzed all retrieved abstracts and obtained the full papers, if the study was dedicated to the connection between COVID-19 and NAFLD.

MOLECULAR SIMILARITIES OF BOTH DISEASES

NAFLD covers a wide spectrum of severity, ranging from bland fatty liver without any inflammation (NAFL) and with little or no tendency to progress all the way to NASH with inflammatory reactions and hepatocyte damage with or without fibrosis. A total of 5% to 20% of patients with NAFLD develop NASH, which undergoes a further transition to higher-grade fibrosis and eventually liver cirrhosis in 10% to 20% of cases[9]. These clinical features of NAFLD are the background for chronic low inflammatory activity of the disease. Intestinal barrier dysfunction plays a major role in triggering and

amplifying these inflammatory processes, leading to translocation of bacteria or bacterial components into the portal circulation and induction of hepatic inflammation[10]. Obesity induced by an unhealthy lifestyle (insufficient exercise and hypercaloric diet) leads to increased secretion of proinflammatory leptin, interleukin (IL)-6, and tumor necrosis factor (TNF)-α from peripheral adipose tissue, while secretion of adiponectin, an inhibitor of human stellate cell proliferation, is decreased[11]. The massive disruption of lipid metabolism due to the disturbed balance between lipolysis, oxidation, secretion, and uptake of lipids between adipose tissue and liver contributes to hepatic steatosis as well as lipotoxicity, affecting key cellular elements such as the endoplasmic reticulum or mitochondrial function[12]. In terms of a vicious cycle, hepatic metabolic pathways (especially β-oxidation) are dysregulated and further reinforce the imbalance in lipid metabolism[13] and thus lipotoxicity. Activation of human stellate cells and cytokine production by Kupffer cells follows, with IL-1β, TNF-α, IL-6, interferon (IFN)-γ, nuclear factor-kappaB, and reactive oxygen species being key extracellular and/or intracellular proinflammatory mediators that maintain chronic low-activity inflammation and induce the development of fibrosis[14,15].

Interestingly, several of these factors also appear to play important roles in COVID-19 pathogenesis in the context of systemic inflammatory response syndrome. IL-1β, TNF-α, IL-6, and IFN-γ are elevated during acute COVID-19 disease[16], and IL-6 in particular may be considered a central cytokine for the hepatic effects of COVID-19 due to its principal role in the negative acute phase response. After acute COVID-19 resolves, chronic systemic inflammatory responses may persist in patients with sequelae after acute disease, although the exact molecular drivers of PASC are largely unknown. Recently, Schultheiß *et al*[17] showed that PASC is associated with chronic elevation of IL-1β, TNF-α, and IL-6 levels. Phetsouphanh *et al*[18] even demonstrated elevation of IFN-γ (and other proinflammatory cytokines) in patients 4 mo after SARS-CoV-2 infection, irrespective of whether they had PASC symptoms.

It is therefore straightforward to speculate that low-activity NAFLD inflammation may be amplified or exacerbated by the acute phase of COVID-19 and chronic systemic inflammatory responses in at least some patients after acute COVID-19, resulting in interactions between the two diseases at the molecular level (see Figure 1).

CLINICAL EFFECTS OF COVID-19 ON THE LIVER AND THEIR MECHANISMS

Because COVID-19 is an aerosol-transmitted disease, clinical symptoms of respiratory disease have always been the primary clinical focus. However, many case series and clinical studies show that COVID-19 also has systemic effects. These include vascular inflammation, thrombosis, and other organ involvement. Liver inflammation is therefore only part of a systemic inflammatory component of SARS-CoV-2 and is almost never the primary clinical symptom.

In a comprehensive review, it was shown that COVID-19 leads to elevations in liver enzymes in approximately 17%-58% of patients[19]. Elevations of transaminases ("hepatitis") dominate, and cholestatic constellations are much less frequent, suggesting predominantly hepatocytic damage[19]. Frequently, this concomitant COVID hepatitis is clinically inapparent. In a recent meta-analysis involving over 77000 patients, the prevalence of clinically overt liver damage was shown to be correlated with the severity of COVID-19. In this analysis, liver damage was described in 40%-47% of severe COVID-19 cases, whereas patients with a milder course were affected in only 10% on average [20]. COVID-19 may trigger acute-on-chronic liver failure in patients with liver cirrhosis due to NAFLD [21]. In contrast, severe hepatic inflammation with impairment of liver function does not seem to occur in patients without advanced preexisting fibrosis[22].

Intracellular uptake of SARS-CoV-2 requires binding of the virus with the spike protein to angiotensin-converting enzyme 2 (ACE2). Further molecular interactions with transmembrane serine protease 2, among others, lead to priming of the S protein and internalization of the virus and its genetic material into the cell[23].

Hepatic tropism of SARS-CoV-2 has been shown recently[24]. However, the exact mechanism of infection of the liver is unclear. Although ACE2 protein expression was observed in the liver, this expression is predominantly located in Kupffer cells (and only at relatively low levels in hepatocytes). In line with that, the SARS-CoV-2 spike protein could be detected in Kupffer and parenchymal cells (for example, hepatocytes)[24,25]. As a potential mechanism of hepatocyte infection, alternative hepatocyte cell entry facilitators are under discussion, *e.g.*, high-density lipoprotein scavenger receptor class B member 1[24] and the asialoglycoprotein receptor[26].

Several mechanisms of liver cell damage are conceivable (see also Figure 1). A direct cytotoxic effect does not appear to be the dominant mechanism of damage in the normal liver. Healthy hepatocytes express almost no ACE2, whereas in liver cirrhosis, ACE2 expression and activity are significantly higher[27]. These molecular regulatory mechanisms tend to argue against direct cytotoxic effects of SARS-CoV-2 in healthy liver but could explain why cirrhotic patients are more susceptible to (further) liver damage or more severe COVID-19 disease overall.

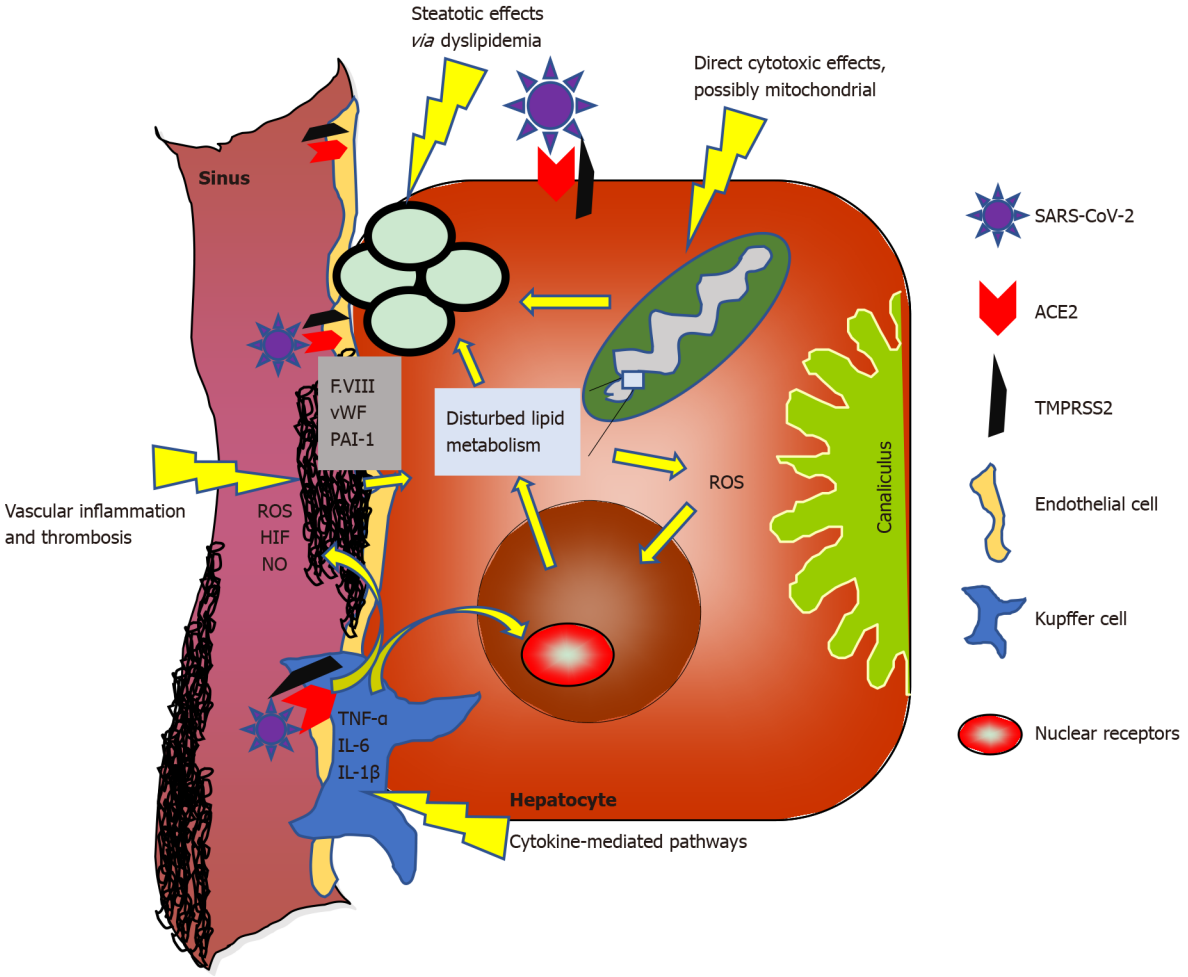

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Figure 1 Possible mechanisms of coronavirus disease-induced liver injury and the interplay between molecular pathways of inflammation in both diseases (in pre-existing non-alcoholic fatty liver disease). F. VIII: Factor VIII; vWF: von Willebrand factor; HIF: Hypoxemia-inducible factor; IL-1β: Interleukin-1β; IL-6: Interleukin-6; NO: Nitric oxide; PAI-1: Plasminogen activator inhibitor 1; ROS: Reactive oxygen species; TNF: Tumor necrosis factor; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; ACE2: Angiotensin converting enzyme-2; TMPRSS2: Transmembrane serine protease 2.

Inflamed hepatocytes (as well as other somatic cells) may exhibit mitochondrial dysfunction in NAFLD or NASH[28,29], which in turn favors ACE2 upregulation[30] and could support viral infection. Conversely, COVID-19 also appears to directly affect mitochondrial function[31]; thus, these effects may amplify each other and induce a more severe course of both diseases.

Another pathogenetic mechanism is the additional fat storage in hepatocytes triggered by SARS-CoV-2. COVID-19 causes dyslipidemia[32] and autopsy studies show a high proportion of steatosis in COVID-19 patients[33-35]. However, it remains unclear in many cases whether steatosis was already preexisting or triggered upon infection. NAFLD patients apparently express ACE2 and various serine proteases at higher levels in the liver[36]; thus, preexisting steatosis may promote COVID-19-induced damage. In turn, COVID-19 may exacerbate steatosis. Whether these dynamics are quantitatively significant effects must remain open for now and deserves future research.

The most significant hypothesis of hepatocyte injury based on clinical characteristics concerns inflammatory cytokine-mediated pathways[37], with commonalities between acutely mediated COVID-19 effects and NAFLD-mediated chronic liver inflammation (especially IL-6; see above). ACE2 expression on Kupffer cells[38] may be the origin of virus-mediated, locally amplified liver inflammation.

Autopsy studies also support possible vascular-associated mechanisms for COVID-19-mediated liver injury[35,39]. One early series of postmortem liver biopsies from patients with COVID-19 reported portal or sinusoidal vascular thrombosis in at least 50% of patients[39]. Patients with COVID-19 exhibit coagulopathy and endotheliopathy, characterized by elevated levels of von Willebrand factor and factor VIII[40]. As factor VIII is produced primarily by liver sinusoidal endothelial cells (LSECs)[41], hypercoagulable LSECs might play an additional role in COVID-19-related liver injury. Of note, endotheliopathy has been reported to be sustained following COVID-19[42], suggesting not only acute but also long-term interactions and consequences of endothelial-mediated inflammation with chronic liver

diseases such as NAFLD. Another direct link between COVID-19 and NAFLD may be *via* plasminogen activator inhibitor 1 (PAI-1). PAI-1 has been shown to be elevated among COVID-19 patients[43]. This role of PAI-1 in COVID-19 liver injury is potentially interesting, especially in NAFLD patients, as elevated PAI-1 has been associated with NAFLD and NASH[44]; therefore, COVID-19-induced PAI-1 elevation may aggravate NAFLD.

The mechanisms described above partly overlap or influence each other, as indicated by the arrows in Figure 1. The pathogenic significance of these molecular associations is not clear in most cases. There are many hints to suggest that the effects of COVID-19 on the liver and especially NAFLD are multifactorial and may also differ individually. Despite these different possible mechanisms, severe liver injury and liver failure are rare even in prediseased liver patients[22].

INFLUENCE OF NAFLD ON COVID-19

Numerous studies of varying quality have addressed the risk for morbidity and mortality of COVID-19 in subjects with NAFLD (Table 1). The studies are extraordinarily heterogeneous, making reliable conclusions difficult. Numerous studies were published only as letters to the editor, not as full articles. Almost all studies are retrospective and include low numbers of patients. Definitions of NAFLD/metabolic syndrome-associated fatty liver disease (MAFLD) vary considerably; in some cases, only blood-based surrogate scores for steatosis and liver fibrosis (such as the hepatic steatosis index, NAFLD fibrosis score, or Dallas steatosis index) were applied. Imaging with ultrasound or computed tomography (CT) is mostly used as the criterion for the presence of fatty liver. Biopsy-proven NAFLD is a rare exception. Importantly, data on alcohol consumption are lacking in many studies. No statements are found on inflammatory activity of fatty liver (*i.e.*, on the presence of NASH). Definitions of severe COVID progression are also inconsistent. Studies that define NAFLD only by scores or by imaging (ultrasound, CT) during the course of hospitalization for COVID-19 cannot provide information about the presence of fatty liver before COVID-19 emergence. Control groups almost invariably contain fewer patients with classic metabolic factors, such as diabetes mellitus and obesity, than the respective NAFLD groups; this metabolic imbalance of study groups cannot easily be controlled by multivariate analysis.

These conditions do not allow confident conclusions to be drawn at this time. Presently, the data suggest that NAFLD alone is not a relevant risk factor for severe COVID-19 progression or mortality. In particular, registry studies from large liver collectives with different etiologies tend not to support a special role for NAFLD[54,63,64,66]. However, available studies also show that the presence of liver fibrosis or cirrhosis is associated with a higher risk of severe COVID-19 disease[61,69]. In this respect, the increase in risk for a more severe course of COVID-19 may not be attributed to NAFLD per se but rather to advanced liver disease irrespective of the underlying etiology in general.

INFLUENCE OF EPIDEMIC HYGIENIC COVID-19 MEASURES ON NAFLD

Disease hygiene measures in the context of the infectious waves probably represent an important factor in the influence of the COVID-19 pandemic on NAFLD. The psychosocial impact of the COVID-19 pandemic resulted in measurable exacerbations of metabolic comorbidities of NAFLD. A United States cohort study examined 111 NAFLD patients and found decreases in physical activity in 51%, weight increase in 34%, and increases in alcohol consumption in 5% during the COVID-19 pandemic[72]. A Spanish study screened over 6000 workers for metabolic factors and found significant increases in body mass index, insulin resistance, and low-density lipoprotein during the pandemic. The average fatty liver index (FLI) as a surrogate for NAFLD increased from 25.2 to 33 in this study[73]. Another Spanish study showed a decrease in physical activity during lockdown with a consecutive increase in FLI and worsening of metabolic status[74]. In an Italian cohort study, 48% of 357 NAFLD patients gained weight during lockdown, and this weight gain was associated with abandonment of a Mediterranean diet and decreased physical activity in univariate analysis and various multivariate models. Interestingly, in PNPLA3-GG polymorphism patients, this genotype represented the only favoring factor for weight gain [75]. A Japanese study examined 973 patients with health checks in 2018 and 2020. In this study, the absolute number of MAFLD patients increased from 261 to 305; however, as the authors identified predominantly higher alcohol consumption as a risk factor for this development, there is actually a definition problem of MAFLD in the strict sense[76]. Overall, these studies show a decrease in physical activity and an increase in weight in the general population. It can be assumed, though not yet clearly shown, that this favors the *de novo* development or exacerbation of steatosis and inflammation in NAFLD, that fibrosis in turn may be further advanced and that the prognosis of the liver disease overall is thus worsened at the end. Long-term studies into these effects of pandemic-associated lifestyle changes are necessary.

