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REVIEW

COVID-19 vaccination and liver disease

Sotaro Ozaka, Takashi Kobayashi, Kazuhiro Mizukami, Kazunari Murakami

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Abstract

Various vaccines against severe acute respiratory syndrome coronavirus 2 have been developed in response to the coronavirus disease 2019 (COVID-19) global pandemic, several of which are highly effective in preventing COVID-19 in the general population. Patients with chronic liver diseases (CLDs), particularly those with liver cirrhosis, are considered to be at a high risk for severe COVID-19 and death. Given the increased rates of disease severity and mortality in patients with liver disease, there is an urgent need to understand the efficacy of vaccination in this population. However, the data regarding efficacy and safety of COVID-19 vaccination in patients with CLDs is limited. Indeed, several organ-specific or systemic immune-mediated side effects following COVID-19 vaccination, including liver injury similar to autoimmune hepatitis, have been recently reported. Although the number of cases of vaccine-related liver injury is increasing, its frequency, clinical course, and mechanism remain unclear. Here, we review the current findings on COVID-19 vaccination and liver disease, focusing on: (1) The impact of COVID-19 in patients with CLD; (2) The efficacy, safety, and risk-benefit profiles of COVID-19 vaccines in patients with CLD; and (3) Liver injury following COVID-19 vaccination.

Key Words: COVID-19 vaccine; Liver disease; Side effect; Liver injury; Immune-related hepatitis; Autoimmune hepatitis

Core Tip: Patients with chronic liver disease (CLD), including cirrhosis, are a high-risk group for severe coronavirus disease 2019 (COVID-19). Presently, the results of several clinical trials for measuring the efficacy and safety of the available COVID-19 vaccines in patients with CLD have been reported. Given the increased rates of severity and mortality of COVID-19 in patients with CLD, the importance of aggressive vaccination in the effective management of severe acute respiratory syndrome coronavirus 2 infection should be emphasized. Although liver injury following COVID-19 vaccination has also been reported, it is infrequent and is not a factor in vaccine hesitancy.

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INTRODUCTION

The December 2019 outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) rapidly spread worldwide and became a global health threat[1,2]. COVID-19 is a respiratory disease caused by SARS-CoV-2, which can damage not only the lungs, but also other organs, including the cardiovascular system, liver, and gastrointestinal tract[3-5]. Vaccines are the most effective prophylaxis against COVID-19, and several vaccines against SARS-CoV-2 have been developed in response to the COVID-19 pandemic, among which vaccines produced mainly by Pfizer-BioNTech, Moderna, and Oxford-AstraZeneca are now widely used[6]. These vaccines are of great importance in controlling severe COVID-19 not only in healthy individuals, but also in high-risk populations, including those with chronic diseases. Chronic liver disease (CLD) is characterized by the gradual destruction of liver tissue over time, and includes liver diseases that are caused by chronic inflammation [chronic viral hepatitis, non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, and autoimmune hepatitis (AIH)] with or without cirrhosis and hepatocellular carcinoma (HCC). Patients with CLD, particularly those with cirrhosis, and liver transplantation (LT) recipients infected with SARS-CoV-2 have been reported to have a higher risk of adverse outcomes than the general population. For example, among 2780 individuals with COVID-19 in the United States, comparison between 250 individuals with liver diseases *vs* the rest of the cases indicated a significantly higher mortality rate in those with liver disease [hazard ratio (HR), 2.8; 95% confidence interval (CI): 1.9-4.0] and a higher mortality risk in those with cirrhosis (HR: 3.0; 95%CI: 1.5-6.0)[7]. In a recent study, the mortality rate of cirrhotic patients with COVID-19 was significantly higher than that of non-cirrhotic patients (HR: 2.38; 95%CI: 2.18-2.59), and was also increased in cirrhotic patients with underlying CLD (HR: 3.31; 95%CI: 2.91-3.77)[8]. Therefore, patients with CLD are considered to be at an increased risk of SARS-CoV-2 infection and worse outcomes. Given the increased rates of severity and mortality in patients with liver disease, there is an urgent need to understand the efficacy and safety of vaccination, as well as the importance of aggressive vaccination in this population. However, since most of the phase 2/3 trials of COVID-19 vaccines mainly recruited healthy individuals, data regarding the efficacy and safety in patients with liver diseases is limited. Presently, the results of several clinical trials for measuring the efficacy and safety of the available COVID-19 vaccines in patients with CLD have been reported.

The safety profiles of each vaccine have also been extensively studied. Common side effects of COVID-19 vaccines include injection site pain, transient fever, headache and fatigue[9,10]. Recently, however, several organ specific or systemic immune-mediated side effects following COVID-19 vaccination have also been reported[11]. These side effects include immune-mediated liver injury resembling AIH.

In this review, we summarized the current knowledge, focusing on the impact of COVID-19 in patients with liver disease, as well as the efficacy and safety of COVID-19 vaccination in these patients. In addition, we analyzed case reports of acute liver injury following COVID-19 vaccination.

OVERVIEW OF COVID-19 VACCINES

Vaccines against SARS-CoV-2 can be categorized based on the platform they are developed on into mRNA, viral vector, inactivated virus, attenuated virus, protein subunit, and recombinant DNA vaccines. The major COVID-19 vaccines currently used worldwide include the vaccines produced by Pfizer-BioNTech, Moderna, and Oxford-AstraZeneca[6]. These, plus the CoronaVac vaccine (Sinovac Biotech) and Janssen-Ad26.COV2.S vaccine (Johnson & Johnson), are the five vaccines registered in the WHO Emergency Use Listing of Qualified Vaccines (Table 1)[12]. Various COVID-19 vaccines have been

Table 1 Summary of coronavirus disease 2019 vaccines

Corporation	Vaccine	Mechanism	Vaccination Group: Number of cases/number of vaccinations (%)	Invaccination Group: Number of cases/number of vaccinations (%)	Efficacy % (95%CI)
Pfizer-BioNTech [18]	BNT162b2	mRNA	8/21720 (0.037)	162/21728 (0.746)	95.0 (90.3-97.6)
Moderna[20]	mRNA-1273	mRNA	11/15210 (0.072)	185/15210 (1.216)	94.1 (89.3-96.8)
Oxford-AstraZeneca[25]	ChAdOx1 nCoV-19 (AZD122)	Vector	27/4440 (0.608)	71/4455 (1.594)	62.1 (41.0-75.7)
Johnson & Johnson[26]	Ad26.COV2.S	Vector	433/19113 (2.265)	883/18924 (4.666)	52.9 (47.1-58.1)
Sinovac Life Science[29]	Corona Vac	Inacctivated	9/6559 (0.137)	32/3470 (0.922)	83.5 (65.4–92.1)

proven to be highly effective and have good safety profiles in healthy populations. The efficacy of vaccines is evaluated based on their: (1) Immunogenicity; (2) Efficacy rate in clinical trials; and (3) Real-world efficacy rate.

mRNA vaccines

The BNT162b2 mRNA (Pfizer-BioNTech) and mRNA-1273 mRNA (Moderna) vaccines are based on mRNA encoding SARS-CoV-2 spike proteins[13]. The injected mRNA is internalized into local host cells and translated, resulting in the production of antigen proteins and antigen-specific immune responses [14,15]. mRNA based vaccines have been shown to be safe and well tolerated in clinical trials. The BNT162b2 mRNA vaccine was administered to healthy adults (18-55 and 65-85 years old) at doses of 10 μg, 30 μg, or 100 μg in a phase 1/2 trial and showed immunogenicity, tolerability, and safety profiles consistent with these doses[16,17]. A phase 3 study of 43548 individuals also showed an efficacy rate of 95% [95%CI: 90.3-97.6; 8 cases of COVID-19 (0.04%) of 21720 in the BNT162b2 group *vs* 162 cases of COVID-19 (0.75%) of 21,728 in the placebo group][18], and a 6-mo follow-up of 44616 adults aged 16 years and older and 2264 participants aged 12 to 15 years showed an efficacy rate of 91.3% (95%CI: 89.0-93.2)[19]. The mRNA-1273 vaccine also showed an efficacy of 94.1% [95%CI: 89.3-96.8; 11 cases of COVID-19 (0.07%) of 15210 in the mRNA-1273 group *vs* 185 cases of COVID-19 (1.22%) of 15210 in the placebo group] in a phase 3 study of 30420 healthy individuals aged 18 or above[20]. It should be noted, however, that the efficacy against the recent epidemic Omicron strain is reduced for both these mRNA vaccines. Neutralizing antibody titers 3 wk after two doses of the BNT162b2 vaccine were significantly lower for the Omicron strain compared with the Wuhan strain[21], and even with the mRNA-1273 vaccine, neutralizing antibody titers against the Omicron strain after two doses of the vaccine were 1/41 to 1/84 of those against the European strain[22]. On the other hand, it was also shown that the neutralizing antibody titer against the Omicron strain increased significantly after the third dose of both vaccines and was almost equivalent to that after two doses[21,22], and the booster dose of mRNA vaccines was also effective against the Omicron strain, with a good efficacy rate of 60 to 70%[23].

Viral vector vaccines

The ChAdOx1 nCoV-19 vaccine (AZD1222), which was developed by Oxford-AstraZeneca, is a viral vector vaccine. Immunity to the spike protein is induced by administration of a chimpanzee adenovirus vector containing the SARS-CoV-2 spike protein gene[24]. A clinical trial of ChAdOx1 nCoV-19 reported an efficacy of 62.1% [95%CI: 41.0-75.7; 27 cases of COVID-19 (0.6%) of 4440 in the ChAdOx1 nCoV-19 group *vs* 71 cases of COVID-19 (1.6%) of 4455 in the control group][25]. The efficacy of Ad26.COV2.S, a single-dose viral vector vaccine produced by Johnson & Johnson, has also been reported. In a phase 3 study, vaccine efficacy after 28 d was 52.9% [95%CI: 47.1-58.1; 433 cases of COVID-19 of 19113 (2.3%) in the Ad26.COV2.S group *vs* 883 cases of COVID-19 of 18924 (4.7%) in the placebo group][26,27].

Inactivated vaccines

Sinovac-CoronaVac is an inactivated vaccine developed by Sinovac Life Sciences and is being used in several countries. Its efficacy and safety were demonstrated in a phase 1/2 study by Zhang *et al*[28] and the phase 3 study of Tanriover *et al*[29]. In a population aged 18-59 years, CoronaVac was shown to be highly effective in preventing symptomatic COVID-19 (83.5% *vs* placebo) and COVID-19-related hospitalizations (100%) at least 14 d after the second dose[29].

IMPORTANCE OF COVID-19 VACCINATION IN PATIENTS WITH LIVER DISEASES

During the COVID-19 pandemic, many cases have been accumulated and the clinical course of COVID-19 in patients with CLD has been characterized. Patients with CLD, including those with cirrhosis or HCC, and LT recipients, are a high-risk group for severe COVID-19[30]. In a large cohort study using electronic health record data from more than 17 million patients in the United Kingdom, which included more than 0.1 million patients with CLD, CLD was a risk factor for death from COVID-19 (HR: 2.39; 95%CI: 2.06-2.77)[31]. A particularly high mortality rate due to COVID-19 has been reported in patients with cirrhosis[7,8,32], and recent prospective data from a multicenter study reported a high mortality rate of 32% among 729 patients with CLD from 29 countries. In particular, patients with decompensated cirrhosis were found to be at a higher risk of hospitalization, mechanical ventilation and death (overall mortality: Child-Pugh-A: 19%, Child-Pugh-B: 32%, Child-Pugh-C: 51%, non-cirrhosis: 8%)[33]. Similarly, in a North American multicenter cohort study, compensated cirrhosis had no effect on mortality in COVID-19, while mortality was increased in patients with decompensated cirrhosis[34]. Furthermore, cirrhotic patients with COVID-19 had a significantly higher mortality rate than patients with COVID-19 alone (30% *vs* 13%, $P = 0.03$) in a multicenter cohort study in the United States and Canada[35]. Patients with CLD, particularly those with cirrhosis, have multiple mechanisms of immune dysfunction that can lead to increased susceptibility to SARS-CoV-2 infection and an abnormal inflammatory response during infection. Thus, it has become clear that liver cirrhosis patients are at an increased risk of adverse COVID-19 outcomes, including death, as has been established by large observational cohorts and population-level data and international registry findings.