Table 1 Studies with data regarding the risk of non-alcoholic fatty liver disease/metabolic syndrome-associated fatty liver disease for severe coronavirus disease 2019

Ref.	Study type and number of NAFLD patients	Results	Appraisal
Zhou *et al*[45], 2020	Retrospective, matched cohorts, *n* = 55 per group	More severe COVID-19 in MAFLD OR = 4.07	Poor matching regarding metabolic status, more male pat in MAFLD group
Targher *et al*[46], 2020	Retrospective, cohort study *n* = 94 (216 w/o MAFLD)	More severe COVID-19 with higher FIB-4 or NFS	No matching, no full paper
Ji *et al*[47], 2020	Retrospective, cohort study *n* = 202	NAFLD 87 % in progressive COVID-19 (*n* = 39) *vs* 26 % in stable COVID-19 (*n* = 163)	Comorbidities highly different between groups, no full paper, NAFLD definition only *via* HSI
Hashemi *et al*[48], 2020	Retrospective, CLD cohort with 55 NAFLD patients (294 w/o CLD/NAFLD)	Presence of CLD and NAFLD higher risk for mechanical ventilation (OR = 2.15) and ICU admission (OR = 2.3), cirrhosis risk factor for mortality	Imbalance in metabolic status, NAFLD diagnosis relying on prior imaging
Huang *et al*[49], 2020	Retrospective, cohort *n* = 86 (194 w/o NAFLD)	Only higher ALT in NAFLD patients, course of disease comparable to controls	NAFLD only defined by HSI, imbalance in metabolic status
Forlano *et al*[50], 2020	Retrospective, cohort *n* = 61 (132 w/o NAFLD)	NAFLD pat with higher CRP, younger age. Fibrosis or cirrhosis no risk for more severe COVID-19	Only hospitalized patients, higher BMI in NAFLD, diagnosis by imaging (US or CT)
Lopez-Mendez *et al* [51], 2021	Retrospective, cohort study *n* = 66 (89 w/o steatosis)	Presence of steatosis (and/or liver fibrosis) not related to severity or mortality of COVID-19	Steatosis only defined by HSI, imbalance on metabolic status
Zheng *et al*[52], 2020	Retrospective, cohort study *n* = 66 (45 with and 21 w/o obesity)	Obesity risk factor for COVID severity in MAFLD patients (OR = 6.3)	Diagnosis of MAFLD by CT and clinical criteria, no controls w/o MAFLD, no full paper
Zhou *et al*[53], 2020	Retrospective, cohort study *n* = 93 (out of 327 total patients)	Younger MAFLD patients with relatively higher risk for severe COVID	No full paper, small number of older patients, CT data
Valenti *et al*[54], 2020	Retrospective, United Kingdom Biobank cohort (Mendelian randomization), total *n* > 500000	No evidence for NAFLD as risk factor for severe COVID-19	Data errors possible, partly little characterization of patients, no full paper
Mahamid *et al*[55], 2021	Retrospective, cohort study *n* = 22 (49 w/o MAFLD)	8/22 with severe COVID-19 *vs* 5/49 w/o MAFLD	CT data, large differences in metabolic status between groups
Chen *et al*[56], 2021	Retrospective, cohort study *n* = 178 (164 w/o hepatic steatosis)	More intubation and vasopressors in steatosis, but lower mortality	Only hospitalized patients, HSI or imaging, rel. high percentage of steatosis in cohort, metabolic status not balanced
Gao *et al*[57], 2021	Retrospective, matched cohorts, *n* = 65	OR = 4.07 for severe COVID-19 only in non-diabetic patients	Poor matching regarding metabolic status, NAFLD diagnosis by CT and clinical criteria, duplicate patients with Zhou *et al*[45]
Marjot *et al*[58], 2021	Retrospective CLD cohort with 322 NAFLD patients	No higher mortality for NAFLD patients in multivariate analysis	Control group matched only to complete CLD cohort, not specifically to NAFLD patients. Unclear definition of NAFLD
Parlak *et al*[59], 2021	Retrospective, cohort study *n* = 55 (288 w/o fatty liver)	Presence of fatty liver risk factor (OR = 3.9) for severe COVID-19	CT data, no data regarding BMI, no data comparison NAFLD *vs* non-NAFLD
Mushtaq *et al*[60], 2021	Retrospective, cohort study *n* = 320 (269 w/o NAFLD)	NAFLD predictor for mild or moderate liver injury, but not for disease severity or mortality	NAFLD only defined by HIS, imbalance on metabolic status, no full paper
Campos-Murguía *et al*[61], 2021	Retrospective, cohort study *n* = 176 (256 w/o MAFLD)	Liver fibrosis, not MAFLD alone, predictor for severity and mortality of COVID-19	CT data, relatively good obesity matching to controls
Kim *et al*[62], 2021	Retrospective, CLD cohort with 456 NAFLD patients	NAFLD no risk factor for severe course or mortality of COVID-19	No control cohort w/o liver disease, tertiary centers only, NAFLD ICD-diagnosis
Simon *et al*[63], 2021	Large Swedish CLD cohort (total *n* = 42320), biopsy confirmed, with unclear number of NAFLD patients	CLD presence as risk factor for hospitalization, but not for severe COVID (including cirrhosis)	Historic cohort with possible drop-outs, underlying CLD in controls may have been missed
Roca-Fernández *et al* [64], 2021	United Kingdom Biobank cohort, with prospective data on infection and hospitalization for COVID	Fatty liver with increased risk for testing COVID-positive, obesity and fatty liver with higher risk for hospitalization, but not obesity alone	Data errors possible, little characterization of patients, small number of patients with severe COVID

Ziaee *et al*[65], 2021	Retrospective Iranian cohort *n* = 218 (357 patients w/o NAFLD, additional control group w/o COVID)	Fatty liver significant more prevalent in COVID group compared to control group (38% *vs* 9%). Longer hospital stay and larger pulmonal involvement in NAFLD patients	Very low percentage of fatty liver in control group. Control group with missing data
Liu *et al*[66], 2022	COVID-19 HGI and United Kingdom Biobank cohorts (Mendelian randomization), retrospective data, total *n* > 2500000	No evidence for NAFLD as risk factor for severe COVID-19	Data errors possible, little characterization of patients, no full paper
Chang *et al*[67], 2022	South Korean COVID-19 cohort with FLI score (total *n* = 3122)	Highest FLI tertile with higher risk for severe COVID-19, but not for higher mortality	No NAFLD-specific case definition, FLI score tertile cutoff low
Vrsaljko *et al*[68], 2022	Prospective cohort study *n* = 120 (96 w/o NAFLD)	NAFLD with higher risk for severe COVID-19 including pulmonary thrombosis	No data regarding fibrosis
Tripon *et al*[69], 2022	Retrospective French cohort *n* = 311 (408 w/o NAFLD)	NAFLD with higher risk for hospitalization, high FIB-4 with higher risk for severe COVID-19	NAFLD only defined by NFS, important data missing in cohort patients
Moctezuma-Velázquez *et al*[70], 2022	Retrospective Mexican cohort *n* = 359 (111 w/o NAFLD)	NAFLD associated with mortality, ICU admission and mechanical ventilation, but CT-determined liver steatosis was not	NAFLD definition based on DSI, small number of control patients, only hospitalized patients
Okuhama *et al*[71], 2022	Retrospective Japanese cohort *n* = 89 (133 w/o fatty liver)	Fatty liver associated with severe COVID-19	CT data, no data regarding dyslipidemia, only hospitalized patients

BMI: Body mass index; CLD: Clinical liver disease; CT: Computed tomography; DSI: Dallas steatosis index; FLI: Fatty liver index; HIS: Hepatic steatosis index; MAFLD: Metabolic syndrome-associated fatty liver disease; NFS: Non-alcoholic fatty liver disease fibrosis score; pat: Patients; US: Ultrasound; NAFLD: Non-alcoholic fatty liver disease; OR: Odds ratio; ICU: Intensive care unit; COVID-19: Coronavirus disease 2019; HGI: Haemoglobin glycation index; ICD: International Classification of Diseases; CRP: C-reactive protein; ALT: Alanine aminotransferase.

CONCLUSION

NAFLD and COVID-19 have both taken a pandemic course in their own ways. Whereas infectious disease essentially causes short-term disease, NAFLD represents a chronic pandemic. Interestingly, the molecular mechanisms of inflammation are similar, although NAFLD is more of a chronic low-activity inflammation while COVID-19 is an acute inflammatory condition. However, in NAFLD patients with ongoing PASC both conditions may chronically interact with unknown mutual effects.

Obesity certainly represents an important unifying clinical factor of both diseases, as obesity is an important risk factor for the development of NAFLD and the severe course of COVID-19. In contrast, the presence of NAFLD per se does not appear to be a relevant risk factor for particularly severe COVID-19. The effects of COVID-19 on liver disease are more complex and still poorly understood. While the direct viral effect on NAFLD may be limited, probably because of the short duration of the acute viral infection, the individual effects associated with lockdowns and isolation are potential risk factors for disease progression due to a reported decrease in physical activity together with an increase in obesity. European Association for the study of the liver position papers provide valuable recommendations for liver patients after the outbreak of the pandemic, including specific recommendations for NAFLD patients[77,78].

FOOTNOTES

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ORIGINAL ARTICLE

Observational Study

Knowledge and attitudes towards the use of histological assessments in ulcerative colitis by gastroenterologists *vs* pathologists

Aviv Pudipeddi, Caroline Fung, Britt Christensen, Robert V Bryant, Kavitha Subramaniam, John Chetwood, Sudarshan Paramsothy, Rupert W Leong

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Abstract

BACKGROUND

Histological remission is increasingly accepted as a treatment endpoint in the

management of ulcerative colitis (UC). However, the knowledge of histology guidelines and the attitudes towards their use in clinical practice by gastroenterologists and pathologists is unknown.

AIM

To evaluate the knowledge of histology guidelines and attitudes towards the use of histology in UC by gastroenterologists and pathologists.

METHODS

A prospective, cross-sectional nationwide survey of gastroenterologists and pathologists who analyse UC specimens was conducted. The survey consisted of 34 questions to assess gastroenterologists' and pathologists' knowledge (score out of 19) and attitudes towards histological assessment in UC. Survey questions were formulated using the European Crohn's and Colitis position paper on histopathology and the British Society of Gastroenterology biopsy reporting guidelines. It included knowledge of histological assessment of disease activity and dysplasia, knowledge of histological scoring systems for ulcerative colitis, uptake of histology scoring systems in routine practice, attitudes towards the role of histological activity, and the use of histological activity in clinical scenarios.

RESULTS

Of 89 responders (77 gastroenterologists, 12 pathologists), there was almost universal acceptance that histological assessment should form part of UC evaluation [95% gastroenterologists, 92% pathologists]. However, gastroenterologists reported that 92% of their pathologists do not use a histological scoring system. Utilisation of a formal histological scoring system was preferred by 77% of gastroenterologists and 58% of pathologists. Both groups lacked awareness of the Geboes Score, Nancy Index and Robarts Histopathological Index scoring systems with 91%, 87%, and 92% of gastroenterologists respectively; and 83%, 83%, and 92% pathologists respectively, being uncertain of scoring systems' remission definitions. Histology knowledge score was not significantly different between gastroenterologists and pathologists [9/19 (IQR: 8-11) *vs* 8/19 (IQR: 7-10), $P = 0.54$]. Higher knowledge scores were predicted by hospital attending gastroenterologists ($P = 0.004$), participation in inflammatory bowel disease (IBD) multidisciplinary teams ($P = 0.009$), and self-declared IBD sub-specialist ($P = 0.03$).

CONCLUSION

Histological remission is a recognised target for both gastroenterologists and pathologists. Despite this, knowledge of histological scoring systems and their utilisation is poor.

Key Words: Histology; Scoring system; Ulcerative colitis; Survey

Core Tip: This manuscript describes, for the first time, the knowledge and attitudes of gastroenterologists and pathologists towards the use of histology in clinical practice. Given the increasing literature and use of histology in trials, there is a need to understand the current perceptions of using histology in the real-world. Using a novel Inflammatory Bowel Disease Knowledge score, we demonstrate that although histology is an accepted endpoint, knowledge is poor, particularly relating to histological scoring systems. As such, these results illustrate a pressing need and opportunity to improve knowledge around histology scores amongst gastroenterologists and pathologists and develop consensus agreements on a reporting approach.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease characterised by a relapsing and remitting course[1]. Disease activity is typically evaluated using clinical, biochemical and endoscopic assessments.

Treatment goals have evolved over time, and current consensus guidelines from the Selecting Therapeutic Targets in Inflammatory Bowel Disease initiative (STRIDE-II) recommend achieving clinical and endoscopic remission[2]. However, up to 40% of patients who achieve these therapeutic endpoints may have persistent histological inflammatory activity[3,4].

Despite endoscopic normalization, ongoing active histological activity may be associated with poorer clinical outcomes including higher clinical relapse rates, corticosteroid requirement, hospitalization, colectomy and development of colorectal neoplasia[3-7]. Although histological remission is currently not a formal treatment target by consensus expert-opinion, STRIDE-II guidelines do recommend that formal histological assessment take place to determine the depth of remission and help prognosticate patient outcomes. Further, it is increasingly incorporated into clinical drug trials, with central reading to reduce bias, to provide objective scoring of inflammatory activity[2]. Standardized histological scoring systems with varying levels of validity have been developed to quantify the degree of microscopic inflammatory activity and provide a more accurate assessment of mucosal inflammation[8-12]. The three most commonly used are the Geboes score, Nancy index and Robarts histopathology index due to evidence of their content validity and reliability in evaluating histological features[13].

Although accepted in modern clinical drug trials and research settings, histological disease activity and scoring systems have not been incorporated in routine clinical practice. It is not known whether gastroenterologists understand these scoring systems or if they welcome their incorporation into routine clinical care. Achieving consensus in a formal reporting scoring system will require agreement by pathologists, but their knowledge of these scoring systems and willingness to use them is also unknown. Many pathologists use written descriptions of UC activity in their reports. Whether this translates to a numerical value, if they favour a particular scoring system, or their attitude towards synaptic reporting of histological activity, is not known. This cross-sectional survey study evaluated gastroenterologist and pathologist knowledge of histological findings and scoring systems, together with their attitudes towards the role of histology in UC management. We hypothesised that based on their dedicated training, knowledge of histological scoring systems would be significantly higher in pathologists than gastroenterologists.

MATERIALS AND METHODS

Study cohort

This was a prospective cross-sectional survey of Australian gastroenterologists and pathologists from July 2021 to January 2022. Gastroenterologists were contacted by proxy through the Gastroenterological Society of Australia, and pathologists who review UC specimens were contacted by their associated gastroenterologists to participate in the survey.

Survey questionnaire and inflammatory bowel disease histology knowledge score

A survey was developed to explore the knowledge and attitudes towards the use of histology in inflammatory bowel disease (IBD) for both gastroenterologists and pathologists. The European Crohn's and Colitis Organisation (ECCO) position paper on histopathology and the British Society of Gastroenterology (BSG) biopsy reporting guidelines were utilised to formulate questions and quantify knowledge[14,15]. The structured survey was designed by a focus group of three gastroenterologists and comprised of 34 questions. It included knowledge of histological assessment of disease activity and dysplasia, knowledge of histological scoring systems for ulcerative colitis, uptake of histology scoring systems in routine practice, attitudes towards the role of histological activity, and the use of histological activity in clinical scenarios (Supplementary Data 1). Questionnaire language and ambiguity were evaluated by the focus group. A novel IBD Histology Knowledge Score was created that was derived from the survey as a tool to measure overall performance and tested for construct validity and discriminant ability (Supplementary Table 1). The IBD Histology Knowledge Score was calculated as the sum of correct responses to survey questions that aligned with the ECCO position paper on histopathology and the BSG reporting guidelines on IBD biopsies[14,15]. The maximum possible score was nineteen. For construct validity, a high-performance score had to represent a good understanding of histological findings. During the development phase, the survey was administered to senior gastroenterologists and pathologists not directly involved in designing the study, and they were deemed as criterion standards. The survey was then administered to gastroenterology fellows, junior resident medical officers and non-medical staff. Senior staff scored significantly higher (P = 0.001) than junior doctors, establishing content validity. Discriminant validity compared the knowledge scores of those who followed published guidelines *vs* those who did not.

Statistical analysis

The IBD Histology Knowledge Score was analysed as a non-parametric continuous variable, described as medians with interquartile ranges and compared using Mann-Whitney *U*-test and Kruskal-Wallis test. Parametric continuous variables were described as means and compared using the *t*-test and ANOVA test. Predictors of the IBD histology knowledge score were determined using linear regression

with backward elimination regression modelling. A *P*-value of < 0.05 was deemed statistically significant. Statistical analyses were performed with SPSS version 27 (SPSS Inc, Chicago, IL, United States).

Ethics approval

The study was approved by the Sydney Local Health District Human Research Ethics Committee (HREC CH62/6/2021-055).

RESULTS

Study cohort

A total of 89 responses were obtained, comprising 77 gastroenterologists and 12 pathologists. The response rate for gastroenterologists was 25% (n = 77/310). Subspecialty breakdown of gastroenterologists is shown in Figure 1. Gastroenterologists listed their predominant work as 31% public hospital staff specialists, 30% private practice, 21% trainee gastroenterologists, 17% visiting medical officers and 1% research-based gastroenterologist. Ninety-four percent of respondents saw > 2 IBD patients each week and 30% saw > 10 patients each week. Forty-five percent of gastroenterologists were involved in a regular IBD multidisciplinary team. Full study cohort characteristics are shown in Table 1.

Of the 12 surveyed pathologists, 83% worked in tertiary teaching hospitals and 17% were solely in private practice. Half of all pathologists were involved in regular IBD multidisciplinary meetings. Full study cohort characteristics are shown in Table 1.

Attitudes towards histology and scoring systems in UC

Histological activity was considered to have an 'emerging' or 'established' role in UC by 40% and 55% of gastroenterologists respectively. Proportions for pathologists were 33% and 58% respectively. Histological remission was considered more important to achieve than endoscopic remission by 65% of gastroenterologists ('somewhat agree' and 'agree') (Table 2).

The proportion of gastroenterologists who want to use a histological scoring system at least 'sometimes' or 'always' was 59%, and 50% for pathologists. Gastroenterologists reported that 92% of their pathologists do not routinely use a histological scoring system, whilst 83% pathologists report not routinely using a scoring system. More than half of gastroenterologists (64%) and pathologists (58%) did not know which scoring systems had undergone the most validation (Table 2).

For the Geboes score, 91% of gastroenterologists and 83% of pathologists did not know the defined histological remission score of '< 2.1'[14]. For the Nancy index, 87% of gastroenterologists and 83% of pathologists did not know the defined histological remission score of '0'[14]. For the Robarts histopathology index (RHI), 92% of gastroenterologists and pathologists did not know the defined histological remission score of '≤ 3'[14] (Table 2 and Figure 2).

Impact of histological activity on treatment decisions in clinical scenarios

The impact of histological disease activity on gastroenterologists' decisions to escalate treatment or de-escalate in particular scenarios is summarized in Table 3. In the setting of clinical and endoscopic remission, but histological activity alone, 10% of gastroenterologists would escalate therapy ('often' or 'always'). When combined with an elevated faecal calprotectin, 30% of gastroenterologists would escalate treatment. A greater proportion of gastroenterologists would de-escalate treatment if two consecutive colonoscopies showed endoscopic and histological remission, compared with a single episode of endoscopic and histological remission (53% *vs* 19% respectively). A greater proportion of gastroenterologists would aim for histological remission if a patient with UC had other risk factors for colon cancer (71%).

IBD histology knowledge score

Gastroenterologists and pathologists had similar IBD histology knowledge scores [8.0 (IQR: 6.5-10.0) *vs* 9.0 (IQR: 7.8-11.0), P = 0.54] (Table 4). Within gastroenterologists, IBD sub-specialists had higher knowledge scores compared with other gastroenterologists [10.5 (IQR: 7.3-14) *vs* 9.0 (IQR: 7.8-10.0), P = 0.02] (Figure 3A). Public hospital staff specialists had higher knowledge scores than visiting medical officers [11.0 (IQR: 9.0-13.0) *vs* 8.0 (IQR: 8.0-9.0), P = 0.003] and those in private practice [11.0 (IQR: 9.0-13.0) *vs* 8.0 (IQR: 6.3-9.8), P = 0.002] (Figure 3B). Gastroenterologists with a PhD had higher knowledge scores than those whose highest level of education was a bachelor degree [11.0 (IQR: 7.0-14.0) *vs* 9.0 (IQR: 8.0-10.0), P = 0.01] (Figure 3C). Involvement in an IBD multidisciplinary team was associated with a higher knowledge score [9.5 (IQR: 8.0-11.0) *vs* 8.0 (IQR: 6.0-10.0), P = 0.002] (Figure 3D).