In addition to cirrhosis, NAFLD, alcoholic liver injury, and HCC are known to be factors affecting COVID-19 severity. Several observational cohort studies revealed a significant increase in the risk of severe COVID-19 in patients with NAFLD. In a Chinese study analyzing 202 COVID-19 cases, NAFLD complications were significantly more frequent in 39 patients whose disease progressed after hospitalization than in non-progressors (87.2% *vs* 25.8%), and the rate of severe disease was significantly higher in patients with NAFLD than in those without NAFLD (44.7% *vs* 6.6%)[36]. A meta-analysis by Pan *et al*[37] showed that NAFLD was associated with more severe COVID-19 [odds ratio (OR): 2.93; 95%CI: 1.87-4.60][37]. Two other meta-analyses have also been reported, both of which showed that NAFLD is a severity factor for COVID-19[38,39]. Patients with NAFLD have decreased hepatic innate immunity with skewed M1/M2 macrophage polarization, as well as increased levels of inflammatory mediators and cytokines. This underlying inflammatory status associated with NAFLD might lead to further exacerbation of the SARS-CoV-2 infection and can lead to a cytokine storm, which greatly increases the mortality rate[37]. Furthermore, it should be noted that diabetes mellitus, obesity, and cardiovascular diseases are also frequently present in the background of NAFLD, and these metabolic disorders might also be factors related to the increased mortality of COVID-19. Regarding alcoholic liver disease, Marjot *et al*[33] reported that it is an independent risk factor for COVID-19 related death (OR: 1.79; 95%CI: 1.03-3.13)[33], and Kim *et al*[34] also reported a 2.4-fold increase in COVID-19 mortality (HR: 2.42; 95%CI: 1.29-4.55) in these patients[34]. Their report also showed that HCC is a risk factor for death from COVID-19 (HR: 3.96; 95%CI: 1.74-8.98)[34]. Since HCC often occurs secondary to cirrhosis, the greater severity of COVID-19 in this patient group is thought to be a result of reduced immunity. Since LT recipients are required to use immunosuppressive agents for a long period of time, they are also considered to be a high-risk group for severe COVID-19. However, many reports from actual cohort studies concluded that LT is not an independent risk factor for COVID-19 related death[40-42]. Webb *et al*[43] compared mortality in 151 COVID-19 patients who underwent LT with 627 healthy subjects without LT, and reported no difference in overall mortality between the two groups (absolute risk difference 1.4%; 95%CI: 7.7-10.4)[43]. On the other hand, their study also reported that gastrointestinal symptoms, such as diarrhea, but not respiratory symptoms, were significantly increased in post-LT patients affected by COVID-19 (30% *vs* 12%, $P < 0.0001$)[43]. In addition, a report from the European Liver Transplant Association of 103 LT recipients affected by COVID-19 showed a significantly higher mortality rate in recipients older than 60 years[44].

Thus, since patients with CLD, especially those with cirrhosis, are a high-risk group for severe COVID-19, aggressive vaccination of this patient group is most important for the effective management of SARS-CoV-2 infection.

EFFICACY AND SAFETY OF COVID-19 VACCINES IN PATIENTS WITH LIVER DISEASES

Given the increased severity and mortality rates of COVID-19 in patients with liver disease, we need to understand the efficacy and safety of vaccination in this population. However, data regarding the efficacy and safety of COVID-19 vaccination in patients with CLD is limited. Previous studies on vaccine efficacy and safety in patients with liver disease that have been reported to date are shown in Table 2[45-65]. It should be noted that most of the reports in Table 2 do not take into account the influence of the omicron variant, which is a limitation of using previous data in the current clinical environment of omicron variant predominance.

Table 2 Literature review of the efficacy and safety of coronavirus disease 2019 vaccines in patients with liver disease

Ref.	Design	Vaccine	Country/region	Number of participants	Value	Major findings (efficacy)	Major findings (safety)
Cirrhosis							
John *et al*[45], 2021	Multicentre retrospective cohort study	BNT162b2 and mRNA-1273	United States	Cirrhosis group (n = 20037); Control (n = 20037)	Efficacy	64.8% decrease in the development of COVID-19 infection after the first dose and a 78.6% decrease after the second dose	NA
Thuluvath *et al*[46], 2021	Prospective cohort study	BNT162b2, mRNA-1273, and AZD1222	United States	LT (n = 62); Cirrhosis (n = 79); CLD (n = 92)	Immunogenicity	Antibody was detectable in 82.2% of LT recipients, 96.2% of cirrhosis and 95.7% of CLD without cirrhosis. 61.3% of LT recipients and 24% CLD with/without cirrhosis had poor antibody responses	No patient had any serious AEs
Ruether *et al*[47], 2022	Prospective cohort study	BNT162b2, mRNA-1273, and AZD1222	Germany	LT (n = 138); Cirrhosis (n = 48); Control (n = 52)	Immunogenicity	Immunological response rates were 36.6%, 65.4%, and 100% in LT, cirrhosis, and controls, respectively	NA
Willuweit *et al*[48], 2022	Prospective cohort study	BNT162b2	Germany	Cirrhosis (n = 166); Control (n = 79)	Immunogenicity	Antibody was detectable in 96% of cirrhosis and 99% of controls. The median SARS-CoV-2 IgG titer was significantly lower in cirrhosis compared to the controls (939 *vs* 1905 BAU/mL, P = 0.0001)	NA
Wang *et al*[49], 2022	Multicentre retrospective cohort study	Inactivated vaccine	China	Compensated-cirrhosis (n = 388); Decompensated cirrhosis (n = 165)	Immunogenicity	Antibodies were detectable in 71.6% and 66.1% in compensated-cirrhosis and decompensated-cirrhosis	The vaccines were well tolerated, most AEs were mild and transient
Ai *et al*[50], 2022	Multicentre prospective cohort study	Inactivated vaccine	China	CLD (n = 284); Compensated cirrhosis (n = 123); Decompensated cirrhosis (n = 30)	Immunogenicity	Antibody detection rates were 76.8% in noncirrhotic CLD group, 78.9% in compensated cirrhotic group, 76.7% in decompensated cirrhotic group, and 90.3% in controls (P = 0.008 *vs* CLD)	There was no significant difference in AE among subgroups
Liver transplant recipients							
Rabinowich *et al*[51], 2021	Multicentre retrospective cohort study	BNT162b2 mRNA vaccine	Israel	LT patients (n = 80); Control (n = 25)	Immunogenicity	Immunogenicity among LT recipients was significantly lower [47.5% (LT) *vs* 100% (control), P < 0.001]	No patient had any serious AEs
Herrera *et al*[52], 2021	Multicentre prospective cohort study	mRNA-1273	Spain	LT recipients (n = 58)	Immunogenicity	93% of patients developed immunologic responses to mRNA-1273 vaccine	No serious AEs were reported in LT recipients
Strauss *et al*[53], 2021	Multicentre retrospective cohort study	BNT162b2 and mRNA-1273	United States	LT recipients (n = 161)	Immunogenicity	Antibody was detectable in 34% (95%CI: 27%-42%) of participants after first dose, and in 81% (95%CI: 74%-87%) after second dose	NA
Nazaruk *et al*[54], 2021	Retrospective cohort study	BNT162b2 mRNA vaccine	Poland	LT recipients (n = 65)	Immunogenicity	Antibody detection rate was 88.9% in LT recipients after the second dose	NA
Timmermann *et al*[55], 2021	Retrospective cohort study	mRNA vaccines	Germany	LT recipients (n = 118)	Immunogenicity	The seroconversion rate was 78.0% in LT recipients. MMF for immunosuppression was risk factors for seroneg-ativity	NA
D'Offizi *et al*[56], 2022	Retrospective cohort study	BNT162b2 and mRNA-	Italy	LT patients (n = 61); Control (n =	Immunogenicity	Immunological response rates 2 wk after 2nd dose	NA