On univariate analysis, subspecialty type (P = 0.005), predominant practice (p=0.004), involvement in an IBD multidisciplinary team (P = 0.002) and a higher level of education (P = 0.02) were all significantly associated with higher IBD histology knowledge scores (Table 5). On multivariate analysis, subspecialty type (P = 0.03), predominant practice (P = 0.005) and involvement in an IBD multidisciplinary team (P =

Table 1 Demographics and study cohort characteristics, *n* (%)

	Gastroenterologists (*n* = 77)	Pathologists (*n* = 12)
Age (yr)		
< 30	4 (5.2)	0 (0.0)
30-40	30 (39.0)	1 (8.3)
41-50	15 (19.5)	4 (33.3)
51-60	19 (24.7)	4 (33.3)
> 60	9 (11.7)	3 (25.0)
Location		
New South Wales	46 (59.7)	8 (66.7)
Victoria	11 (14.3)	2 (16.7)
Queensland	11 (14.3)	2 (16.7)
Western Australia	8 (10.4)	0 (0.0)
Australian Capital Territory	1 (1.3)	0 (0.0)
Highest level of education		
Bachelor of medicine/bachelor of surgery	51 (66.2)	11 (91.7)
Masters	10 (13.0)	0 (0.0)
PhD	16 (20.8)	1 (8.3)
What is your predominant practice		
Staff specialist	24 (31.2)	10 (83.3)
University academic work	1 (1.3)	0 (0.0)
Visiting medical officer	13 (16.9)	0 (0.0)
Private practice	23 (29.9)	2 (16.7)
In training program	16 (20.8)	0 (0.0)
How many IBD patients do you see each week		
0-1	5 (6.5)	N/A
2-5	31 (40.3)	N/A
6-10	18 (23.4)	N/A
> 10	23 (29.9)	N/A
Involved in regular IBD multidisciplinary meeting		
Yes	35 (45.5)	6 (50.0)
No	42 (54.5)	6 (50.0)

IBD: Inflammatory bowel disease; N/A: Not applicable.

0.009) remained significant predictors for higher IBD histology knowledge scores (Table 5).

DISCUSSION

Therapeutic goals in UC have evolved from achieving clinical response to attaining objective targets of resolution of inflammation beyond symptoms such as biochemical and endoscopic remission. However, histological remission outside of the research setting has yet to be adopted by gastroenterologists and pathologists. Our study revealed firstly that histological activity is a recognised treatment goal for gastroenterologists who wish to use histology results in combination with other endpoints to guide management decisions. Secondly and conversely, despite this awareness and use of histology, there is a poor knowledge of histological scoring systems in UC not only by gastroenterologists, but by pathologists as well. As such there is an opportunity to develop consensus guidelines incorporating

Table 2 Attitudes towards histology and histological scoring systems, *n* (%)

	Gastroenterologists (*n* = 77)	Pathologists (*n* = 12)
The role of histological activity in IBD is		
Not established	3 (3.9)	1 (8.3)
Preliminary	1 (1.3)	0 (0.0)
Emerging	31 (40.3)	4 (33.3)
Established	42 (54.5)	7 (58.3)
Histological remission is more important to achieve than endoscopic remission		
Disagree	4 (5.2)	N/A
Somewhat disagree	13 (16.9)	N/A
Neither agree nor disagree	10 (13.0)	N/A
Somewhat agree	36 (46.8)	N/A
Agree	14 (18.2)	N/A
What histological scoring system does your pathologist routinely or frequently use in their reports		
Geboes	2 (2.6)	0 (0.0)
Nancy index	3 (3.9)	1 (8.3)
RHI	1 (1.3)	0 (0.0)
They do not routinely use a scoring system	71 (92.2)	10 (83.3)
Other		IBD-DCA score (*n* = 1)
I would like to use a histological scoring system for my IBD patients		
Never	8 (10.4)	4 (33.3)
Rarely	10 (13.0)	1 (8.3)
Occasionally	14 (18.2)	1 (8.3)
Sometimes	23 (29.9)	3 (25.0)
Always	22 (28.6)	3 (25.0)
Which scoring systems have undergone the most validation		
Modified Riley score	1 (1.3)	1 (8.3)
Geboes score	13 (16.9)	3 (25.0)
Nancy index	20 (26.0)	5 (41.7)
RHI	9 (11.7)	3 (25.0)
Truelove and Richards score	5 (6.5)	0 (0.0)
Not sure	49 (63.6)	7 (58.3)
What Geboes score is considered histological remission		
< 1.1	2 (2.6)	1 (8.3)
< 2.1	7 (9.1)	2 (16.7)
< 3.1	4 (5.2)	0 (0.0)
< 4.1	1 (1.3)	0 (0.0)
Not sure	63 (81.8)	9 (75.0)
What Nancy index is considered histological remission		
0	10 (13.0)	2 (16.7)
≤ 1	4 (5.2)	3 (25.0)

≤ 2	0 (0.0)	0 (0.0)
≤ 3	0 (0.0)	0 (0.0)
Not sure	63 (81.8)	7 (58.3)
What Robarts histopathology index is considered histological remission		
≤ 2	4 (5.2)	1 (8.3)
≤ 3	6 (7.8)	1 (8.3)
≤ 4	0 (0.0)	0 (0.0)
≤ 5	0 (0.0)	1 (8.3)
Not sure	67 (87.0)	9 (75.0)

RHI: Robarts histopathology index; IBD: Inflammatory bowel disease; N/A: Not applicable.

Table 3 Impact of histological disease activity on treatment management in clinical scenarios, *n* (%)

Scenario	Never	Not often	Sometimes	Often	Always
If a patient is in clinical and endoscopic remission, but has histological activity, then I will escalate medical therapy	14 (18.2)	35 (45.5)	20 (26.0)	5 (6.5)	3 (3.9)
If a patient is in clinical and endoscopic remission, but has an elevated faecal calprotectin (> 100 μg/g) and histological activity, then I will escalate medical therapy	4 (5.2)	18 (23.4)	31 (40.3)	19 (24.7)	5 (6.5)
If a patient is in clinical, endoscopic and histological remission, (but prior colonoscopy showed Mayo 1 endoscopic disease), then I will de-escalate medical therapy	7 (9.1)	19 (24.7)	36 (46.8)	15 (19.5)	0 (0.0)
If a patient is in clinical remission, with their last 2 colonoscopies showing endoscopic and histological remission, then I will de-escalate medical therapy	2 (2.6)	2 (2.6)	31 (40.3)	38 (49.4)	4 (5.2)
If a patient with ulcerative colitis has other risk factors for colon cancer, then I will aim to achieve histological remission	0 (0.0)	7 (9.1)	14 (18.2)	27 (35.1)	29 (37.7)

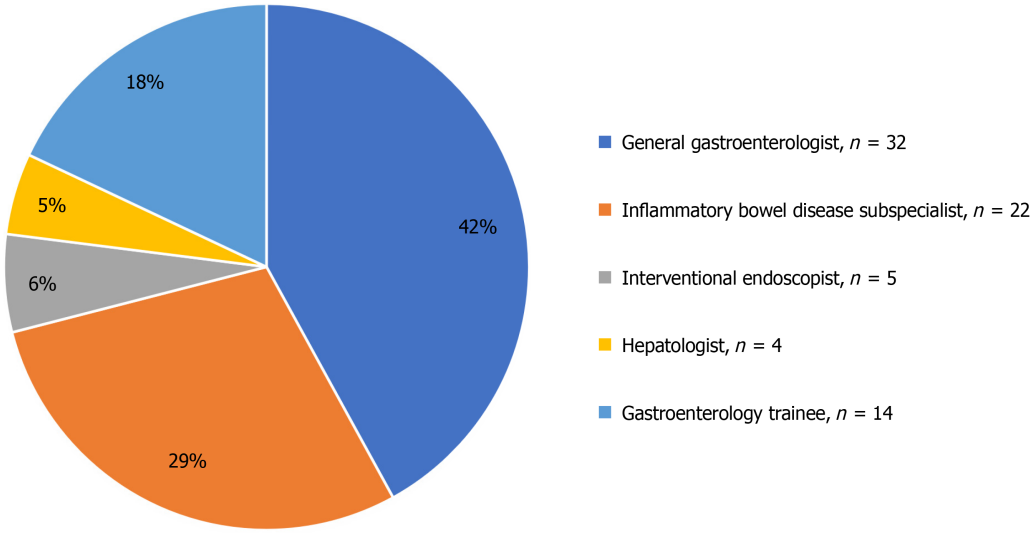

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Figure 1 Subspeciality characteristics of gastroenterologists.

gastroenterologists and pathologists that are adopted by the respective societies to further this evolving field.

Our study showed 95% of gastroenterologists believe histological activity plays a role in the management of UC, with 76% wanting to use a histological scoring system in clinical practice. Further evidence on the role of UC histological activity scores is required as only a small proportion of gastroenterologists currently make treatment decisions based solely on histological activity. In UC patients with clinical and endoscopic remission but ongoing histological disease activity, 10% of gastroenterologists

Table 4 Inflammatory bowel disease histology knowledge scores

	Gastroenterologists (*n* = 77)	Pathologists (*n* = 12)
IBD histology knowledge score [median (IQR)]	9.0 (7.8-11.0)	8.0 (6.5-10.0)
Type of subspecialist		
General gastroenterologist	8.0 (7.0-9.0)	N/A
IBD subspecialist	10.5 (7.3-14)	N/A
Interventional endoscopist	9.0 (4.5-9.8)	N/A
Hepatologist	10.5 (8.5-11)	N/A
Gastroenterology trainee	8.5 (6.0-10.0)	N/A
Predominant practice		
Staff specialist	11.0 (9.0-13.0)	N/A
Visiting medical officer	8.0 (8.0-9.0)	N/A
Private practice	8.0 (6.3-9.8)	N/A
In training program	8.5 (6.0-10.0)	N/A
Highest level of education		
Bachelor degree	9.0 (8.0-10.0)	N/A
Masters	8.0 (7.0-11.0)	N/A
PhD	11.0 (7.0-14.0)	N/A
Involved in regular IBD multidisciplinary meeting	35 (45.5%)	6 (50.0%)
Yes	9.5 (8.0-11.0)	N/A
No	8.0 (6.0-10.0)	N/A

IQR: Interquartile range; N/A: Not applicable; IBD: Inflammatory bowel disease.

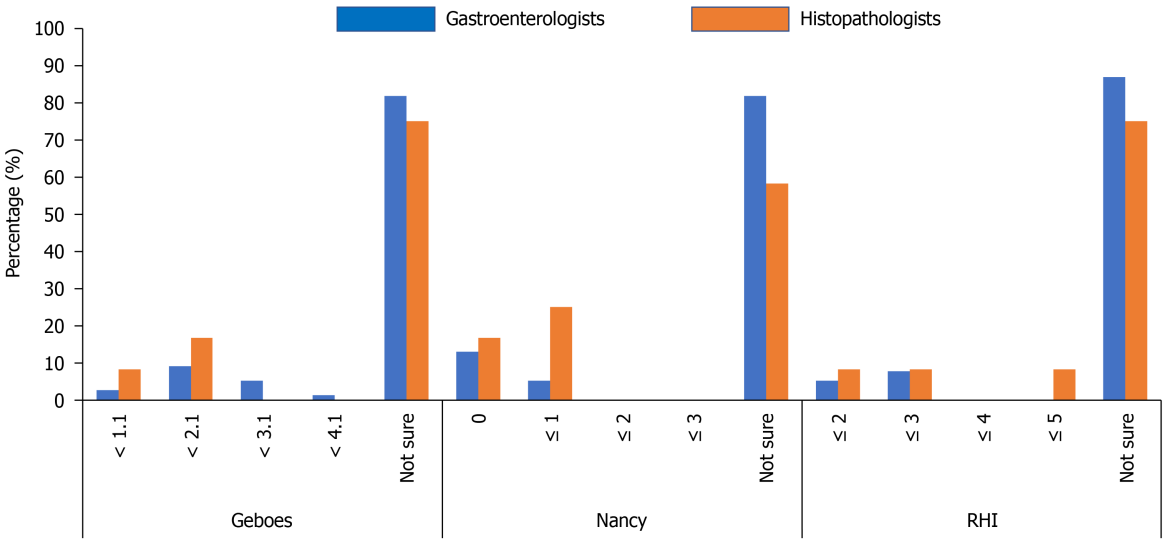
Table 5 Significant predictors of inflammatory bowel disease histology knowledge score for gastroenterologists on univariate and multivariate analyses

	Univariate analysis *P* value	Multivariate analysis *P* value
Type of subspecialty	0.005	0.03
Predominant practice	0.004	0.005
Involvement in IBD MDT	0.002	0.009
Highest level of education	0.02	

IBD: Inflammatory bowel disease; MDT: Multidisciplinary team.

would escalate medical therapy. However, when histological activity coincides with elevated faecal calprotectin, 30% were prepared to escalate treatment. These decisions match the current STRIDE-II guidelines given that histological activity is not currently an accepted target, but shows that gastroenterologists are prepared to include this endpoint as a treatment target[2]. Histological remission becomes even more important if a patient with UC had other risk factors for colon cancer, with 72% prepared to escalate treatment, given that histological activity increases the risk of colorectal neoplasia (odds ratio 3.0, 95%CI: 1.4-6.3)[5]. Therefore, when UC subjects have greater colonic disease extent, more prolonged duration of UC, presence of primary sclerosing cholangitis, or presence of a family history of colorectal cancer, gastroenterologists might escalate treatment in the presence of histological disease activity irrespective of symptoms.

Despite the awareness of the importance of histology in UC, our survey demonstrated a lack of knowledge of histological scoring systems by gastroenterologists. Clinical trials have used Nancy index, RHI and the Geboes score but recent European Crohn's and Colitis Organisation (ECCO) guidelines recommended the use of the Nancy index and RHI for randomised clinical trials, and the Nancy index for clinical practice given its ease of use[14]. Gastroenterologists did not know which scoring systems



Percentage of gastroenterologists	2.6	9.1	5.2	1.3	81.8	13	5.2	0	0	81.8	5.2	7.8	0	0	87
Percentage of histopathologists	8.3	16.7	0	0	75	16.7	25	0	0	58.3	8.3	8.3	0	8.3	75

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Figure 2 Knowledge of histological remission definitions for scoring systems by gastroenterologists and pathologists. RHI: Robarts histopathology index.

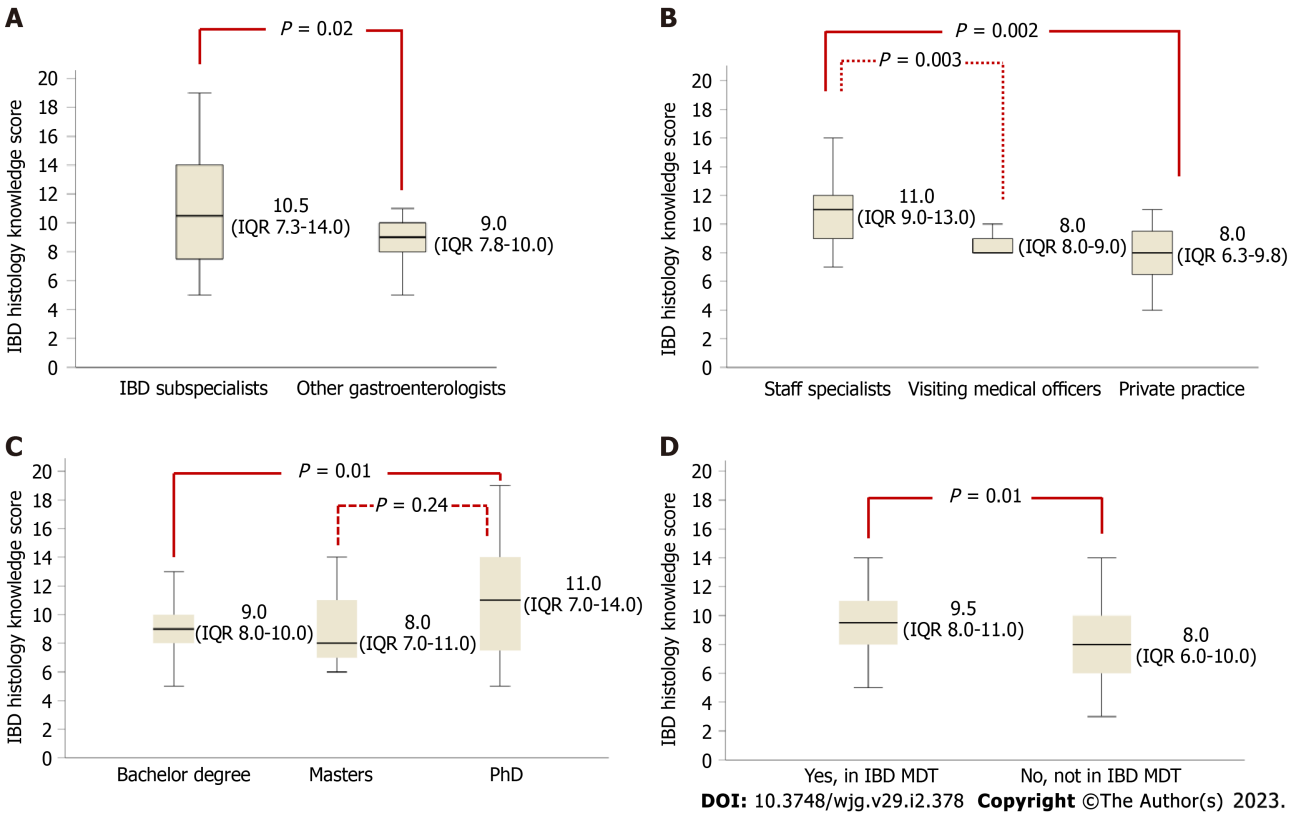

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Figure 3 Comparisons of inflammatory bowel disease histology knowledge score for gastroenterologists. A: Subspecialty type; B: Predominant practice; C: Highest education level; and D: Involvement in inflammatory bowel disease multidisciplinary team. IBD: Inflammatory bowel disease; IQR: Interquartile range; MDT: Multidisciplinary team.

had undergone the most validation, or were unaware of the histological remission scores for the Geboes score (91%), Nancy index (87%) and RHI (92%). Despite the increasing interest and evolving role of histological scoring systems in UC, there is an opportunity to educate gastroenterologists about these

scoring systems and how to apply them in clinical practice. Predictors for higher knowledge included employment as a public hospital staff specialist and involvement in an IBD multidisciplinary team. As such, it is likely working in public hospitals within an IBD team would lead to increased exposure to the understanding of common histological scoring systems in UC. Conversely, gastroenterologists working in private practice would have less exposure to these scoring systems and their utility in UC management, contributing to lower knowledge scores.