		1273		51)		were 47.5% (LT) and 100% (control) ($P < 0.001$)	
John *et al*[57], 2022	Multicentre retrospective cohort study	BNT162b2 and mRNA-1273	United States	LT patients (n = 1133); Control (n = 791)	Efficacy	Vaccination with 2 doses of an mRNA vaccine was associated with a 64% decrease in COVID-19 infection and 87% decrease in COVID-19–related death in LT recipients	NA
Davidov *et al* [58], 2022	Retrospective cohort study	BNT162b2 mRNA vaccine	Israel	LT patients (n = 76); Control (n = 174)	Immunogenicity	Immunological response rates 2 wk after 2nd dose were 72.0% (LT) and 94.2% (control) ($P < 0.001$)	AEs were reported by 51% LT recipients. No serious events were reported
Sakai *et al* [59], 2022	Retrospective cohort study	BNT162b2	Japan	LT patients (n = 56); Control (n = 42)	Immunogenicity	LT recipients showed a lower seroconversion rate (44/56; 78.6%) than healthy controls (41/42; 97.6%)	NA
Calleri *et al* [60], 2022	Retrospective cohort study	BNT162b2 and mRNA-1273	Italy	Pre-LT patients (n = 89)	Immunogenicity	In the 89 pre-LT patients, seroconversion rate was 94.4% (23 d after vaccination), and 92.0% (68 d after vaccination)	No serious AEs were reported in participants
Viral hepatitis and NAFLD							
Xiang *et al* [61], 2021	Retrospective cohort study	Inactivated vaccine	China	CHB patients (n = 149)	Immunogenicity	The seroconversion rate was 87.2% in CHB	No serious AEs were reported in participants
He *et al*[62], 2022	Cross-sectional observational study	Inactivated vaccine	China	CHB patients (n = 362); Control (n = 87)	Immunogenicity	The seroconversion rates of SARS-CoV-2 antibodies were similar between CHB patients and healthy controls	The incidence was similar between CHB patients and controls. No serious AE
Wang *et al* [63], 2021	Multicentre retrospective cohort study	Inactivated vaccine	China	NAFLD patients (n = 381)	Immunogenicity	The inactivated COVID-19 vaccine was good immunogenicity (95.5%) in patients with NAFLD	AEs within 7 d and within 28 d totaled 95 (24.9%) and 112 (29.4%), respectively. No serious AEs were recorded
Autoimmune liver disease							
Duengelhoef *et al*[64], 2022	Prospective cohort study	BNT162b2, mRNA-1273, and AZD1222	Germany	AIH (n = 103); PSC (n = 64); PBC (n = 61); Control (n = 95)	Immunogenicity	Seroconversion was measurable in 97% of AIH and 99% of PBC/PSC patients, respectively. In 14% of AIH patients antibody levels were lower compared to PBC/PSC or controls	NA
Schneider *et al* [65], 2022	Prospective cohort study	BNT162b2 mRNA vaccine	Austria	AIH (n = 12); Control (n = 24)	Immunogenicity	Patients of AIH and healty controls acquired sufficient antibodies after third vaccination	NA

AE: Adverse event; AIH: Autoimmune hepatitis; CHB: Chronic hepatitis B; CLD: Chronic liver disease; LT: Liver transplant; MMF: Mycophenolate mofetil; NA: Not available; NAFLD: Non-alcoholic fatty liver disease; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis; CI: Confidence interval.

Response to vaccination in liver cirrhosis patients

Given the higher COVID-19 related mortality in individuals with decompensated cirrhosis, it is important to prioritize vaccination in this group. Patients with cirrhosis have previously shown hyporesponsiveness to hepatitis B virus and pneumococcal vaccines[66,67], and were also considered to be less responsive to COVID-19 vaccines[68]. Indeed, cirrhosis has been reported to be a high-risk factor (3.0-fold) for COVID-19 related deaths after COVID-19 mRNA vaccination in a large United Kingdom cohort study. A defect in acquired immunity in patients with cirrhosis probably predicts a low response to vaccination in this patient population[69]. However, a recent study in the United States found that 75%

of CLD patients without cirrhosis and 77% of those with cirrhosis had adequate antibody responses to COVID-19 vaccination[46]. In that study, antibody responses to the BNT162b2 mRNA vaccine and mRNA-1273 mRNA vaccine were favorable (64.4% and 76.4%, respectively), whereas those to the Ad26.COV2.S vaccine (Johnson & Johnson) were low (15.8%). In a study analyzing the immune response of 110 patients with cirrhosis after two doses of the BNT162b2 mRNA vaccine, while the antibody initial acquisition rate was 96%, which was not significantly different from that in the control group (99%) (P = 0.04), the antibody titer showed a rapid and significant reduction in patients with cirrhosis[48]. This suggests that although the initial results after vaccination with the COVID-19 BNT162b2 vaccine are favorable in patients with cirrhosis, it should be noted that the antibody response deteriorates rapidly over time, and, hence, the timing of the booster shot needs to be addressed in the near future.

The immunogenicity of COVID-19 vaccines in patients with cirrhosis appears to be inferior to that in healthy individuals, although real-world cohort studies showed that BNT162b2 mRNA and mRNA-1273 vaccines reduce the development of COVID-19 infection by 64.8% after the first dose and 78.6% after the second dose[45]. Furthermore, a recent report on BNT162b2 mRNA, mRNA-1273, and Ad26.COV2.S vaccines has shown significantly reduced COVID-19-related mortality in vaccinated patients with cirrhosis (HR: 0.21; 95%CI: 0.11-0.42)[70]. In the report on the Sinovac-CoronaVac vaccine, the immunogenicity in patients with CLD was lower (77.3%) compared to that in healthy controls (90.3%), although the presence or absence of cirrhosis in CLD patients did not affect the antibody retention rate [non-cirrhotic CLD (76.8%), compensated cirrhosis (78.9%), and decompensated cirrhosis (76.7%)]. The safety in each group was as good as in healthy controls in that report [healthy controls (16.0%), non-cirrhotic CLD (15.5%), compensated cirrhosis (16.3%), and decompensated cirrhosis (20.0%)][50]. In a study examining the efficacy of the Ad26.COV2.S vaccine, the vaccine efficacy in patients with cirrhosis was 64% (OR: 0.36; 95%CI: 0.20-0.62, P = 0.005), which was non-inferior to the results of a phase 3 trial[71].

Regarding safety, Cao *et al*[72] reported that of 85 patients with decompensated cirrhosis who received at least one dose of a SARS-CoV-2 vaccine, only one patient (1.2%) had an adverse reaction requiring hospitalization[72]. Bakasis *et al*[73] also reported that there was no significant difference in the safety of mRNA vaccines given to 87 patients with liver diseases including cirrhosis, and 40 controls [73]. The limitations of these studies[50,71-73], however, include a relatively small sample size of participants who received the vaccines and a short follow-up period. Research with larger sample sizes is, thus, required in the future.

Hence, although each vaccine is slightly inferior in immunogenicity in patients with cirrhosis compared with healthy individuals, they can be safely used with adequate efficacy in patients with cirrhosis. However, there is insufficient data to allow recommendation of one vaccine over another.

LT

COVID-19 outcomes in LT recipients are not necessarily worse than those in the general population, although they have a higher rate of admission to the intensive care unit. Thus, COVID-19 vaccination should be prioritized for these patients, since the benefits far outweigh the potential risks. Vaccination for LT recipients is an interesting area of research, with a series of reports on its efficacy and safety.

Several reports of low humoral and cellular responses after COVID-19 vaccination in LT recipients suggest that vaccine-induced immunity in this patient subgroup is lower than in the general population. As shown in Table 2, LT recipients exhibit significantly attenuated humoral and cellular immunity 2 wk after mRNA vaccination compared to healthy individuals[56,58], lower seroconversion rates[59] and significantly lower immunogenicity[51]. Indeed, combination immunosuppressive therapy, including mofetil mycophenolate, is reportedly a predictor of reduced responsiveness to vaccination[55,58,59]. The results of such vaccine responses in LT recipients were similar to the results of other studies in kidney transplant recipients[74], lung transplant recipients[75], and allogeneic hematopoietic stem-cell transplant recipients[76]. On the other hand, a recent multicenter cohort study showed that mRNA vaccines reduce SARS-CoV-2 infection rate, occurrence of symptomatic COVID-19, and mortality in LT recipients as well as in patients with cirrhosis[70]. While the immunogenicity of mRNA vaccines in LT recipients appears to be attenuated compared with that in healthy individuals, the disease status of COVID-19 is greatly attenuated in vaccinated LT recipients compared to unvaccinated recipients. Additionally, the value and safety of routine immunization in liver transplant recipients has been well established, and current guidelines recommend pre-transplant vaccination whenever possible[77]. However, since LT recipients also show a rapid decrease in antibody titers after mRNA vaccination[59], and because booster doses are reportedly extremely safe in post-organ transplant patients [78], it is recommended that all LT recipients receive a third or fourth booster dose if they have low or insufficient antibody titers[47].

HCC

HCC patients are considered a high risk group for severe COVID-19[34]. Nevertheless, there are no cohort studies investigating the immunogenicity and safety of COVID-19 vaccines in patients with HCC. According to the American Association for the Study of Liver Diseases, patients with HCC receiving locoregional or systemic therapy should also be considered for vaccination without

interruption of treatment[79]. Most patients treated for solid tumors, including HCC, show an adequate humoral response against SARS-CoV-2 after two vaccine doses. However, vaccination during chemotherapy tends to be associated with lower antibody levels, resulting in a suboptimal response in a small percentage of patients[80,81]. Furthermore, the concentration of neutralizing antibodies decreases over time, further reducing immunity[82]. Based on these reports, many countries are prioritizing a third vaccine dose in patients with solid tumors.

Viral hepatitis and NAFLD

With regard to viral hepatitis and NAFLD, several cohort studies evaluating the efficacy of inactivated vaccines have been reported from China. Xiang *et al*[61] analyzed the seroprevalence of anti-spike protein antibodies and neutralizing antibodies in chronic hepatitis B (CHB) patients who received two doses of inactivated vaccines, and reported high seropositivity rates of 87.2% and 74.5%, respectively [61]. This report showed that nucleotide analog therapy has no effect on vaccine-induced immune responses, suggesting that vaccination should be performed even during treatment of CHB. He *et al*[62] also reported that seroconversion rates of SARS-CoV-2 antibodies after 1, 2, and 3 mo in patients with CHB who received an inactivated vaccine were comparable to those in healthy controls[62]. No serious adverse reactions were reported in either study.Next, in a multicenter study of the safety and immunogenicity of inactivated vaccines in 381 individuals with NAFLD, the incidence of adverse reactions within 7 d and 28 d after vaccination was 24.9% and 29.4%, respectively. Neutralizing antibodies were detectable in 364 (95.5%) patients, and titers of neutralizing antibodies were shown to persist for a long time[63]. Given that NAFLD is a risk factor for severe COVID-19[37-39], active vaccination of this patient population would be ideal.

AIH

There have been two reports on the immunogenicity of COVID-19 vaccines in patients with AIH. Duengelhoef *et al*[64] reported that 91 of 94 (97%) AIH patients who received the second dose of a vaccine achieved seroconversion, although AIH patients showed impaired spike-specific T-cell responses and lower antibody levels at 7 mo compared with healthy individuals or patients with primary biliary cholangitis (PBC)/primary sclerosing cholangitis (PSC) (641 *vs* 1020 *vs* 1200 BAU/mL, respectively). These results were not related to the use of immunosuppressive medications, suggesting that the underlying immune abnormality of AIH might be involved in this diminished response[64]. In addition, a study that followed the antibody responses after mRNA vaccination in patients with AIH and healthy controls showed that anti-SARS-CoV-2 antibodies were sufficiently produced after the second and third vaccinations in both groups[65]. Therefore, early booster doses of vaccines should be considered in patients with AIH. Although none of the patients in these reports had serious adverse reactions, it is noteworthy that several case reports have been published reporting an increased risk of developing AIH-like syndromes after SARS-CoV-2 vaccination.