Few studies have evaluated pathologists' views on histological activity, but most believe that they have a role in evaluating UC. However, pathologists' knowledge of UC histology was comparable to gastroenterologists [median knowledge score 8.0 (IQR: 6.5-10.0) *vs* 9.0 (IQR: 7.8-11.0) $P = 0.54$]. Similar to gastroenterologists, they also lacked knowledge of histological scoring systems and their remission definitions. There is an opportunity, therefore, to improve the utilisation of histological activity scoring for both pathologists and gastroenterologists. A harmonised approach to histological assessment in UC is lacking[16]. Future directions should include the development of histology consensus guidelines in consultation with pathologists to ensure homogeneity in reporting across hospitals to permit comparability of mucosal biopsies across different sites.

This study has several limitations. First, responder bias may have played a role, whereby responders having greater knowledge were more likely to take part on the survey. However, this would indicate a greater unawareness of histological activity scoring in the assessment of UC and a greater need for education and a harmonized approach towards the adoption of a scoring system. Secondly, a smaller respondent number for pathologists was surveyed. However, we demonstrated statistically that pathologists did not differ in their knowledge of histological scoring systems in UC despite expertise in reading biopsy histology. Thirdly, the results may lack worldwide generalisability given the survey was sent to Australian health professionals.

Strengths of this study included: (1) Being the first to report gastroenterologists' knowledge and attitudes towards the use of histology in UC; (2) recruitment of pathologists to compare their awareness against gastroenterologists; and (3) to target respondents nationwide to demonstrate generalisability.

CONCLUSION

The study highlights that while there is an acknowledgment of the importance of histological assessment in UC, there is a lack of knowledge of histological scoring systems. It indicates areas of educational need in the field of UC histology, and the importance of including pathologists in developing future consensus guidelines on the use of histology in clinical practice.

ARTICLE HIGHLIGHTS

Research background

The role of histology in ulcerative colitis has evolved over time. Histological activity despite endoscopic remission is associated with poorer clinical outcomes, and various histological scoring systems have been developed. However, the knowledge and attitudes towards the use of histology in the management of ulcerative colitis by gastroenterologists and pathologists is unknown.

Research motivation

Although there has been an increasing literature into the use of histology in ulcerative colitis, it is unknown whether this has translated into knowledge and use by gastroenterologists and pathologists in clinical practice.

Research objectives

The main objective was to evaluate the knowledge of histology guidelines and attitudes towards the use of histology in ulcerative colitis by gastroenterologists and pathologists.

Research methods

A prospective, cross-sectional survey of gastroenterologists and pathologists was conducted in Australia. The survey was formulated by using peer-reviewed guidelines.

Research results

Of 89 responders (77 gastroenterologists, 12 pathologists), there was almost complete acceptance that histological assessment should form part of ulcerative colitis evaluation (95% gastroenterologists, 92% pathologists). However, the majority of both groups lacked awareness of the Geboes score, Nancy index and Robarts histopathological index. Higher knowledge scores were predicted by public hospital attending gastroenterologists and involvement in an inflammatory bowel disease meeting.

Research conclusions

Histological remission is a recognised target for both gastroenterologists and pathologists. However knowledge of histological scoring systems was poor.

Research perspectives

Future research should involve the development of consensus guidelines in consultation with pathologists on the use of histology in ulcerative colitis management. This should include an agreement on a standardised scoring system to ensure homogenity in reporting across hospitals to permit comparability of biopsies.

FOOTNOTES

Author contributions: Pudipeddi A and Leong RW designed the research study. Pudipeddi A, Chetwood J, Paramsothy S and Leong RW performed the research and collected data. Pudipeddi A, Chetwood J and Leong RW analysed the data. Pudipeddi A drafted the manuscript. Fung C, Christensen B, Bryant RV and Subramaniam K edited the manuscript; All authors have read and approve the final manuscript.

Institutional review board statement: The study was approved by the Sydney Local Health District Human Research Ethics Committee (HREC CH62/6/2021-055).

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

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Data sharing statement: The data underlying this article will be shared on reasonable request to the corresponding author.

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SYSTEMATIC REVIEWS

Third-line and rescue therapy for refractory *Helicobacter pylori* infection: A systematic review

Pedro Vieira de Moraes Andrade, Yan Mosca Monteiro, Ethel Zimberg Chehter

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Abstract

BACKGROUND

Due to increasing resistance rates of *Helicobacter pylori* (*H. pylori*) to different antibiotics, failures in eradication therapies are becoming more frequent. Even though eradication criteria and treatment algorithms for first-line and second-line therapy against *H. pylori* infection are well-established, there is no clear recommendation for third-line and rescue therapy in refractory *H. pylori* infection.

AIM

To perform a systematic review evaluating the efficacy and safety of rescue therapies against refractory *H. pylori* infection.

METHODS

A systematic search of available rescue treatments for refractory *H. pylori* infection was conducted on the National Library of Medicine's PubMed search platform based on Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. Randomized or non-randomized clinical trials and observational studies evaluating the effectiveness of *H. pylori* infection rescue therapies were included.

RESULTS

Twenty-eight studies were included in the analysis of mean eradication rates as rescue therapy, and 21 of these were selected for analysis of mean eradication rate as third-line treatment. For rifabutin-, sitafloxacin-, levofloxacin-, or metronidazole-based triple-therapy as third-line treatment, mean eradication rates of 81.6% and 84.4%, 79.4% and 81.5%, 55.7% and 60.6%, and 62.0% and 63.0% were found in intention-to-treat (ITT) and per-protocol (PP) analysis, respectively. For third-line quadruple therapy, mean eradication rates of 69.2% and 72.1% were found for bismuth quadruple therapy (BQT), 88.9% and 90.9% for bismuth quadruple therapy, three-in-one, Pylera® (BQT-Pylera), and 61.3% and 64.2% for non-BQT) in ITT and PP analysis, respectively. For rifabutin-, sitafloxacin-, levofloxacin-, or metronidazole-based triple therapy as rescue therapy, mean eradication

rates of 75.4% and 78.8%, 79.4 and 81.5%, 55.7% and 60.6%, and 62.0% and 63.0% were found in ITT and PP analysis, respectively. For quadruple therapy as rescue treatment, mean eradication rates of 76.7% and 79.2% for BQT, 84.9% and 87.8% for BQT-Pylera, and 61.3% and 64.2% for non-BQT were found in ITT and PP analysis, respectively. For susceptibility-guided therapy, mean eradication rates as third-line and rescue treatment were 75.0% in ITT and 79.2% in PP analysis.

CONCLUSION
We recommend sitafloxacin-based triple therapy containing vonoprazan in regions with low macrolide resistance profile. In regions with known resistance to macrolides or unavailability of bismuth, rifabutin-based triple therapy is recommended.

Key Words: *Helicobacter pylori*; Refractory infection; Third-line therapy; Rescue therapy; Eradication; Treatment

Core Tip: The eradication of *Helicobacter pylori* is widely discussed given the high prevalence and incidence of its infection. Even with established criteria in the V Maastricht Consensus for the eradication of infection and treatment algorithms for choosing first-line and second-line therapeutic regimens, therapeutic failure is frequent. Therefore, establishing safe, effective, and accessible third-line and rescue therapies for patients in need of eradication is necessary in the management of such infection. Due to this need, the present systematic review performed a systematic review evaluating the efficacy and safety of rescue therapies against refractory *Helicobacter pylori* infection.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative microaerophilic bacterium with a wide genomic diversity, which are the product of mutations, recombination, migrations, and genetic drift that favored the emergence of multiple populations and subpopulations of this bacterium[1-3]. *H. pylori* is a microorganism of global relevance, infecting about 50% of the world population[4].

Exclusive or multifactorial infection by *H. pylori* is associated with the onset of multiple diseases. The exclusive action of *H. pylori* through its virulence factors is related to the development of peptic ulcer, duodenal ulcer, gastritis, and consequently, dyspepsia[5-7]. Although *H. pylori* infection is often the primary cause of gastric cancers, the development of this pathological process results from a multifactorial interaction between bacterial, host, and environmental factors[8]. Furthermore, *H. pylori* can also stimulate lymphocytic infiltration in the gastric mucosa, which combined with high-risk genotypes may be associated with a neoplastic transformation into mucosa-associated lymphoid tissue lymphoma[9,10].

According to the IV Brazilian Consensus on Infection by *H. pylori* and the Maastricht V/Florence Consensus, the eradication of *H. pylori* is recommended in cases of peptic ulcer, mucosa-associated lymphoid tissue lymphoma, atrophic gastritis, after gastric cancer resection and in patients with first-degree relatives with gastric cancer. However, in addition to its adverse effects, the eradication of *H. pylori* can result in changes in the stomach, intestine, pancreas, and other systems and allow the colonization of other bacteria. Therefore, the risk/benefit ratio of this therapy must be evaluated by the physician[11,12].

Treatment of *H. pylori* infection is based on a combination of antimicrobials and antisecretory agents that promote an increase in gastric pH, enabling the action of antimicrobials. The increasing rates of *H. pylori* resistance to the classes of antimicrobials commonly used in conventional therapeutic regimens has reduced the effectiveness of these drugs, and failures in eradication therapies have become increasingly frequent. In an attempt to combat the growing resistance to antimicrobials, new therapeutic regimens have been used as an alternative to conventional regimens. The association of bismuth, the use of new classes of antisecretory agents such as the competitive inhibitor of potassium channels, and the adoption of new antimicrobials have acted as an alternative to standard therapeutic regimens.

Although the Maastricht V/Florence Consensus presents very well-established criteria for the eradication of infection and treatment algorithms for the choice of first-line and second-line therapeutic regimens (Figure 1) against *H. pylori* infection, there is no clear recommendation for third-line and rescue regimens in refractory *H. pylori* infection. Given the need to establish safe, effective, and accessible therapies for patients, the aim of this study was to evaluate the efficacy and safety of third-line and rescue therapies in refractory *H. pylori* infection.

MATERIALS AND METHODS

Although no review protocol was registered, the present review was conducted in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2009 guideline, from a survey of available rescue treatments for refractory *H. pylori* infection in scientific articles on the PubMed search platform of the National Library of Medicine. The search was performed between April 22, 2021 and August 20, 2021. Different descriptors were used throughout the study for maximization of the database, namely: *Helicobacter pylori* multidrug resistance and rescue therapy; *H. pylori* multiresistant and rescue treatment; *Helicobacter pylori* multidrug resistance and rescue treatment; *Helicobacter pylori* rescue therapy; *Helicobacter pylori* and third line treatment; and fourth line therapy and *Helicobacter pylori* . After applying the inclusion and exclusion criteria, the selected articles were analyzed in two stages: first by two independent reviewers and later by the senior reviewer in order to minimize the possibility of errors and bias by the authors.

Information from articles selected and approved in both stages was extracted by reviewers independently to ensure reliable data detection and collection. A statistical analysis was performed from relevant data to the objective of this review to compare the results found in the studies. In addition to the analysis of eradication rates both by intention to treat (ITT) and per protocol (PP), a comparative analysis on adverse effects found in the different therapeutic approaches was also performed to assess their feasibility in clinical practice.

Due to the heterogeneity pool of objectives in the articles (most of them evaluated different classes or combinations of antibiotics), the level of evidence, grade rating, and bias analysis required in the PRISMA protocol could not be analyzed. Therefore, some items of the PRISMA checklist could not be applied. All articles selected according to our inclusion and exclusion criteria were included in this review, despite their PRISMA rating grade, evidence level, or bias. It was equally challenging to present their risk of bias, outcome level assessment, and strength of evidence, even with a two-phase analysis. Therefore, some of this information may be lacking in this review, but all articles included were analyzed in detail to minimize the inclusion of low evidence information.

Inclusion criteria

The present review included randomized or non-randomized clinical trials and observational studies that evaluated the efficacy of rescue therapies in refractory *H. pylori* infection published from 2014 onwards in the search platforms defined by the authors.

Exclusion criteria

Exclusion criteria adopted in the selection of articles of the present study were the following: Studies with pediatric patients; studies exclusively with patients who had only one failed eradication attempt; studies including patients with two or more previous failed eradication therapies, in which eradication rates for these patients were not specified; studies that did not fully discriminate the therapeutic approach used; studies without evidence of infection by *H. pylori* using methods of high sensitivity and specificity (13C-UBT and/or biopsy); and studies in which there was no subsequent follow-up of patients.

RESULTS

Selection of articles

The initial search in the PubMed database resulted in 751 potential articles. After excluding those published before January 1, 2014, 362 articles remained. After temporal delimitation and reading the abstracts of the remaining articles, 271 articles did not contain relevant information about rescue treatment. Of the 91 remaining articles containing relevant information on rescue treatment, 38 were excluded after a double check with reviewers and the senior reviewer because they were duplicates and/or statistical information related to eradication rates of third-line and rescue therapies was lacking. Articles without data on adverse effects but with eradication rates were included in this review. At the end of this stage, 53 articles were selected for analysis and read in full by the authors. Finally, 25 articles were excluded in the final stage because they did not meet the inclusion criteria in full or met any of the exclusion criteria, leaving 28 articles for inclusion. The selection process is presented in the PRISMA

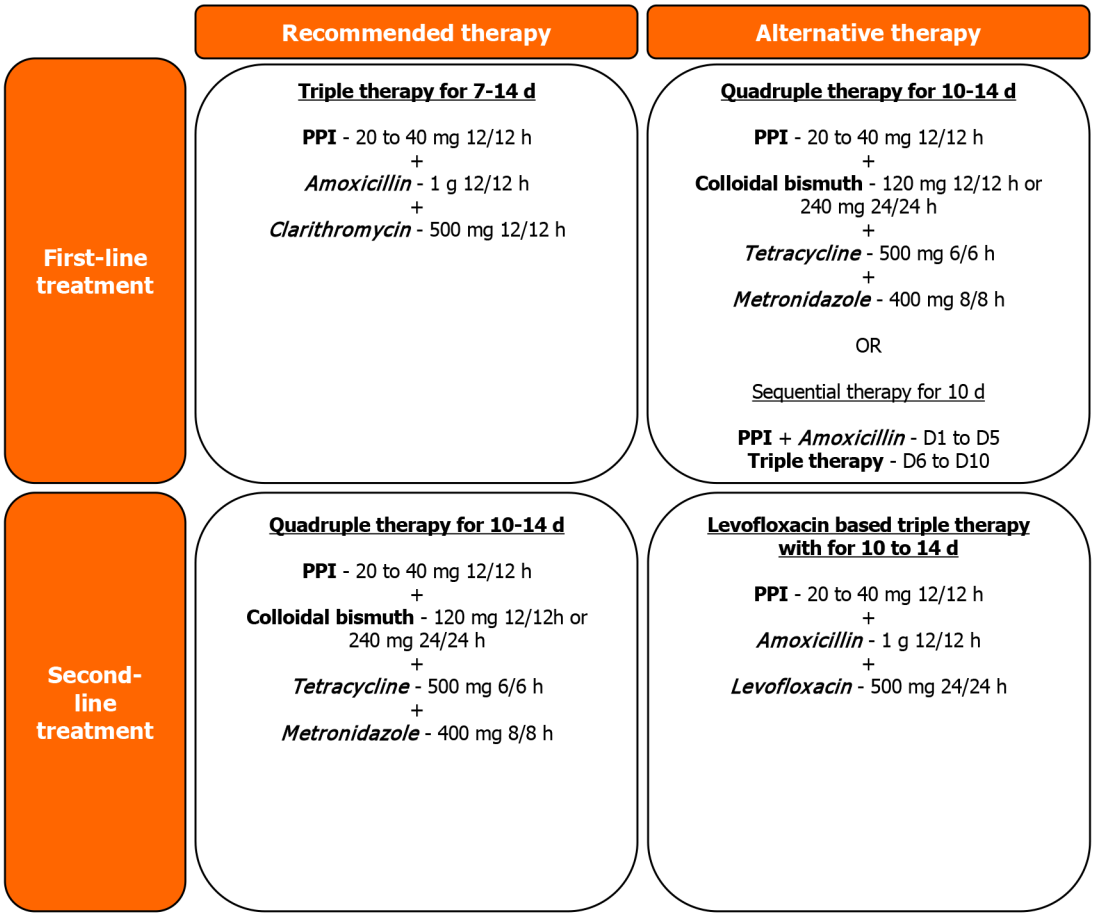

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Figure 1 Therapeutic regimens recommended by Maastricht V/Florence consensus report as first-line and second-line treatment. PPI: Proton pump inhibitor.

diagram of included articles. PRISMA flow diagram reported in Figure 2.

Eradication rate

The different approaches used in the selected articles and their eradication rates can be seen in Table 1 [13-40].

Among the 28 selected articles, different active principles and therapeutic approaches were used as rescue treatment, achieving different eradication rates. Twenty-one studies were selected for analysis of the mean eradication rate as third-line treatment. Regarding the analysis of mean eradication rates of rescue therapies, studies containing patients with two or more previous failed eradications were included; the 28 studies presented in Table 1 were used. The analysis of eradication rates of regimens used as third-line treatment and rescue therapy were stratified into three subgroups based on the therapeutic regimens used, namely triple therapy, quadruple therapy, and susceptibility-guided therapy (SGT). Note that in the analysis of mean eradication rates of therapies performed in our study, therapeutic regimens were not discriminated based on the duration and dosage of the drugs used. In the absence of studies evaluating the effectiveness of therapeutic approaches as fourth-line or more, the third-line was considered as rescue therapy.

Triple therapy: Eradication rates found for triple therapy as third-line treatment were 81.6% and 84.4% for rifabutin-based regimens, 79.4% and 81.5% for sitafloxacin-based regimens, 55.7 % and 60.6% for levofloxacin-based regimens, and 62.0% and 63.0% for metronidazole-based regimen by ITT and PP, respectively (Figure 3). Regarding triple therapy as rescue treatment, mean eradication rates of 75.4% and 78.8% were found for rifabutin-based regimens, 79.4% and 81.5% for sitafloxacin-based regimens, 55.7% and 60.6% for levofloxacin-based regimens, and 62.0% and 63.0% for metronidazole-based regimen by ITT and PP, respectively (Figure 4).