LIVER INJURY AFTER COVID-19 VACCINATION

Adverse effects of COVID-19 vaccines

Vaccination in patients with liver diseases is generally considered safe and effective and should be strongly recommended. The common side effects of COVID-19 vaccination include injection site pain, fever, headaches, and fatigue. All these side effects are mild and typically resolve 1-3 d after vaccination. However, recent worldwide dissemination of COVID-19 vaccines and post-marketing surveillance have led to an increasing number of reports of several organ-specific immune-related diseases, including myocarditis, immune thrombotic thrombocytopenia, Guillain-Barré syndrome, and pancreatitis, among others[11,83-85]. It is speculated that an abnormal immune response following vaccination is involved in the development of such diseases. Acute liver injury was not previously reported in clinical trials on COVID-19 vaccination because the sample size was insufficient to detect rare adverse events after vaccination. Recently, a large-scale population-based study on acute liver injury occurring after COVID-19 vaccination was reported from Hong Kong. In that study, among 2343288 COVID-19 vaccine recipients, acute liver injury within 56 d after the first and second vaccine dose occurred in 307 and 521 (335 and 334 *per* 100000 person-years) individuals vaccinated with BNT162b2, and 304 and 474 (358 and 403 *per* 100000 person-years) of those who received CoronaVac. The incidence of acute liver injury within 56 d of SARS-CoV-2 infection, on the other hand, was 32997 cases *per* 100000 person-years, indicating that the incidence of acute liver injury after COVID-19 vaccination was much lower than that after SARS-CoV-2 infection. It was also concluded that compared to the non-exposure period, no increased risk of acute liver injury was observed in the 56-d risk period following the first (IRR: 0.800; 95%CI: 0.680-0.942) and second (IRR: 0.944; 95%CI: 0.816-1.091) BNT162b2 dose, and the first (IRR: 0.689; 95%CI: 0.588-0.807) and second (IRR: 0.905, 95%CI: 0.781-1.048) CoronaVac dose. Thus, COVID-19 vaccines do not seem to increase the risk of acute liver injury[86]. However, since there have been case reports of acute liver injury requiring hospitalization after COVID-19 vaccination, this is an adverse effect that should not be overlooked. The current review aimed to increase awareness of this rare

adverse effect to promote its early recognition.Review of case reports on liver injury after COVID-19 vaccinationSince the summer of 2021, several case reports of liver injury after COVID-19 vaccination have been reported. The clinical and histological findings of most patients resembled AIH and the reported cases responded well to corticosteroid therapy. Previous cases of AIH-like acute liver injury after COVID-19 vaccinations are shown in Table 3[87-109]. Twenty-three reports (28 cases) of acute liver injury secondary to COVID-19 vaccines have been published in the PubMed database as of July 2022 (Table 3). The median age at the time of diagnosis was 61 (range 27-80) years, with a predominance of women (79%, females: 22, males: 6). Eight patients (29%) had no underlying disease, whereas nine patients (32%) had been diagnosed with other immune disorders (Hashimoto's disease: 5, PSC: 2, PBC: 1, sarcoidosis: 1). One patient was three months postpartum, and another patient was taking hormonal therapy due to menstrual irregularities. Liver injury occurred after vaccination following the BNT162b2 vaccine in 11 (39%) of the cases, mRNA-1273 vaccine in nine (32%), ChAdOx1 nCoV-19 vaccine in seven (25%), and CoronaVac vaccine in one case (4%). Seventeen patients (61%) developed liver injury after the first dose, 10 patients (36%) after the second dose, and two patients (7%) after the booster shot. The median time from vaccination to the onset of acute liver injury was 11 (range 3-35) d. The most common symptom was jaundice, while other symptoms, such as fatigue and anorexia, were also frequently observed. The most common pattern of liver injury was hepatocellular injury, with transaminase levels exceeding 1000 U/L in many cases. The mean alanine aminotransferase level was 848.2 (± 465.0) U/L, mean aspartate aminotransferase level was 1031.5 (± 578.3) U/L, and mean total bilirubin level was 9.08 (± 5.76) mg/dL. Serum immunoglobulin G (IgG) levels were measured in 25 patients and were elevated in 22 patients (88%). Anti-nuclear antibodies (ANA) were positive in 22 (82%) of 27 patients, while anti-smooth muscle antibodies and anti-mitochondrial antibodies (AMA) were elevated in some cases. Liver biopsy was performed in all cases. According to the simplified international diagnostic criteria published by the international AIH-group, seven cases were "typical" of AIH and 20 cases were "compatible with" AIH. Only one patient had poor findings of typical AIH and was diagnosed with drug-induced liver injury. Steroids as first-line treatment were used in 26 of the patients (93%), of whom 10 received prednisone, 14 received prednisolone, one received budesonide, and one was given methyl-prednisolone intravenously. Azathioprine was concomitantly used in five patients. The overall outcomes with corticosteroid therapy were favorable, and improvement was seen in 27 patients (96%). Only one male patient reported from India progressed to liver failure and died despite five cycles of plasma exchange[99].

All of the patients developed acute liver injury soon after COVID-19 vaccination, with findings consistent with AIH on liver biopsy, and responded well to corticosteroids. These case reports strongly suggest that the association between vaccination and the onset of AIH-like liver injury might be more than coincidental. However, it is difficult to establish a causal relationship between vaccination and liver injury with certainty. Indeed, post-pregnancy status, use of statins, and concomitant history of autoimmune diseases included in the reported cases are likely major confounding factors. It should be noted that almost all the reported cases lacked pre-vaccination laboratory data, and hence, the presence of pre-existing hepatitis cannot be ruled out. Indeed, Cao *et al*[110] reported that a patient with vaccine-induced AIH had advanced fibrosis on liver biopsy, suggesting the presence of CLD prior to vaccination [110].

Furthermore, a large international case series that provided evidence for the hepatotoxic potential of COVID-19 vaccines has recently been reported[111]. In that study, data from 18 countries on 87 patients who developed liver injury after COVID-19 vaccination were retrospectively collected. The median age at diagnosis was 48 (range 18-79) years and 63% were female. The median time from vaccination to the onset of liver injury was 15 (range 3-65) d. Liver injury occurred after vaccination with BNT162b2 in 59% of the cases, mRNA-1273 in 18% and ChAdOx1 nCoV-19 in 23%. When elevated IgG and autoantibody positivity were used to define immune-mediated hepatitis, 57% of the patients had immune-mediated hepatitis. Corticosteroids were mainly used in cases of severe liver injury and immune-mediated hepatitis (53%). In this study, there was one case of liver failure requiring LT, while the remaining cases had a good prognosis. There were no differences in the severity of liver injury, the rate of immune-mediated hepatitis, or the rate of steroid usage depending on the type of vaccine. Responses to treatment and outcomes were favorable in all groups. These results were generally consistent with the characteristics of the previous case reports shown in Table 3.