Quadruple therapy: Eradication rates found for quadruple therapy as third-line treatment were 69.2% and 72.1% for bismuth quadruple therapy (BQT), 88.9% and 90.9% for bismuth quadruple therapy, three-in-one Pylera® (BQT-Pylera®), and 61.3% and 64.2% for non-BQT by ITT and PP, respectively (Figure 5). Regarding quadruple therapy as rescue treatment, mean eradication rates of 76.7% and 79.2%

Table 1 Eradication rates by therapeutic regimen

Year	Ref.	Type of study	Rescue therapy	Duration	Eradication rate
2014	Lim *et al*[13]	Randomized clinical trial	Group A: lansoprazole (30 mg, 12/12 h), amoxicillin (1 g, 8/8 h), and rifabutin 150 mg (12/12 h)	7 d	ITT: 78.1%; PP: 80.6%
2014	Lim *et al*[13]	Randomized clinical trial	Group B: lansoprazole (60 mg, 12/12 h), amoxicillin (1 g, 8/8 h), and rifabutin 150 mg (12/12 h)	7 d	ITT: 96.3%; PP: 100%
2014	Furuta *et al* [14]	Randomized clinical trial	RAS: rabeprazole (10 mg, 8/8 h or 12/12 h), amoxicillin (500 mg, 6/6 h), sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 84.1%; PP: 86.4%
2014	Furuta *et al* [14]	Randomized clinical trial	RAS: rabeprazole, amoxicillin (500 mg, 6/6 h), sitafloxacin (100 mg, 12/12 h)	14 d	ITT: 88.9%; PP: 90.9%
2014	Furuta *et al* [14]	Randomized clinical trial	RMS: rabeprazole, metronidazole (250 mg, 12/12 h), sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 90.9%; PP: 90.9%
2014	Furuta *et al* [14]	Randomized clinical trial	RMS: rabeprazole, metronidazole (250 mg, 12/12 h), sitafloxacin (100 mg, 12/12 h)	14 d	ITT: 87.2%; PP: 91.1%
2014	Gisbert *et al* [15]	Prospective multicenter observational study	PPI (standard dose, 12/12 h), bismuth subcitrate (120 mg 8/8 h or 240 mg, 12/12 h), tetracycline (250 mg, 6/6 h or 500 mg 8/8 h or 500 mg, 6/6 h), and metronidazole (250 mg, 8/8 h or 250 mg, 6/6 h or 500 mg, 8/8 h or 500 mg, 6/6 h)	7-14 d	ITT: 65.0%; PP: 67.0%
2014	Okimoto *et al*[16]	Randomized clinical trial	RAL: rabeprazole (10 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), levofloxacin (500 mg, 24/24 h)	10 d	ITT: 45.8%; PP: 45.8%
2014	Okimoto *et al*[16]	Randomized clinical trial	RA: rabeprazole (10 mg, 6/6 h) and amoxicillin (500 mg, 6/6 h)	14 d	ITT: 40.7%; PP: 45.8%
2015	Paoluzi *et al* [17]	Randomized clinical trial	Esomeprazole (20 mg, 12/12 h), levofloxacin (500 mg, 12/12 h), doxycycline (100 mg, 12/12 h)	7 d	ITT: 40.0%; PP: 43.0%
2015	Paoluzi *et al* [17]	Randomized clinical trial	Esomeprazole (20 mg, 12/12 h), levofloxacin (500 mg, 12/12 h), doxycycline (100 mg, 12/12 h), *Lactobacillus casei* DG (24 billion units)	7 d	ITT: 54%; PP: 55%
2016	Muller *et al* [18]	Non-randomized clinical trial	Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, 6/6 h), omeprazole (20 mg, 12/12 h)	10 d	ITT: 83.0%; PP: 87.0%
2016	Mori *et al* [19]	Randomized clinical trial	Third-line: esomeprazole (20 mg, 6/6 h), amoxicillin (500 mg, 6/6 h), and rifabutin (300 mg, 24/24 h)	10 d	ITT: 83.3%; PP: 81.8%
2016	Mori *et al* [19]	Randomized clinical trial	Third-line: esomeprazole (20 mg, 6/6 h), amoxicillin (500 mg, 6/6 h), and rifabutin (300 mg, 24/24 h)	14 d	ITT: 94.1%; PP: 91.7%
2016	Mori *et al* [19]	Randomized clinical trial	Fourth-line: esomeprazole (20 mg, 6/6 h), amoxicillin (500 mg, 6/6 h), and rifabutin (300 mg, 24/24 h)	10 d	ITT: 77.9%; PP: 77.9%
2016	Mori *et al* [19]	Randomized clinical trial	Fourth-line: esomeprazole (20 mg, 6/6 h), amoxicillin (500 mg, 6/6 h), and rifabutin (300 mg, 24/24 h)	14 d	ITT:90.9%; PP: 90.9%
2016	Mori *et al* [20]	Randomized clinical trial	Esomeprazole (20 mg, 12/12 h), amoxicillin (500 mg, 6/6 h), and sitafloxacin (100 mg, 12/12 h)	10 d	ITT: 81.0%; PP: 82.0%
2016	Mori *et al* [20]	Randomized clinical trial	Esomeprazole (20 mg, 12/12 h), metronidazole (250 mg, 12/12 h), and sitafloxacin (100 mg, 12/12 h)	10 d	ITT: 72.4%; PP: 76.4%
2016	Chen *et al* [21]	Randomized clinical trial	Lansoprazole (30 mg, 12/12 h), potassium bismuth subcitrate (220 mg, 12/12 h), metronidazole (400 mg, 6/6 h), and amoxicillin (1 g, 8/8 h)	14 d	ITT: 88.5%; PP: 93.7%
2016	Chen *et al* [21]	Randomized clinical trial	Lansoprazole (30 mg, 12/12 h), potassium bismuth subcitrate (220 mg, 12/12 h), metronidazole (400 mg, 6/6 h), and tetracycline (500 mg, 6/6 h)	14 d	ITT: 87.2%; PP: 95.3%
2016	Noh *et al*[22]	Non-randomized clinical trial	PPI (standard dose, 12/12 h), levofloxacin (500 mg, 24/24 h), and amoxicillin (1 g, 12/12 h)	7 d	ITT: 58.3%; PP: 58.3%
2016	Noh *et al*[22]	Non-randomized clinical trial	PPI (standard dose, 12/12 h), levofloxacin (500 mg, 24/24 h), and amoxicillin (1 g, 12/12 h)	10 d	ITT: 62.5%; PP: 68.2%
2016	Noh *et al*[22]	Non-randomized clinical trial	PPI (standard dose, 12/12 h), levofloxacin (500 mg, 24/24 h), and amoxicillin (1 g, 12/12 h)	14 d	ITT: 73.7%; PP: 93.3%
2016	Hirata *et al* [23]	Non-randomized clinical trial	Esomeprazole (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 83.0%; PP: 83.0%
2017	Rodríguez de Santiago *et al*[24]	Multicenter observational prospective study	Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, 3 capsules, 6/6 h) and esomeprazole (40 mg, 12/12 h) or omeprazole (40 mg, 12/12 h)	10 d	ITT: 80.2%; PP: 84.4%

2017	Costa *et al* [25]	Single-center observational retrospective study	SGT	-	ITT: 59.5%; PP: 61.5%
2017	Puig *et al* [26]	Multicenter observational prospective study	Esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 8/8 h), and metronidazole (500 mg, 8/8 h)	14 d	ITT: 62.0%; PP: 63.0%
2018	Fiorini *et al* [27]	Non-randomized clinical trial	Esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), rifabutin (150 mg, 24/24 h)	12 d	PP: 87.9%
2018	Liou *et al* [28]	Randomized clinical trial	Clinical trial 1: sequential susceptibility-guided therapy: esomeprazole (40 mg, 12/12 h) and amoxicillin (1 g, 12/12 h), for the first 7 d followed by metronidazole (500 mg, 12/12 h) and levofloxacin (250 mg, 12/12 h) or clarithromycin (500 mg, 12/12 h) or doxycycline (100 mg, 12/12 h), for another 7 d. Sequential empirical therapy: esomeprazole (40 mg, 12/12 h) and amoxicillin (1 g, 12/12 h) for the first 7 d, followed by metronidazole (500 mg, 12/12 h) and doxycycline (100 mg, 12/12 h), for another 7 d	14 d	SGT ITT: 81.0%, PP: 80.0%; Sequential empirical therapy ITT: 60.0%, PP: 60.0%
2018	Liou *et al* [28]	Randomized clinical trial	Clinical trial 2: sequential SGT: esomeprazole (40 mg, 12/12 h) and amoxicillin (1 g, 12/12 h) for the first 7 d followed by metronidazole (500 mg, 12/12 h) and levofloxacin (250 mg, 12/12 h) or clarithromycin (500 mg, 12/12 h) or tetracycline (500 mg, 12/12 h) for another 7 d. Sequential empirical therapy: esomeprazole (40 mg, 12/12 h) and amoxicillin (1 g, 12/12 h) for the first 7 d followed by metronidazole (500 mg, 12/12 h) and tetracycline (100 mg, 12/12 h) for another 7 d	14 d	SGT ITT: 78.0%, PP: 78.4%; Sequential empirical therapy ITT: 72.2%, PP: 74.4%
2018	Huang *et al* [29]	Non-randomized clinical trial	SGT: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h) and tetracycline (500 mg, 6/6 h) or metronidazole (500 mg, 8/8 h) or levofloxacin (500 mg, 24/24 h)	14 d	ITT: 81.4%; PP: 89.7%
2018	Huang *et al* [29]	Non-randomized clinical trial	Empirical quadruple therapy: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), tetracycline (500 mg, 6/6 h), and metronidazole (500 mg, 8/8 h)	14 d	ITT: 51.8%; PP: 58.3%
2019	Saito *et al* [30]	Non-randomized clinical trial	Esomeprazole (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), and sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 54.2%; PP: 56.5%
2019	Saito *et al* [30]	Non-randomized clinical trial	Vonoprazan (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), and sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 93.0%; PP: 93.0%
2019	Sue *et al*[31]	Randomized clinical trial	Vonoprazan (20 mg, 12/12 h) amoxicillin 750 mg, (12/12 h), and sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 75.8%; PP: 83.3%
2019	Sue *et al*[31]	Randomized clinical trial	Lansoprazole (30 mg, 12/12 h) or rabeprazole (10 mg, 12/12 h) or esomeprazole (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), and sitafloxacin 100 mg, 12/12 h)	7 d	ITT: 53.3%; PP: 57.1%
2019	Ribaldone *et al*[32]	Non-randomized clinical trial	Fifth-line: rifabutin (150 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and omeprazole (20 mg, 12/12 h), esomeprazole (40 mg, 12/12 h), pantoprazole (40 mg, 12/12 h) rabeprazole (40 mg, 12/12 h), or lansoprazole (30 mg, 12/12 h)	14 d	ITT: 71.5%; PP: 72.7%
2020	Liu *et al*[33]	Single center observational retrospective study	*Lactobacilli acidophilus* (1g, 8/8 h), esomeprazole (20mg, 12/12 h), potassium bismuth subcitrate (220 mg, 12/12 h), tetracycline (750 mg, 12/12 h), and furazolidone (100 mg, 12/12 h)	*Lactobacilli acidophilus* for 14 d and the others for 10 d	ITT: 92.0%; PP: 91.8%
2020	Sugimoto *et al*[34]	Single center observational retrospective study	Vonoprazan (20mg, 12/12 h), amoxicillin (500 mg, 6/6 h), and sitafloxacin (100 mg, 12/12 h)	7 d	ITT: 87.5%; PP: 87.5%
2020	Saracino *et al*[35]	Single center observational retrospective study	Third-line: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and rifabutin (150 mg, 24/24 h)	12 d	ITT: 56.1%; PP: 68.5%
2020	Saracino *et al*[35]	Single center observational retrospective study	Fourth-line: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and rifabutin (150 mg, 24/24 h)	12 d	ITT: 54.5%; PP: 63.1%
2020	Saracino *et al*[35]	Single center observational retrospective study	Fifth-line or more: esomeprazole (40 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and rifabutin (150 mg, 24/24 h)	12 d	ITT: 24.4%; PP: 30.3%
2020	Saracino *et al*[35]	Single center observational retrospective	Third-line: Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, 6/6 h) and esomeprazole (20 mg, 12/12 h)	10 d	ITT: 87.5%; PP: 91.3%

		study			
2020	Saracino *et al*[35]	Single center observational retrospective study	Fourth-line: Pylera® (140 mg potassium bismuth subcitrate, 125 mg de metronidazole, 125 mg tetracycline, 3 capsules, 6/6 h) and esomeprazole (20 mg, 12/12 h)	10 d	ITT: 83.9%; PP: 89.6%
2020	Saracino *et al*[35]	Single center observational retrospective study	Fifth-line or more: Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, 3 capsules, 6/6 h) and esomeprazole (20 mg, 12/12 h)	10 d	ITT: 71.9%; PP: 74.2%
2020	Hirata *et al* [36]	Non-randomized clinical trial	Fourth-line: vonoprazan (20 mg, 12/12 h), amoxicillin (750 mg, 12/12 h), and rifabutin (150 mg, 12/12 h)	10 d	ITT: 100.0%; PP: 100.0%
2020	Ji *et al*[37]	Randomized clinical trial	Susceptibility-guided quadruple therapy: rabeprazole (10 mg, 12/12 h), colloidal bismuth (200 mg, 12/12 h), 2 sensitive antibiotics	14 d	PP: 86.49%
2020	Ji *et al*[37]	Randomized clinical trial	Rabeprazole (10 mg, 12/12 h), colloidal bismuth (200 mg, 12/12 h), amoxicillin (1 g, 12/12 h), levofloxacin (500 mg, 24/24 h), or furazolidone (100 mg, 12/12 h)	14 d	PP: 82.4%
2020	Mori *et al* [38]	Non-randomized clinical trial	Esomeprazole (20 mg, 12/12 h), amoxicillin (500 mg, 6/6 h), and sitafloxacin (100 mg, 12/12 h)	10 d	ITT: 81.6%; PP: 81.6%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth and tetracycline-based quadruple therapy: PPI, bismuth salts (120 mg, 6/6 h or 240 mg, 12/12 h), metronidazole (500 mg, 8/8 h), and tetracycline (500 mg, 6/6 h)	10 d	ITT: 66.0%; PP: 66.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth and tetracycline-based quadruple therapy: PPI, bismuth salts (120 mg, 6/6 h or 240 mg, 12/12 h), metronidazole (500 mg, 8/8 h), and tetracycline (500 mg, 6/6 h)	14 d	ITT: 82.0%; PP: 83.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth and doxycycline-based quadruple therapy: PPI, bismuth salts (120 mg, 6/6 h or 240 mg, 12/12 h), metronidazole (500 mg, 8/8 h), and doxycycline (100 mg, 12/12 h)	10 d	ITT: 63.0%; PP: 63.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth and doxycycline-based quadruple therapy: PPI, bismuth salts (120 mg, 6/6 h or 240 mg, 12/12 h), metronidazole (500 mg, 8/8 h), and doxycycline (100 mg, 12/12 h)	14 d	ITT: 70.0%; PP: 71.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth-based quadruple therapy, three-in-one, Pylera®: PPI and Pylera®	10 d	ITT: 88.0%; PP: 88.0%
2020	Nyssen *et al* [39]	Multicentric observational retrospective study	Bismuth-based quadruple therapy, three-in-one, Pylera®: PPI and Pylera®	14 d	ITT: 100.0%; PP: 100.0%
2020	Kuo *et al*[40]	Non-randomized clinical trial	Rifabutin (150 mg, 12/12 h), amoxicillin (1 g, 12/12 h), and esomeprazole (40 mg, 12/12 h)	10 d	ITT: 77.5%; PP: 79.5%

ITT: Intention to treat; PP: Per protocol; PPI: Proton pump inhibitor; RAS: Rabeprazole, amoxicillin, and sitafloxacin; RMS: Rabeprazole, metronidazole, and sitafloxacin; SGT: Susceptibility-guided therapy.

were found for BQT, 84.9% and 87.8% for BQT-Pylera®, and 61.3% and 64.2% for non-BQT regimens by ITT and PP, respectively (Figure 6).

SGT: Eradication rates found for SGT as third-line treatment and rescue therapy were 75% and 79.2% by ITT and PP, respectively.

Adverse effects: From the reading of selected articles, information on adverse effects found in different therapeutic approaches was extracted, as shown in Table 2. The mean adverse effects rate for rifabutin-, sitafloxacin-, levofloxacin-, and metronidazole-based triple therapy in patients with two or more previous failed eradications was 53.70%, 52.36%, 13.93%, and 58.00%, respectively. With respect to adverse effects for BQT, BQT-Pylera, and non-BQT regimens, mean rates were 34.0%, 65.0%, and 45.0%, respectively. The safety of SGT was not evaluated in the present study since the choice of the therapeutic regimen was dependent on results obtained by susceptibility and genotypic resistance tests.