There is also a concern that hepatitis might be exacerbated by vaccination of patients originally diagnosed with AIH. Shroff *et al*[112] reported that six of 16 patients who developed vaccine-induced liver injury previously had AIH. They concluded that hepatotoxicity could be induced after vaccination by autoimmune induction[112]. However, the number of patients described in previous reports[87-109] (Table 3) is extremely small compared with the overall vaccinated population worldwide. The current review, thus, aimed to increase awareness about this rare adverse effect in order to promote its early recognition.

Mechanism of liver injury after COVID-19 vaccination

The mechanisms underlying the development of liver injury following COVID-19 vaccination remain unclear. As shown in Table 3, liver injury following COVID-19 vaccination is clinically and pathologically similar to AIH, suggesting that immune abnormalities associated with vaccination contribute to

Table 3 Reported cases of liver injury following coronavirus disease 2019 vaccination

Ref.	Age/sex	Past history	Vaccine	Onset	AST/ALT (U/L)	Total bilirubin (mg/ dL)	IgG	Antibody	Biopsy	Diagnose	Treatment	Outcome
Bril *et al*[87]	35/F	Third month postpartum	BNT162b2	7 d after the1st dose	754/2001	4.8	Normal	ANA: 1:1280	Interface hepatitis, rosette formation, eosinophil infiltration	Typical for AIH	Prednisone (20 mg/d)	Improved
Lodato *et al*[88]	43/F	Dyslipidemia	BNT162b2	15 d after the 1st dose	52/51	17.54	Normal	ANA: negative	Moderate portal inflammatory infiltrate with interface hepatitis, biliary injury	Compatible with AIH	Methyl-prednisolone (1 mg/kg/d)	Improved
Vuille-Lessard *et al*[89]	76/F	Hashimoto's disease, urothelial carcinoma	mRNA-1273	3 d after the 1st dose	811/579	3.8	Increased	ANA: 1:1280, AMA: 1:1280	Interface hepatitis, plasma cells infiltration, pseudorosettes	Compatible with AIH	Prednisolone (40 mg/d) + azathioprine	Improved
Londoño *et al*[90]	41/F	Premature ovarian failure	mRNA-1273	7 d after the 2nd dose	993/1312	2.3	Increased	ANA: 1:80	Interface hepatitis with a lymphoplasmacytic infiltrate	Typical for AIH	Prednisone (1 mg/kg)	Improved
Rocco *et al*[91]	80/F	Hashimoto's disease	BNT162b2	7 d after the 3rd dose	1401/1186	10.5	Increased	ANA: 1:160	Interface hepatitis with a lymphoplasmacytic infiltrate	Typical for AIH	Prednisone	Improved
McShane *et al*[92]	71/F	Osteoarthritis	mRNA-1273	4 d after the 1st dose	-/1067	15.7	Increased	ASMA: 1:2560	Interface hepatitis, eosinophil infiltration	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Clayton-Chubb *et al*[93]	36/M	Hypertension	AZD1222	26 d after the 1st dose	633/1774	9.9	Normal	ANA: 1:160	Interface hepatitis	Compatible with AIH	Prednisolone (60 mg/d)	Improved
Tan *et al*[94]	56/F	None	mRNA-1273	35 d after the 1st dose	1124/1701	5.9	Increased	ANA: positive, ASMA: Positive	Interface hepatitis, rosette formation, eosinophil infiltration	Compatible with AIH	Budesonide	Improved
Ghielmetti *et al*[95]	63/M	Type 2 diabetes, ischemic heart disease	mRNA-1273	7 d after the 1st dose	1127/1038	11.9	Increased	ANA: 1:640	Inflammatory portal infiltrate with interface hepatitis	Typical for AIH	Prednisone (40 mg/d)	Improved
Zhou *et al*[96]	36/F	Ulcerative colitis, primary sclerosing cholangitis	mRNA-1273	11 d after the 1st dose	581/588	1.4	Increased	ANA: 1:2560	Interface hepatitis with a lymphoplasmacytic infiltrate, rosette, eosinophil	Compatible with AIH	Prednisone (50 mg/d)	Improved
Garrido *et al*[97]	65/F	JAK2 V617F-positive polycythemia	mRNA-1273	14 d after the 1st dose	1056/1092	1.1	Increased	ANA: 1:100	Interface hepatitis	Compatible with AIH	Prednisolone (60 mg/d)	Improved
Goulas *et al*[98]	52/F	None	mRNA-1273	14 d after the 1st dose	350/936	9.06	Increased	ANA: 1:320, ASMA: positive	Portal, periportal inflammation, rosette formation	Typical for AIH	Prednisolone (50 mg/d) + azathioprine	Improved
Rela *et al*[99]	38/F	Hypothyroidism	AZD1222	20 d after the 1st dose	1101/1025	14.9	Increased	ANA: positive	Multiacnar hepatic necrosis and periportal neocholangiolar proliferation	Compatible with AIH	Prednisolone (30 mg/d)	Improved
Rela *et al*[99]	62/M	None	AZD1222	13 d after the 2nd dose	1361/1094	19.2	NA	ANA: negative	Portal neocholangiolar proliferation and mild to moderate	Compatible with AIH	Prednisolone (30 mg/d) + plasma	Death