Table 2 Adverse effects

Ref.	Therapeutic scheme	Adverse effects, *n*	Total rate
Okimoto *et al* [16], 2014	Dual therapy: rabeprazole and amoxicillin	*n* = 24. Loose stools/diarrhea: 5 (20.8%); nausea: 1 (4.2%); skin rash: 0 (0%)	25%
Okimoto *et al* [16], 2014	Triple therapy: rabeprazole, amoxicillin, and levofloxacin	*n* = 24. Loose stools/diarrhea: 5 (20.8%); nausea: 0 (0%); skin rash: 1 (4.2%)	25%
Lim *et al*[13], 2014	Triple therapy: lansoprazole, amoxicillin, and rifabutin	Group A (*n* = 32). Epigastric pain: 3 (9.3%); epigastric discomfort: 2 (6.2%); asthenia: 1 (3.1%); nausea: 1 (3.1%); change in urine color: 1 (3.1%); drowsiness: 1 (3.1%); lip discomfort: 1 (3.1%); treatment was discontinued because of adverse effects: 1 (3.1%)	15.5%
Lim *et al*[13], 2014	Triple therapy: lansoprazole, amoxicillin, and rifabutin	Group B (*n* = 27). Epigastric pain: 1 (3.7); epigastric discomfort: 1 (3.7); asthenia: 0 (0%); nausea: 1 (3.7); urine color change: 0 (0%); drowsiness: 0 (0%), lip discomfort: 0 (0%)	
Furuta *et al* [14], 2014	Triple therapy: rabeprazole, sitafloxacin, and amoxicillin or metronidazole	RAS, 7 d (*n* = 44). Diarrhea: 9 (20.4%); loose stools: 20 (45.4%)	65.9%
Furuta *et al* [14], 2014	Triple therapy: rabeprazole, sitafloxacin, and amoxicillin or metronidazole	RAS, 14 d (*n* = 45). Diarrhea: 12 (26.6%); loose stools: 17 (37.7%)	64.4%
Furuta *et al* [14], 2014	Triple therapy: rabeprazole, sitafloxacin, and amoxicillin or metronidazole	RMS, 7 d (*n* = 44). Diarrhea: 8 (18.2%); loose stools: 17 (38.6%)	56.8%
Furuta *et al* [14], 2014	Triple therapy: rabeprazole, sitafloxacin, and amoxicillin or metronidazole	RMS, 14 d (*n* = 47). Diarrhea: 12 (25.5%); loose stools: 26 (55.3%)	86.3%
Paoluzi *et al* [17], 2015	Triple therapy: esomeprazole, levofloxacin, and doxycycline	*n* = 142. Swelling:; flavor perversion; mild diarrhea; treatment was discontinued because of adverse effects: 1 (0.7%)	7.7%
Mori *et al* [19], 2016	Triple therapy: esomeprazole, amoxicillin, and rifabutin	10-d group (*n* = 12). Fever: 2 (16.6%); diarrhea: 0 (0%); headache: 3 (25%); liver dysfunction: 2 (16.6%); loose stools: 2 (16.6%); urine discoloration: 1 (8.3%); allergy: 1 (8.3%); leukopenia: 1 (8.3%); stomatitis: 1 (8.3%); dysgeusia: 1 (8.3%); vertigo: 0 (0%); fatigue: 0 (0%); photophobia: 0 (0%); treatment was discontinued because of adverse effects: 1 (8.3%)	75%
Mori *et al* [19], 2016	Triple therapy: esomeprazole, amoxicillin, and rifabutin	14-d group (*n* = 17). Fever: 6 (35%); diarrhea: 5 (29.4%); headache: 3 (17.7%); liver dysfunction: 3 (17.7%); loose stools: 2 (11.8%); urine discoloration: 3 (17.7%); allergy: 2 (11.8%); leukopenia: 1 (5.9%); stomatitis: 0 (0%); dysgeusia: 0 (0%); vertigo: 1 (5.9%); fatigue: 1 (5.9%); photophobia: 1 (5.9%); treatment was discontinued because of adverse effects: 5 (29.4%)	94.1%
Mori *et al* [20], 2016	Triple therapy: esomeprazole, amoxicillin, and sitafloxacin	EAS (*n* = 63). Diarrhea: 11 (17.5%); loose stools: 9 (14.2%); constipation: 1 (1.5%); abdominal pain: 3 (4.8%); dyspepsia: 2 (3.2%); dysgeusia: 7 (11.1%); stomatitis: 3 (4.8);; allergy: 2 (3.2%); pruritus: 1 (1.5%); headache: 0 (0%); fatigue: 1 (1.5%); fever: 0 (0%); treatment was discontinued because of adverse effects: 1 (1.5%)	47.6%
Mori *et al* [20], 2016	Triple therapy: esomeprazole, metronidazole, and sitafloxacin	EMS (*n* = 58). Diarrhea: 15 (25.8%); loose stools: 8 (13.8%); constipation: 2 (3.4%); abdominal pain: 2 (3.4%); dyspepsia: 1 (1.7%); dysgeusia: 5 (8.6%); stomatitis: 2 (3.4%); ; allergy: 1 (1.7%); pruritus: 1 (1.7%); headache: 2 (3.4%); fatigue: 0; fever: 1 (1.7%); treatment was discontinued because of adverse effects: 1 (1.7%)	51.7%
Noh *et al*[22], 2016	Triple therapy: PPI, amoxicillin, and levofloxacin	-	-
Hirata *et al* [23], 2016	Triple therapy: esomeprazole, amoxicillin, and sitafloxacin	*n* = 30. Diarrhea: 5 (15.7%); rash: 1 (3.3%); asthma attack: 1 (3.3%); stomatitis: 1 (3.3%); cystitis: 1 (3.3%)	26.6%
Puig *et al*[26], 2017	Triple therapy: esomeprazole, amoxicillin, and metronidazole	*n* = 68. Diarrhea: 13 (20.0%); swelling: 3 (4.0%); dyspepsia: 14 (21.0%); taste disturbance: 23 (35.0%); nausea/vomiting: 10 (15.0%); asthenia: 4 (6.0%); others: 3 (4.0%); treatment was discontinued because of adverse effects: 2 (3.0%)	58.0%
Fiorini *et al* [27], 2018	Triple therapy: esomeprazole, amoxicillin, and rifabutin	*n* = 254. Nausea or vomiting: 6 (2.5%); abdominal pain: 13 (5.4%); mild diarrhea: 12 (5.1%); headache: 4 (1.6%); itching: 4 (1.6%); taste change: 4 (1.6%); myalgia: 1 (0.5%)	18.3%
Saito *et al* [30], 2019	Triple therapy: esomeprazole, amoxicillin, and sitafloxacin	-	-
Saito *et al* [30], 2019	Triple therapy: vonoprazan, amoxicillin, and sitafloxacin	-	-
Sue *et al*[31], 2019	Triple therapy: vonoprazan, amoxicillin, and sitafloxacin	*n* = 33. Diarrhea: 16 (50%); dysgeusia: 5 (15%); nausea: 1 (4%); anorexia: 3 (8%); abdominal pain: 5 (15%); heartburn: 6 (19%); headache: 4 (12%); eructations: 12 (35%); general malaise: 5 (16%);	-

		abdominal swelling: 12 (35%); urticaria: 3 (8%); treatment was suspended because of adverse effects: 2 (6.0%)	
Sue *et al*[31], 2019	Triple therapy: lansoprazole or rabeprazole or esomeprazole, amoxicillin, and sitafloxacin	*n* = 30. Diarrhea: 15 (51%); dysgeusia: 5 (17%); nausea: 5 (17%); anorexia: 4 (13%); abdominal pain: 6 (21%); heartburn: 4 (13%); headache: 2 (8%); eructations: 2 (8%); general malaise: 2 (8%); abdominal swelling: 6 (21%); urticaria: 2 (8%)	-
Ribaldone *et al*[32], 2019	Triple therapy: omeprazole or esomeprazole or pantoprazole or rabeprazole or lansoprazole, amoxicillin, and rifabutin	*n* = 302. Abdominal/epigastric pain: 9 (3.0%); nausea/vomiting: 7 (2.3%); diarrhea: 2 (0.7%); fatigue: 1 (0.3%); headache: 1 (0.3%); oral candidiasis: 1 (0.3%); allergy: 1 (0.3%); treatment was discontinued because of adverse effects: 4 (1.3%)	7.3%
Sugimoto *et al*[34], 2020	Triple therapy: vonoprazan, amoxicillin, and sitafloxacin	*n* = 32. Diarrhea: 4 (12.5%); loose stools: 2 (6.2%); abdominal pain: 2 (6.2%); allergic reaction: 0 (0%); others: 1 (3.1%)	28.1%
Saracino *et al*[35], 2020	Triple therapy: esomeprazole, amoxicillin, and rifabutin	*n* = 270. Diarrhea: 21 (9.3%); abdominal pain: 20 (8.8%); nausea: 17 (7.7%); headache: 15 (6.6%); dyspepsia: 14 (6.0%); treatment was discontinued because of adverse effects: 3 (1.3%)	46.4%
Saracino *et al*[35], 2020	BQT-Pylera: Pylera® and esomeprazole	*n* = 153. Nausea: 43 (29.7); drowsiness: 35 (24.1%); asthenia: 33 (22.8%); dyspepsia: 28 (19.3%); diarrhea: 26 (17.9%); treatment was discontinued because of adverse effects: 8 (5.2%)	65.5%
Hirata *et al*[36], 2020	Triple therapy: vonoprazan, amoxicillin, and rifabutin	*n* = 19. Diarrhea: 4 (21.0%); headache: 1 (5.2%); taste change: 1 (5.2%); ear fullness: 1 (5.2%)	42.0%
Gisbert *et al*[15], 2014	BQT: Bismuth, PPI, tetracycline, and metronidazole	*n* = 192. Nausea: 24 (12%); abdominal pain: 22 (11%); metallic taste: 17 (8.5%); diarrhea: 16 (8%); asthenia: 15 (7.5%); vomiting: 13 (6.5%); headache: 2 (1%); oral injury: 1 (0.5%); dizziness: 1 (0.5%); myalgia: 1 (0.5%)	22.0%
Chen *et al*[21], 2016	BQT: bismuth, lansoprazole, metronidazole, and amoxicillin	*n* = 156. Flavor distortion: 2 (1.3%); dyspepsia: 2 (1.3%); nausea: 30 (19.2%); vomiting: 4 (2.6%); abdominal pain: 1 (0.7%); swelling: 8 (5.1%); diarrhea: 1 (0.7%); dizziness: 10 (6.4%); headache: 2 (1.3%); skin rash: 3 (1.9%); fatigue: 2 (1.3%); fever: 1 (0.7%); treatment was discontinued because of adverse effects: 8 (5.2%)	34.0%
Rodríguez de Santiago *et al*[24], 2017	BQT-Pylera: Pylera® and esomeprazole or omeprazole	*n* = 101. Dyspepsia: 43 (43.9%); asthenia: 35 (35.7%); dysgeusia: 34 (34.7%); nausea: 26 (26.5%); abdominal pain: 25 (25.5%); abdominal swelling: 20 (20.4%); hyporexia: 19 (19.4%); diarrhea: 14 (14.3%); headache: 13 (13.3%); myalgia: 13 (13.3%); heartburn: 7 (7.1%); flatulence: 8 (8.1%); urticaria/eczema: 5 (5.1%); paresthesia: 4 (4.1%); arthralgia: 4 (4.1%); drowsiness: 3 (3.1%); cough: 3 (3.1%); depression: 3 (3.1%); oral aphthous ulcers: 2 (2.7%); itching: 2 (2.7%); mucous candidiasis: 2 (2.7%); insomnia: 1 (1.4%); constipation: 1 (1.4%); hypertensive crisis: 1 (1.4%)	67.3%
Huang *et al*[29], 2018	N-BQT: esomeprazole, amoxicillin, tetracycline, and metronidazole	*n* = 24. Abdominal pain: 3 (12.5%); nausea/vomiting: 3 (12.5%); constipation: 1 (4.2%); dizziness: 1 (4.2%); headache: 1 (4.2%); skin rash: 0 (0%); diarrhea: 0 (0%)	29.2%
Huang *et al*[29], 2018	Susceptibility-guided therapy	*n* = 39, Abdominal pain: 3 (7.7%); nausea/vomiting: 3 (7.7%); constipation: 2 (5.1%); dizziness: 0 (0%); headache: 1 (2.6%); skin rash: 1 (2.6%); diarrhea: 0 (0%)	25.6%
Liu *et al*[33], 2020	BQT: bismuth, esomeprazole, tetracycline, furazolidone, and *Lactobacillus acidophilus*	*n* = 50. Loose stools: 1 (2.0%); dizziness: 4 (8.0%); skin rash: 2 (4.0%); foot joint pain: 1 (2.0%); dry mouth: 3 (6.0%)	20.0%
Ji *et al*[37], 2020	BQT: bismuth, rabeprazole, amoxicillin, levofloxacin or furazolidone	*n* = 191. Abdominal pain: 4 (2.09%); constipation: 2 (1.04%); nausea: 11 (5.7); diarrhea: 5 (2.6%); flatulence: 7 (3.6%); skin rash: 5 (2.6%); pruritus: 1 (0.5%); dysgeusia: 1 (0.5%); headache: 3 (1.6%); anorexia: 0 (0%); dizziness: 8 (4.1%); dyspepsia: 6 (3.1%); drowsiness: 0 (0%); Fever: 2 (1.0%); paresthesia: 1 (0.5%); tachycardia: 2 (1.0%); vomiting: 1 (0.5%); fatigue: 2 (1.0%); suspended treatment because of adverse effects: 6 (3.1%)	20.4%
Ji *et al*[37], 2020	Susceptibility-guided therapy	*n* = 163. Abdominal pain: 8 (4.9%); constipation: 2 (1.2%); nausea: 10 (6.2%); diarrhea: 3 (1.8%); flatulence: 9 (5.5%); skin rash: 2 (1.2%); pruritus: 3 (1.8%); dysgeusia: 6 (3.7%); headache: 2 (1.2%); anorexia: 1 (0.6%); dizziness: 3 (1.8%); dyspepsia: 1 (0.6%); drowsiness: 1 (0.6%); fever: 1 (0.6%); paresthesia: 0 (0%); tachycardia: 0 (0%); vomiting: 0 (0%); fatigue: 0 (0%); treatment was discontinued because of adverse effects: 2 (1.2%)	23.3%
Nyssen *et al*[39], 2020	BQT-Pylera: Pylera® and PPI	*n* = 275. Nausea: 45 (16.0%); metallic taste: 13 (4.7%); diarrhea: 44 (16.0%); vomiting: 27 (9.8%); fatigue: 33 (12.0%); abdominal pain: 22 (8.0%); anorexia: 32 (12.0%)	42.0%
Nyssen *et al*[39], 2020	BQT: bismuth, PPI, metronidazole, and tetracycline	*n* = 85. nausea: 35 (41.0%); metallic taste: 30 (35.0%); diarrhea: 22 (26.0%); vomiting: 15 (18.0%); fatigue: 10 (12.0%); abdominal pain: 5 (5.9%); anorexia: 6 (7.1%)	52.0%
Nyssen *et al*[39], 2020	BQT: bismuth, PPI, metronidazole, and doxycycline	*n* = 94. nausea: 12 (13.0%); metallic taste: 5 (5.3%); diarrhea: 3 (3.2%); vomiting: 3 (3.2%); fatigue: 4 (4.3%); abdominal pain: 4 (4.3%); anorexia: 0 (0%)	30.0%
Costa *et al*[25], 2017	Susceptibility-guided therapy	*n* = 42. Abdominal pain: 7 (16.7%); diarrhea: 5 (11.9%); nausea: 4 (9.5%); vomiting: 3 (7.1%); change in taste sensation: 1 (2.3%); treatment was discontinued because of adverse effects: 2 (4.7%)	35.7%
Liou *et al*[28], 2018	Susceptibility-guided therapy	Clinical Trial 1 (*n* = 21). Rash: 0 (0%); dizziness: 4 (19.0%); headache: 1 (4.8%); taste distortion: 0 (0%); swelling: 1 (4.8%); abdominal pain: 0 (0%); nausea: 1 (4.8%); vomiting: 0 (0.0%); diarrhea: 2 (9.5%); constipation: 0 (0.0%)	42.9%

Liou *et al*[28], 2018	Susceptibility-guided therapy	Clinical Trial 2 (n = 202). Skin rash: 5 (2.5%); dizziness: 25 (12.4%); headache: 8 (4.0%); taste distortion: 7 (3.5%); swelling: 22 (10.9%); abdominal pain: 9 (4.5%); nausea: 38 (18.8%); vomiting: 14 (6.9%); diarrhea: 4 (2.0%); constipation: 1 (0.5%)	51.0%
Liou *et al*[28], 2018	N-BQT: esomeprazole, amoxicillin, metronidazole, and tetracycline	Clinical Trial 2 (n = 202). Skin rash: 3 (1.5%); dizziness: 18 (8.9%); headache: 11 (5.5%); taste distortion: 9 (4.5%); swelling: 11 (5.5%); abdominal pain: 6 (3.0%); nausea: 30 (14.9%); vomiting: 6 (3.0%); diarrhea: 14 (6.9%); constipation: 4 (2.0%)	50.5%
Liou *et al*[28], 2018	N-BQT: esomeprazole, amoxicillin, metronidazole, and doxycycline	Clinical Trial 1 (n = 20). Rash: 0 (0%); dizziness: 3 (15.0%); headache: 2 (10.0%); taste distortion: 1 (5.0%); swelling: 0 (0%); abdominal pain: 2 (10.0%); nausea: 1 (5.0%); vomiting: 0 (0%); diarrhea: 3 (15.0%); constipation: 1 (5.0%)	55.0%

BQT: Bismuth quadruple therapy; EAS: Esomeprazole, amoxicillin, and sitafloxacin; EMS: Esomeprazole, metronidazole, and sitafloxacin; N-BQT: Non-bismuth quadruple therapy; PPI: Proton pump inhibitor; RAS: Rabeprazole, amoxicillin, and sitafloxacin; RMS: Rabeprazole, metronidazole, and sitafloxacin.

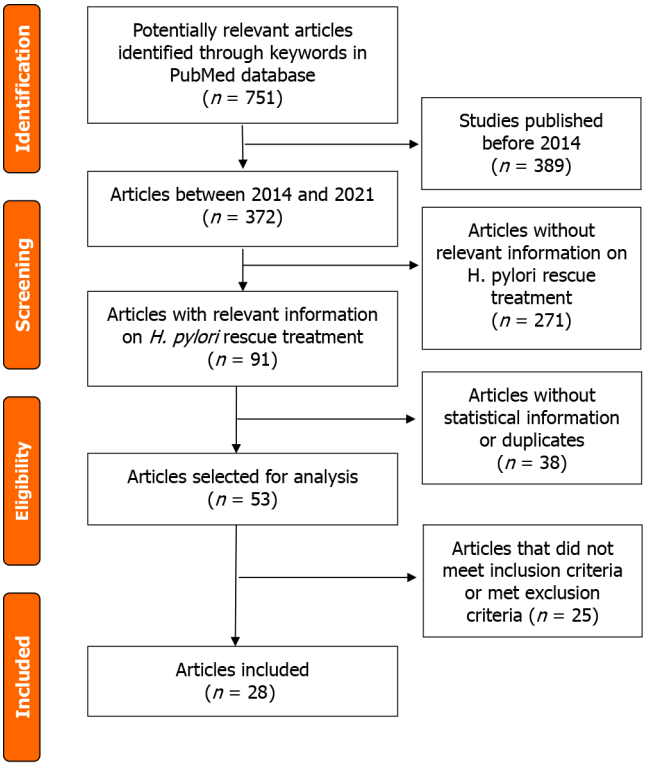

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Figure 2 Preferred Reporting Items for Systematic reviews and Meta-Analyzes flow diagram. *H. pylori*: *Helicobacter pylori*.

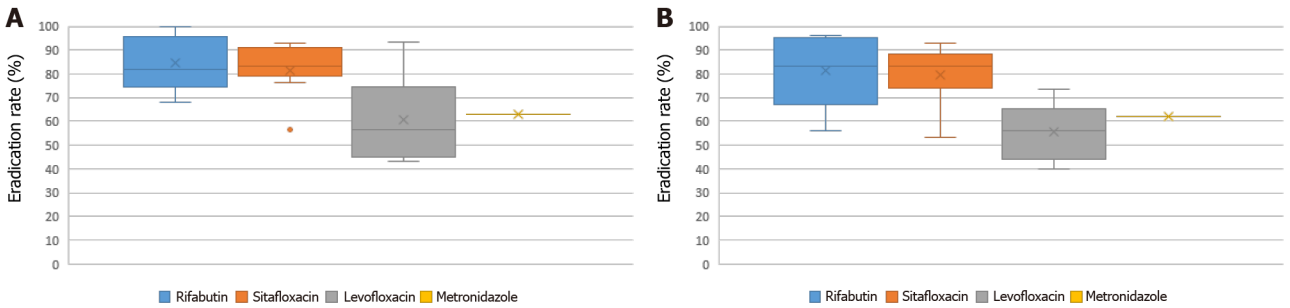

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Figure 3 Third-line triple therapy eradication rates. A: Triple therapy as third line (per protocol); B: Triple therapy as third line (intention to treat).

DISCUSSION

Given the high prevalence and incidence of *H. pylori* infection, its eradication is widely discussed in the

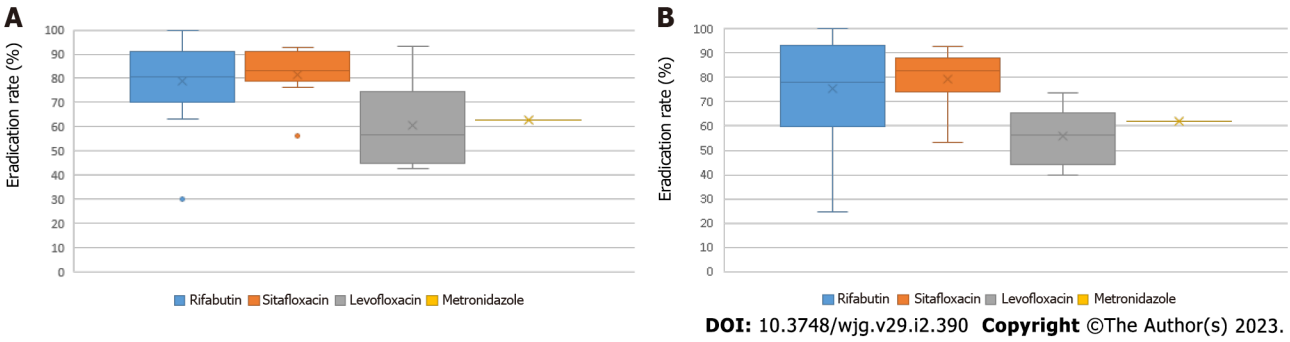

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Figure 4 Triple therapy eradication rates as rescue treatment. A: Triple therapy as rescue treatment (per protocol); B: Triple therapy as rescue treatment (intention to treat).

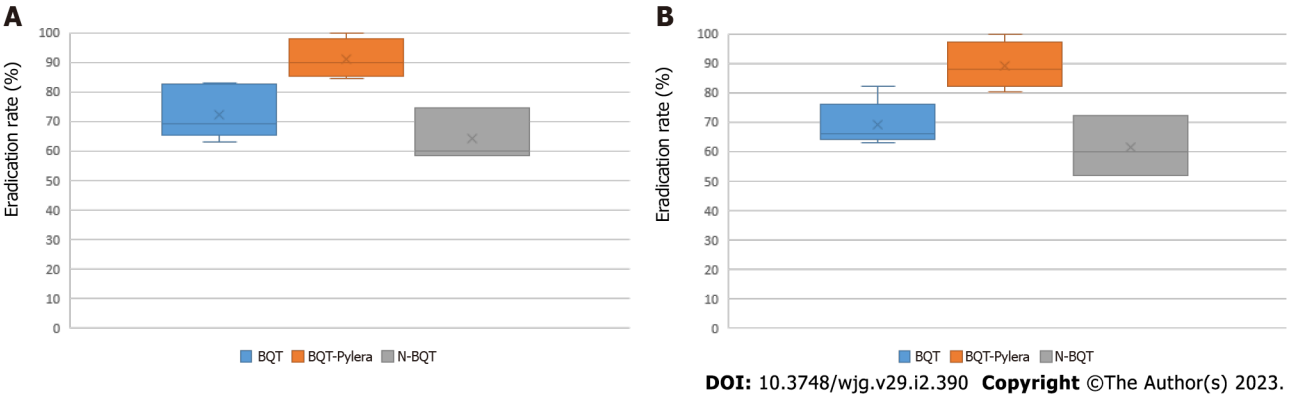

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Figure 5 Third-line quadruple therapy eradication rates. A: Quadruple therapy as third-line treatment (per protocol); B: Quadruple therapy as third-line treatment (intention to treat). BQT: Bismuth quadruple therapy; N-BQT: Non-bismuth quadruple therapy.

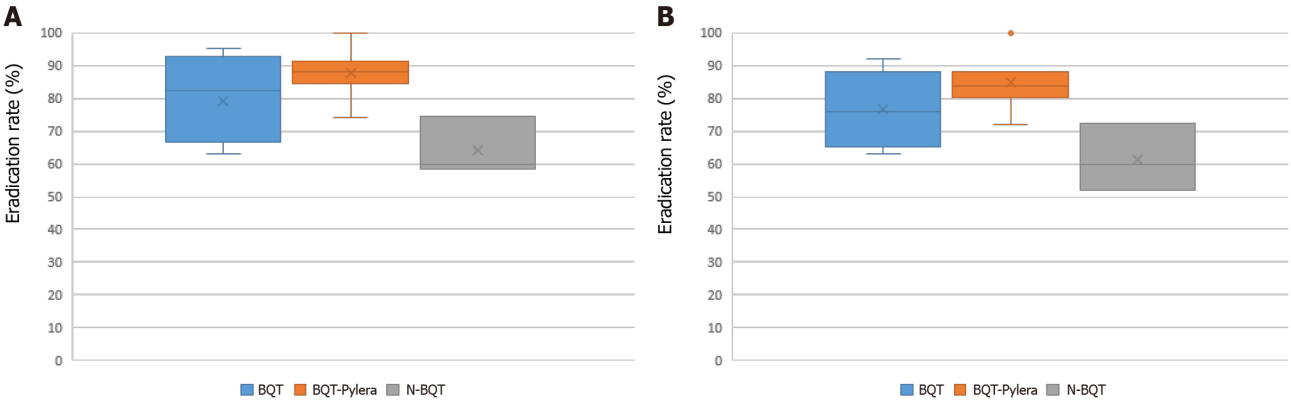

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Figure 6 Quadruple therapy eradication rates as rescue treatment. A: Quadruple therapy as rescue treatment (per protocol); B: Quadruple therapy as rescue treatment (intention to treat). BQT: Bismuth quadruple therapy; N-BQT: Non-bismuth quadruple therapy.

current scenario. Even with very well-established criteria for eradicating the infection and treatment algorithms for choosing first-line and second-line regimens against *H. pylori* infection, therapeutic failure is still very frequent. Possible causes responsible for failure to eradicate *H. pylori* include factors related to the microorganism, host, or the treatment itself, such as poor adherence of patients because of adverse effects and complexity of therapeutic regimens[41-43]. Thus, it is necessary to establish safe, effective, and accessible third-line and rescue therapies for patients.

The Maastricht V/Florence Consensus states that after failure of a first-line therapy containing clarithromycin and BQT second-line, SGT or an empirical therapy based on fluoroquinolones should be used or a combination of different antibiotics with bismuth in regions with a profile of known fluoroquinolone resistance. In cases of failure of first-line treatment based on triple or quadruple

therapy without bismuth and second-line treatment containing fluoroquinolones, the use of BQT as third-line is recommended. After failure to use BQT as first-line and therapy containing fluoroquinolones as second-line, the use of clarithromycin-based triple therapy or quadruple therapy is recommended. However, given the low level of evidence and recommendation of all these statements, their incorporation in clinical practice is difficult[12].

Triple therapy

Rifabutin: Rifabutin-based triple therapy regimens have been widely discussed as an alternative for the rescue treatment of *H. pylori* infection. In the present review, most rifabutin-based triple therapy regimens used rifabutin 300 mg (150 mg twice daily or 300 mg once daily) plus amoxicillin (variable daily dosage) and a proton pump inhibitor (PPI) (variable daily dosage) lasting 7-14 d. The mean overall eradication rate of these third-line regimens was 81.6% and 84.4% by ITT and PP, respectively. Regarding the use of rifabutin-based triple therapy as a rescue regimen, *i.e.* in patients with two or more previous failed eradications, the mean overall eradication rate was 75.4% and 78.8% by ITT and PP, respectively.

In the prospective study conducted by Lim *et al*[13], the effectiveness of rifabutin-based triple therapy was evaluated according to the PPI dosage. In this study, patients who received a rifabutin-based triple therapy regimen with higher doses of PPIs had eradication rates of 96.3% and 100% by ITT and PP, respectively, whereas patients who received standard dose PPIs showed eradication rates of 78.1% and 80.6% by ITT and PP, respectively. In turn, Mori *et al*[19] performed a comparative analysis between the duration of rifabutin-based triple therapy regimens. In this study, longer duration regimens had higher eradication rates compared to shorter duration regimens, and eradication ranged from 83.3% to 94.1% and from 81.8% to 91.7% by ITT and PP, respectively. Both studies were in line with the review performed by Gisbert *et al*[42], which suggested increasing the dose of PPIs and the duration of the therapeutic regimen as a strategy for optimizing rifabutin-based treatment.

On the other hand, in studies conducted by Ribaldone *et al*[32] and Saracino *et al*[35], lower eradication rates than those of the other studies included in the present review were found, with values of 71.5% and 68.5% by ITT and 72.7% and 56.1% by PP, respectively. These results corroborate the mean eradication rate found in the same study conducted by Gisbert *et al*[42] in 2020, in which, based on an analysis of 678 patients using rifabutin-based triple therapy, an eradication rate of 69% was found for this regimen as third-line treatment. Regarding rifabutin-based triple therapy as fourth-line treatment, in the prospective study by Hirata *et al*[36] from 2020, an association between amoxicillin, rifabutin, and vonoprazan (a competitive inhibitor of potassium) was used in patients who used sitafloxacin-based third-line. The eradication rate found by Hirata *et al*[36] was 100% by ITT and PP.

Regarding adverse effects related to rifabutin-based triple therapy, the literature presents controversial consequences of this regimen. In our review, an average of 53.7% of patients using this approach had at least one adverse effect. Although most cases are related to mild and transient adverse effects, such as gastrointestinal discomfort, there is a lot of divergence between studies. In therapies with prolonged use of rifabutin, for example, Mori *et al*[19] reported a high rate of adverse effects, with 94.1% of patients having at least one effect and discontinuation of treatment by 29.4% of patients. On the other hand, Ribaldone *et al*[32] reported that only 7.3% of patients had at least one adverse effect, and treatment was discontinued by 1.3% of patients. In addition, the use of rifabutin is associated with serious adverse effects such as myelotoxicity[41]. However, only one of the studies included in this review[19] presented patients with myelotoxicity, and 6.8% of patients had transient leukopenia with recovery of hematological patterns after 1 wk of treatment.

The mean eradication rates of rifabutin-based triple therapies found in the present review are encouraging. However, the heterogeneity of studies, whether related to eradication rates or adverse effects, makes it difficult to assess the real efficacy and safety of using rifabutin-based triple therapy as third-line treatment and rescue regimen. In addition, rifabutin is used mainly for the treatment of tuberculosis and other mycobacteria, especially in the context of immunodeficiency or HIV infection, and a possible acquisition of resistance to rifabutin is a limitation to its widespread use. Resistance to rifabutin has been reported in patients with low CD4 lymphocyte counts and when intermittent dosages were used[44]. Although the use of rifabutin in the management of refractory *H. pylori* infection involves a risk, rifabutin-based therapies act as an important alternative for third-line treatment and rescue regimens, especially in regions of previously known resistance to quinolones.

Sitafloxacin: Sitafloxacin is a quinolone with low minimum inhibitory concentration for *H. pylori* that has been used as rescue therapy[14]. In the present study, most sitafloxacin-based triple therapy regimens had a treatment regimen with sitafloxacin 200 mg (100 mg twice daily) plus amoxicillin (750 mg twice daily or 500 mg four times daily) or metronidazole (250 mg twice daily) and a PPI (variable daily dose) or vonoprazan (20 mg twice daily) for 7-14 d. The mean overall eradication rate of these regimens as third-line treatment was 79.4% and 81.5% by ITT and PP, respectively.

Although eradication rates in the studies included in the present review showed satisfactory results for the use of sitafloxacin-based triple therapy as third-line treatment, results were not homogeneous between studies. In a retrospective study, Saito *et al*[30] compared the efficacy of using sitafloxacin-based therapy associated with amoxicillin and esomeprazole or vonoprazan for 7 d as third-line

treatment. In this study, the eradication rate found in the sitafloxacin-esomeprazole association was 54.2% and 56.5% by ITT and PP, respectively. The same therapeutic regimen was used in two other studies showing discrepant eradication rates. While in the prospective study by Hirata *et al*[23], eradication rates of 83.0% by ITT and PP were found, in the randomized clinical trial performed by Sue *et al*[31], eradication rates were 53.3% by ITT and 57.1% by PP.

In a systematic review[45] from 2021, 12 clinical trials were analyzed. A mean eradication rate of 80.6% was found for sitafloxacin-based therapies containing PPIs or vonoprazan for a period of 7 d, corroborating the findings in the present study. Regarding the heterogeneity of studies included in our review, discrepancies may be based on the presence of bacterial strains with mutation in the *gyrA* gene. Mutations in this gene are responsible for conferring resistance to quinolones, leading to a lower eradication rate. The relevance of the *gyrA* gene mutation status in eradication rates of quinolone-containing regimens was expressed in the review by Mori *et al*[46]. Thus, it is recommended to identify the mutation in the *gyrA* gene before using regimens containing quinolones such as sitafloxacin, especially in regions of known previous resistance to quinolones.

Although equivalent therapeutic regimens between different studies present heterogeneous eradication rates, there is agreement regarding no statistically significant difference between the efficacy of the association of sitafloxacin with metronidazole or amoxicillin and between 7-d and 10-d duration regimens. In two studies, eradication rates between regimens containing sitafloxacin-amoxicillin and sitafloxacin-metronidazole as third-line treatment were compared, finding similar results. Furuta *et al*[14] found eradication rates for the use of amoxicillin and metronidazole, respectively, of 84.1% and 90.9% by ITT and 86.4% and 90.9% by PP for 7-d regimens and 88.9% and 87.2% by ITT and 90.9% and 91.1% by PP for 14-d regimens. Similarly, for a 10-d regimen, Mori *et al*[20] found eradication rates of 81% and 72.4% by ITT and 82% and 76.4% by PP for amoxicillin and metronidazole, respectively. Regarding the duration of therapeutic regimens, in the present study, eradication rates of 73.1%, 78.3%, and 88.0% by ITT and 74.8%, 80.0%, and 91.0% by PP were found in regimens of 7 d, 10 d, and 14 d, respectively. Both the results related to the duration of regimens and the results related to the association of sitafloxacin with amoxicillin or metronidazole were in agreement with data presented by Mori *et al*[46] in a review conducted in 2020. In this study, eradication rates of 82.0% and 76.4% were found for 10-d regimens containing amoxicillin or metronidazole, respectively, with no statistically significant difference between therapeutic regimens. In addition, as in the present review, no statistically significant difference was found between eradication rates of sitafloxacin-based treatments in regimens of 7-d and 10-d duration. Thus, the choice between the association of sitafloxacin with amoxicillin or metronidazole should be based on the availability of drugs, knowledge of previously used regimens, and the presence of penicillin allergy. The choice of therapeutic regimens with a 7-d duration is also recommended to obtain greater adherence to treatment.

The present review also showed that triple therapy based on sitafloxacin plus vonoprazan is more effective than regimens containing conventional PPIs. Two studies conducted in 2019 compared the efficacy between regimens containing vonoprazan and regimens containing PPIs. Among 63 patients involved in one of the studies[31], 33 used a regimen containing vonoprazan and 31 used a regimen containing PPIs, with eradication rates of 75.8% by ITT and 83.3% by PP with the use of vonoprazan and 53.3% by ITT and 57.1% by PP with the use of PPIs. The superiority of vonoprazan in relation to PPIs was also observed in the study by Saito *et al*[30], in which, among 81 patients with two previous failed therapies, 93.0% of those who used vonoprazan obtained successful eradication of *H. pylori*, while with the use of esomeprazole, eradication rates were 54.2% by ITT and 56.5% by PP. In a review[45] from 2021, a comparative analysis between therapies containing PPIs or vonoprazan was performed, finding eradication rates of 70.1% and 88.9%, respectively, demonstrating the superiority of regimens containing vonoprazan. Therefore, the association of sitafloxacin with vonoprazan is recommended for greater treatment efficacy when available.

Regarding adverse effects related to sitafloxacin-based triple therapy, an overall adverse event rate of 52.4% was found in our review. However, most adverse effects found were mild and transient gastrointestinal disorders. The intensity and duration of these adverse effects were also evaluated in two reviews[45,46] that reported mild and transient effects. Therefore, the use of sitafloxacin-based regimens as third-line treatment may act as a safe and effective alternative for the eradication of refractory *H. pylori* infection.

Levofloxacin: The mean eradication rate found for levofloxacin-based triple therapy as third-line treatment was 55.7% by ITT and 60.6% by PP. This unsatisfactory eradication rate was homogeneous among studies included in the present review, with the exception of a non-randomized clinical trial[22] that compared the efficacy of levofloxacin-based regimens with 7-d, 10-d, and 14-d duration as third-line treatment. In this clinical trial, from the use of a levofloxacin-based triple therapy for a 14-d period, an eradication rate of 73.7% by ITT and 93.3% by PP was reported. However, for 7-d and 10-d regimens, eradication rates were 58.3% and 62.5% by ITT and 58.3% and 68.2% by PP, respectively. These unsatisfactory rates were also found by Okimoto *et al*[16] in 2014 and by Paoluzi *et al*[17] in 2015 (see Table 1).

In a prospective observational study[47], 500 patients in third-line treatment were followed, reporting an eradication rate of 75.0% for levofloxacin-based triple therapy, which was different from the findings of the present review. However, this divergence can be explained by the increasing resistance to

levofloxacin, which acts as an important factor in the failure of therapeutic regimens, as demonstrated by the meta-analysis performed by Chen *et al*[21]. The overall adverse effect rate related to levofloxacin-based triple therapy found in our review was 13.9%. Most adverse effects related to levofloxacin-based triple therapy regimens reported gastrointestinal tract disturbances of mild intensity and transient nature. As in one of the studies[22] in the present review, the follow-up of treatment-related adverse effects was not performed, and it was not included in the overall mean rate of adverse effects. Therefore, the safety of this therapeutic regimen in a 14-d regimen has not been evaluated.

Although BQT is recommended as second-line treatment by most guidelines, levofloxacin-based triple therapy is proposed as a potential alternative by the Maastricht V/Florence Consensus[12]. In addition to being associated with a wide incidence of adverse effects, BQT is also difficult to use because of the availability of bismuth in different regions. The association of these factors, together with the efficacy and safety of levofloxacin-based second-line therapies demonstrated in the systematic review by Gisbert *et al*[48], allow the use of these regimens as second-line treatment in regions with no bismuth availability or in regions with previously known resistance to clarithromycin regimens. Thus, the use of levofloxacin-based triple therapy as a third-line treatment and rescue therapy is not recommended in 7-d and 10-d regimens given the possibility of its use as a second-line treatment and low treatment efficacy. In turn, 14-d regimens require randomized clinical trials for a more accurate assessment of the efficacy and safety of this regimen as third-line treatment and rescue therapy.

Quadruple therapy

BQT: In the present review, BQT regimens featured a treatment regimen with bismuth subcitrate (variable dose) plus a PPI (variable dose) and two antibiotics (amoxicillin, metronidazole, tetracycline, levofloxacin, furazolidone, and doxycycline, variable dose) with 7-14 d duration (see Table 1). The mean overall eradication rate of these third-line regimens was 69.2% by ITT and 72.1% by PP, with the mean overall rate of rescue treatment being 76.7% by ITT and 79.2% by PP.

In the multicenter observational study by Gisbert *et al*[15], the effectiveness and safety of BQT was investigated in 200 patients with two previous failed eradications with clarithromycin- and levofloxacin-based regimens. In this study, administration of a BQT regimen as third-line resulted in a common eradication rate of 65.0% by ITT and 67.0% by PP for regimens of 7 d, 10 d, and 14 d, with no increase in therapeutic efficacy with the extension of regimens. In contrast, in the study carried out by Nyssen *et al*[39], eradication rates of 66.0% by ITT and PP for a 10-d regimen and 82.0% by ITT and 83.0% by PP for a 14-d regimen were found, reporting an increase in therapeutic efficacy with prolonged regimens. An observational study by Hsu *et al*[49] reported eradication rates of 84.0% by ITT and PP for a 10-d BQT. Thus, the expansion of the effectiveness of therapeutic regimens based on their prolongation presents heterogeneous results among studies included in our review. More comparative studies should be performed with the objective of evaluating a possible optimization of regimens based on the increase in their duration.

In addition to this possible optimization of the quadruple therapy by increasing the regimen duration, the association of different antimicrobials, such as furazolidone proved to be effective, as demonstrated by Ji *et al*[37] and Liu *et al*[33] in 2020. Similarly, a non-inferiority randomized clinical trial [21] reported satisfactory and similar eradication rates between conventional BQT and an alternative BQT containing amoxicillin, although the alternative therapy reported better adherence and safety. These studies highlight the need to perform clinical trials comparing different combinations of antimicrobials in order to accurately assess the effectiveness of these regimens.

The evaluation of the efficacy of BQT regimens in our study showed heterogeneous results given the multiple antimicrobial combinations used in therapeutic regimens. The association of these different regimens, which were equivalently accounted to find the overall mean eradication rate of BQT, acts as a limitation of our study. Therefore, the use of BQT as third-line treatment and rescue therapy requires further investigation regarding the combination of antimicrobials and duration of regimens since in our review the mean eradication rates were unsatisfactory and heterogeneous.

In addition to the conventional use of BQT, the use of BQT-Pylera was also evaluated. In the present review, three-in-one quadruple therapy regimens featured a treatment regimen with Pylera® (140 mg potassium bismuth subcitrate, 125 mg metronidazole, 125 mg tetracycline, three capsules, 6/6 h) plus a PPI (variable dose) lasting 10-14 d. The mean overall eradication rate of this regimen as third-line was 88.9% and 90.9% by ITT and PP, respectively, while the mean overall rate of rescue treatment was 84.9% by ITT and 87.8% by PP.

In the study performed by Nyssen *et al*[39] in 2020, 222 patients with two previous failed eradications used BQT-Pylera for 10 d and 5 patients for 14 d, and eradication was observed in 88.0% of patients by ITT and PP, and 100% by ITT and PP, respectively. Both Rodríguez de Santiago *et al*[24] in 2017 and Saracino *et al*[35] in 2020 found similar eradication rates for the use of BQT-Pylera for a period of 10 d, with 80.2% and 87.5% by ITT and 84.4% and 91.3% by PP, respectively. Although eradication rates found in the present review are satisfactory, it is important to evaluate optimization strategies for these regimens.

The review performed by Liou *et al*[43] suggested the increase in the dose of PPIs and the prolongation of therapeutic approaches for 14-d regimens as optimization strategies for the treatment of refractory *H. pylori* infections. However, in our review, no statistical differences were observed in the

use of these strategies for BQT-Pylera. The increase in the dose of PPI used was evaluated in the study by Rodríguez de Santiago *et al*[24], and it did not report results superior to those found by Saracino *et al*[35] in a regimen of equivalent duration. Regarding the prolongation of the therapeutic approach, even though the comparative evaluation performed by Nyssen *et al*[39] was encouraging, it had only 5 patients in the 14-d regimen. Hence, further comparative studies are needed to determine the most effective duration of this treatment regimen as triple and rescue therapy.

Regarding adverse effects of BQT, an overall rate of 34.0% was found in conventional therapy and a rate of 65.0% for BQT-Pylera. Among studies included in the present review, Rodríguez de Santiago *et al*[24] reported that 97.0% of patients had at least one adverse effect, and despite the high proportion, no impact was reported on treatment adherence related to these events. Note that most adverse effects found by the studies were mild and transient gastrointestinal disorders, which did not pose a significant limitation to the use of these therapeutic approaches. Thus, the use of BQT, either conventional or BQT-Pylera, is an effective and safe alternative for its use as rescue therapy. However, BQT is recommended by consensus[11,12] as second-line after failure of a first-line containing clarithromycin or as first-line in regions with clarithromycin resistance greater than 15%. Thus, the use of this approach as third-line, despite showing encouraging rates, is limited not only by the use of these therapies as first-line or second-line but also by the limited availability of bismuth salts in multiple regions.

Non-BQT: In the present review, non-BQT regimens featured a treatment regimen with a PPI (variable dose, see Table 1) plus amoxicillin (1 g, 12/12 h), metronidazole (variable dose, see Table 1), and tetracycline (variable dose, see Table 1) or doxycycline (100 mg, 12/12 h) for 14 d. The mean overall eradication rate of these regimens as third-line was 61.3% and 64.2% by ITT and PP, respectively.

In the clinical trial by Huang *et al*[29], the use of a non-BQT was analyzed in a sequential regimen with tetracycline in 27 patients, finding an eradication rate of 51.8% and 58.3% by ITT and PP, respectively. Ineffective eradication rates were also found by Liou *et al*[28] in a clinical trial from 2018. In this study, two clinical trials comparing quadruple therapies containing tetracycline or doxycycline were performed, resulting in eradication rates of 72.2% and 60.0% by ITT and 74.4% and 60.0% by PP, respectively. Regarding the safety of this therapeutic approach, the mean rate of adverse effects of 44.90% was found and most were mild and transient gastrointestinal effects.

Our results show that the use of non-BQT is safe but ineffective as third-line treatment. As only two studies with this therapeutic regimen were included in our review and none of them evaluated the use of this therapy in patients with three or more previous failed eradications, more clinical trials are needed for a more accurate assessment of the efficacy and safety of these regimens as third-line treatment and rescue therapy.

SGT

In the present review, SGT as third-line treatment had an overall mean eradication rate of 75.0% and 79.2% by ITT and PP, respectively, and these same values were found for the use of this therapy as rescue treatment. These findings, in turn, are in line with the systematic review performed by Puig *et al*[50], which found moderate results with a mean eradication rate of 72.0% by ITT and PP.

The reviews carried out by Liou *et al*[43] and Puig *et al*[50] agree with the recommendation of the Maastricht V/Florence Consensus, which suggests that SGT is recommended after failure of a second-line therapy whenever possible. However, both reviews have reservations regarding the use of this therapy as third-line treatment and as rescue therapy since the adoption of this regimen must account for the availability of tests, costs, and the patient's preference. In addition to these limitations, comparative studies between SGT and empirical regimens are limited, acting as a further obstacle to assess the practical use of this therapeutic approach as third-line and rescue therapy.

Two studies[28,37] included in the present review concluded there is no superiority in the use of SGT compared to regimens based on drug history. In the randomized clinical trial performed by Liou *et al*[28], the effectiveness of an empirical quadruple therapy and an SGT was compared in two trials. In the first clinical trial, eradication rates of 81.0% and 60.0% by ITT and 80.0% and 60.0% by PP were found for SGT regimens and empirical therapy, respectively. In the second clinical trial, eradication rates were 78.0% and 72.2% by ITT and 78.4% and 74.4% by PP, for SGT regimens and empirical therapy, respectively, concluding the non-superiority of SGT compared to empirical therapy. In contrast, the comparative clinical trial developed by Huang *et al*[29] in 2018 found the superiority of SGT in relation to empirical therapy. In this study, the eradication rate of the group that performed susceptibility tests was 81.4% and 89.7% by ITT and PP, respectively, while for the empirical group, rates were 51.8% and 58.3% by ITT and PP, respectively.

Thus, although there are not enough comparative studies to determine the real effectiveness of SGT as rescue therapy and the results presented were heterogeneous, the present review agrees with the recommendation of the Maastricht V/Florence Consensus[12]. Even if results do not show superiority in relation to empirical therapy, the use of susceptibility and genotypic resistance tests should be performed whenever possible as they provide an alternative to the growing bacterial resistance to antimicrobials.

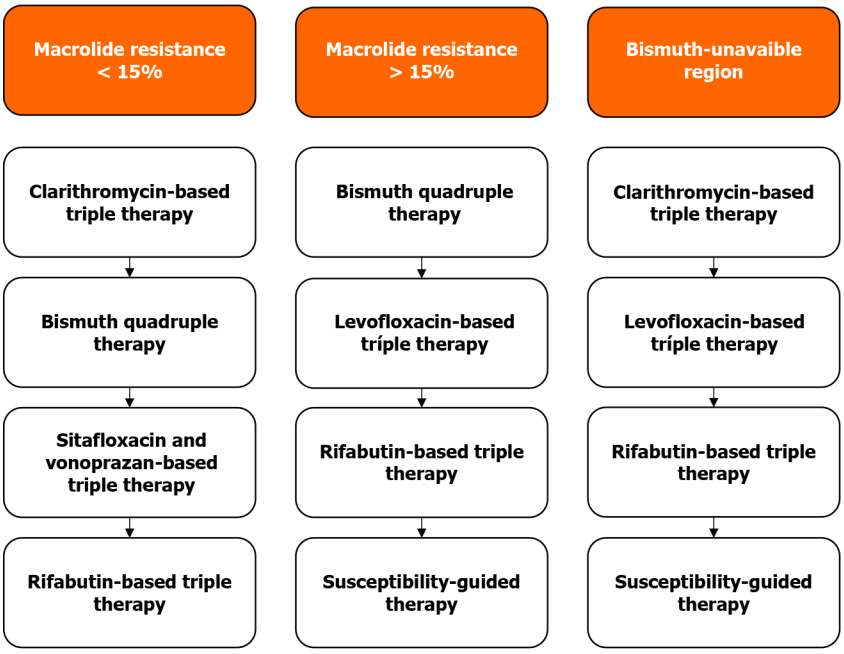

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Figure 7 Recommendation diagram.

CONCLUSION

The present review highlighted the need to carry out a greater range of comparative studies on third-line treatment and rescue regimens in refractory *H. pylori* infection, given the increasing resistance to antimicrobials and reduction in eradication rates of therapeutic regimens. In view of recommendations of the Maastricht V/Florence Consensus, after two previous failed eradication attempts, our study also recommends performing susceptibility tests whenever possible. However, given the difficulties related to test availability, costs, and patient preference, this therapeutic approach is not always an option. Thus, the establishment of effective and safe empirical therapies is fundamental for the management of refractory *H. pylori* infection.

Among the therapeutic regimens evaluated as alternatives to third-line treatment and rescue therapy, rifabutin- or sitafloxacin-based triple therapies as well as BQT-Pylera were shown to be safe and effective. On the other hand, BQT or non-BQT and levofloxacin-based triple therapy did not present satisfactory eradication rates or presented limitations regarding their use. Therefore, although safe, their use in therapeutic management should be avoided. Note that studies related to BQT showed heterogeneous results, and further investigations regarding its use as third-line treatment and rescue therapy are necessary. Furthermore, it is also necessary to develop studies evaluating both the efficacy and the safety of regimens with levofloxacin for 14 d. As in the present review, encouraging results were found when using this regimen as third-line treatment.

From the comparison between therapeutic approaches that obtained satisfactory results as third-line treatment, the alternative with better eradication rates was the rifabutin-based triple therapy, with a mean overall eradication rate of 84.4% for third-line treatment. However, the use of rifabutin as third-line presents the risk of development of resistance by *Mycobacterium tuberculosis* as a possible limitation, and its use as third-line treatment and rescue therapy is encouraged in specific situations. Based on the encouraging results found in our study, triple therapy based on sitafloxacin containing vonoprazan is recommended as third-line treatment in regions with a low profile of macrolide resistance, and the association with amoxicillin or metronidazole should be based on availability of drugs, knowledge of previously used regimens, and the presence of allergy to penicillin since this approach had an eradication rate of at least 83.3%. Based on the promising results reported from the comparison between conventional PPIs and vonoprazan, it is important that new clinical trials are developed in order to assess the efficacy of regimens with different associations between antimicrobials and vonoprazan.

In regions with previously known resistance to macrolides or low availability of bismuth, quinolone-based therapies are used as second-line treatment, and the use of sitafloxacin-based therapies as third-line treatment and rescue therapy is not recommended. In these cases, rifabutin-based triple therapy should be used, and in cases of therapeutic failure, an evaluation of the susceptibility profile should be chosen. These recommendations can be seen in the recommendation diagram (Figure 7).

As a final consideration, despite the satisfactory mean eradication rates found with BQT-Pylera, BQTs are recommended by guidelines as second-line treatment after failure of a first-line containing clarith-

romycin or as first-line in regions with greater than 15% clarithromycin resistance, limiting its use as third-line treatment and rescue therapy. Note that the combination of three-in-one therapy drugs is related to the increase in positive outcomes in eradication, and this combination of BQT should be used instead of standardized BQTs as first- or second-line, when available.

ARTICLE HIGHLIGHTS

Research background

The eradication of *Helicobacter pylori* (*H. pylori*) is widely discussed given the high prevalence and incidence of its infection and since therapeutic failure is frequent establishing safe, effective, and accessible third-line and rescue therapies for patients in need of eradication is necessary in the management of such infection.

Research motivation

Even though eradication criteria and treatment algorithms for first-line and second-line therapy against *H. pylori* infection are well-established, there is no clear recommendation for third-line and rescue therapy in refractory *H. pylori* infection.

Research objectives

To evaluate the efficacy and safety of rescue therapies against refractory *H. pylori* infection and to establish safe, effective, and accessible third-line and rescue therapies for patients in need of eradication.

Research methods

A systematic search of available rescue treatments for refractory *H. pylori* infection was conducted on the National Library of Medicine's PubMed search platform based on Preferred Reporting Items for Systematic reviews and Meta-Analyses guidelines. Different descriptors were used throughout the study for maximization of the database, namely: *Helicobacter pylori* multidrug resistance and rescue therapy; *H. pylori* multiresistant and rescue treatment; *Helicobacter pylori* multidrug resistance and rescue treatment; *Helicobacter pylori* rescue therapy; *Helicobacter pylori* and third line treatment; and fourth line therapy and *Helicobacter pylori*. Upon reliable data detection and collection, a statistical analysis was performed to compare eradication rates both by intention to treat and per protocol, and adverse effects found in the different therapeutic approaches to assess their feasibility in clinical practice.

Research results

Twenty-eight studies were included in the analysis of mean eradication rates as rescue therapy, and 21 of these were selected for mean eradication rate analysis as third-line treatment. Rifabutin-, sitafloxacin-, levofloxacin-, and metronidazole-based triple therapies, bismuth quadruple therapy (BQT), BQT, three-in-one, Pylera® (BQT-Pylera), non-BQT, and susceptibility-guided therapy were assessed. Furthermore, sitafloxacin-based and rifabutin-based triple therapies achieved higher efficacy than other therapeutic approaches.

Research conclusions

We managed to create a recommendation flowchart regarding rescue therapies in different situations, such as regions with previously known resistance to macrolides and in areas where bismuth is unavailable. These results can aid the clinical management of the *H. pylori* infection and furthermore prevent an increase in resistance rates to different antibiotics.

Research perspectives

New clinical trials should be developed in order to assess the efficacy of regimens with different associations between antimicrobials and vonoprazan, based on the promising results reported from the comparison between conventional proton pump inhibitors and vonoprazan.

FOOTNOTES

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LETTER TO THE EDITOR

Celiac disease screening in patients with cryptogenic cirrhosis

Janaina Luz Narciso-Schiavon, Leonardo Lucca Schiavon

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Abstract

We write a letter to the editor commenting the article "Who to screen and how to screen for celiac disease". We discuss the present literature on cirrhosis and celiac disease (CD) and recommend screening and treating CD in individuals with cryptogenic cirrhosis.

Key Words: Celiac disease; Liver cirrhosis; Liver failure; Aspartate aminotransferase; Alanine aminotransferase

Core Tip: We discuss reasons for recommendation of celiac disease screening in patients with cryptogenic cirrhosis.

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TO THE EDITOR

We read with interest the article by Singh *et al*[1]. Liver cirrhosis is a disease with potential morbidity, which can progress to decompensation, hepatocellular carcinoma and death. A high proportion (9.15%) of patients with cryptogenic hypertransaminasemia is affected by asymptomatic celiac disease (CD)[2]. It has been proposed that the hepatic manifestation of CD is a nonspecific chronic hepatitis[3], called by some authors celiac hepatitis[4]. A higher prevalence of CD has been demonstrated in

individuals with autoimmune hepatitis[5], and anti-actin antibodies may be present in both diseases, as they are reliable for the diagnosis of type-1 autoimmune hepatitis[6] and can also be associated with severe intestinal mucosa damage in CD patients[7]. This could support an immunological link between CD and liver injury. Despite these findings, it is not known for sure whether liver disease associated with celiac has the potential to progress to liver cirrhosis, although CD is twice as common in individuals with cirrhosis of the liver as in the general population[8,9]. In this sense, studies suggest that CD can be a cause of cryptogenic cirrhosis[10,11]. Most importantly, it has been reported that a gluten-free diet (GFD) treatment can reverse the decompensation of cirrhosis and remove the patient from liver transplantation waiting list[12-14]. Joshi *et al*[9] evaluated 84 patients with chronic liver disease, and 13% were diagnosed with CD. An improvement in liver function tests and Child-Pugh score was observed after GFD treatment. Demir *et al*[10] reported five cases of children with cryptogenic cirrhosis and CD. Treatment with GFD leaded to clinical and biochemical improvement, followed by a decrease in liver and spleen size. The most important sample was reported by Wakim-Fleming *et al*[8]. They have evaluated 204 patients with biopsy proven cirrhosis of different causes, and 2.5% were diagnosed with CD. After a GFD, patients with CD showed a return to normal levels of their celiac antibodies, small bowel biopsy and liver enzymes, and none received a liver transplant[8]. The European Society for the Study of Celiac Disease states that patients with unexplained elevation of liver enzymes should be assessed for CD and recognizes that CD can be associated with severe liver disease and even liver failure[15].

For the aforementioned reasons, and because liver cirrhosis has a high potential for morbidity and mortality, we recommend screening and treating CD in individuals with cryptogenic cirrhosis[16]. And one should consider screening for celiac antibodies in patients with decompensated cirrhosis on the liver transplantation waiting list, whatever are the mechanisms involved in the deterioration of liver function.

FOOTNOTES

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