								inflammation		exchange	
Palla *et al*[100]	40/F	Sarcoidosis	BNT162b2	28 d after the 2nd dose	4 times upper limit of normal	NA	Increased	ANA: 1:640	Interface hepatitis with plasma cells infiltration	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Mann *et al* [101]	61/F	Irritable bowel disease, cholecystectomy	BNT162b2	9 d after the 2nd dose	37/37	6.2	NA	ANA: negative	Scattered inflammatory cells consisting of lymphocyte and few eosinophils	Drug induced liver injury	Conservative treatment	Improved
Avci *et al*[102]	61/F	Hashimoto's disease, hypertension	BNT162b2	28 d after the 1st dose	455/913	11.8	Increased	ANA: 1:100, ASMA: 1:100	Interface hepatitis	Compatible with AIH	Prednisolone (40 mg/d) + azathioprine	Improved
Torrente *et al* [103]	46/F	Hypothyroidism, anemia	AZD1222	21 d after the 1st dose	241/353	Normal	Increased	ANA: 1:160	Lymphoplasmacytic portal infiltrate	Compatible with AIH	Prednisone (30 mg/d) + azathioprine	Improved
Ghorbani *et al* [104]	62/M	None	Corona Vac	3 d after the 2nd dose	722/435	8	NA	ANA: negative, ASMA: negative	Interface hepatitis, infiltration of lymphocytes and eosinophils in portal tract	Compatible with AIH	Ursodeoxycholic acid	Improved
Kang *et al*[105]	27/F	None	BNT162b2	7 d after the 2nd dose	1004/1478	8.6	Increased	ANA: 1:80	Interface hepatitis with a lymphoplasmacytic infiltrate, rosette, eosinophil	Typical for AIH	Prednisolone (40 mg/d)	Improved
Camacho-Domínguez *et al*[106]	79/M	None	AZD1222	15 d after the 1st dose	2003/1994	11.9	Increased	ANA: 1:80	Interface hepatitis with a lymphocytic infiltrate, eosinophil	Compatible with AIH	Prednisone (30 mg/d) + azathioprine	Improved
Shahrani *et al* [107]	59/F	Dyslipidemia	AZD1222	12 d after the 2nd dose	962/1178	7.5	Increased	NA	Lympho-plasmacellular portal infiltrate	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Shahrani *et al* [107]	63/F	Ulcerative colitis, primary sclerosing cholangitis	AZD1222	14 d after the 1st dose	505/354	18.6	Increased	ANA: positive	Interface hepatitis	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Shahrani *et al* [107]	72/F	None	BNT162b2	10 d after boostershot	1452/2280	1.7	Increased	ANA: negative, AMA: positive	Infiltration of lymphocytes and plasma cells in portal tract	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Zin Tun *et al* [108]	47/M	None	mRNA-1273	3 d after the 1st dose; A few days after the 2nd dose	NA/1048	11.3	Increased	ANA: positive	Interface hepatitis with a lymphoplasmatic infiltrate, rosette, emperipolesis	Typical for AIH	Prednisolone (40 mg/d)	Improved
Suzuki *et al* [109]	80/F	Gastroesophageal reflux esophagitis	BNT162b2	10 d after the 2nd dose	995/974	3.5	Increased	ANA: 1:40	Lymphoplasmacytic infiltration in the portal area, interface hepatitis	Compatible with AIH	Prednisone (0.8 mg/kg/d)	Improved
Suzuki *et al* [109]	75/F	Dyslipidemia	BNT162b2	4 d after the 2nd dose	1085/820	17.7	Increased	ANA: 1:80	Lymphoplasmacytic infiltration in the portal area, interface hepatitis	Compatible with AIH	Prednisone (1.0 mg/kg/d)	Improved
Suzuki *et al* [109]	78/F	Primary biliary cholangitis	BNT162b2	7 d after the 1st dose	401/542	1.3	Increased	ANA: 1:80	Lymphoplasmacytic infiltration in the portal area, interface	Compatible with AIH	Prednisone (0.6 mg/kg/d)	Improved

	hepatitis

AIH: Autoimmune hepatitis; AMA: Anti-mitochondrial antibodies; ANA: Anti-nuclear antibodies; ASMA: Anti-smooth muscle antibodies; JAK: Janus kinase; NA: Not available; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; IgG: Immunoglobulin G.

its development. However, the association between vaccines and the development of autoimmune diseases is controversial and most studies related to this have been inconclusive[113].

Molecular mimicry and bystander activation have been hypothesized as possible mechanisms by which vaccines can trigger autoimmune reactions. In the antigen-specific mechanism of molecular mimicry, it is hypothesized that similarities between certain pathogenic elements contained in the vaccine and specific human proteins cause cross-reactions. It is believed that the injurious antibodies produced by this mechanism destroy human proteins and cause organ damage[114]. In support of this hypothesis, it has been reported that antibodies against the spike protein of SARS-CoV-2 have cross-reactivity against many human tissue antigens[115]. Although the target antigen of the autoimmune response in hepatocytes and specific autoantibodies in AIH have not yet been identified, Vojdani *et al* [115] reported that anti-SARS-CoV-2 protein antibodies cross-react with liver microsomal antigen, pyruvate dehydrogenase peptide E2, and mitochondrial M2 antigen[115]. It has also been shown that anti-SARS-CoV-2 spike protein antibodies cross-react with human tissue antigens, resulting in a marked increase in autoimmune markers such as ANA and AMA[116]. This suggests that COVID-19 vaccination might induce autoimmune reactions based on molecular mimicry in liver tissues, resulting in AIH-like liver injury (Figure 1). Bystander activation, on the other hand, is an antigen non-specific mechanism, in which self-antigens are released extracellularly by vaccination and are taken up by antigen-presenting cells. Then, autoreactive T cells are activated by type I interferon (IFN) and recognize the presented self-antigen, which is hypothesized to attack normal cells[117]. Bystander activation has also been proposed as one of the mechanisms of autoimmune disease development after vaccination. Furthermore, there is a rapid increase in type I IFN expression and oxidative stress coupled with effective anti-SARS-CoV-2 neutralizing antibody production after vaccination in healthy individuals[118]. Therefore, the side effects of COVID-19 vaccines are thought to be only a by-product of a transient burst of type I IFN generation with induction of an effective immune response[119]. It has also recently been hypothesized that the COVID-19 vaccine triggers autoimmune diseases *via* induction of age-associated B cells (ABCs) [120]. The number of ABCs, a rare subset of B cells that express CD11c and T-bet, increases with age in healthy individuals, and is increased early in patients with infectious diseases and autoimmune disorders[121]. In the pathogenesis of autoimmune diseases, ABCs are implicated in generating IgG, in increasing antigen presentation to T cells, and in germinal center formation. Moreover, ABCs are hyper-responsive to Toll-like receptor (TLR) 7 signaling, and are capable of generating autoreactive antibody-secreting plasmablasts. COVID-19 vaccines use TLR7/8 agonists as adjuvants, which might stimulate ABCs, leading to the triggering of post-vaccination autoimmune syndromes[122]. Activation of TLR7 can lead to the production of type I IFN, which is an important cytokine in the development of autoimmune disorders, such as rheumatic diseases and systemic lupus erythematosus[123]. It was previously shown in a mouse model that lipid nanoparticles, which are one of the potent adjuvants of mRNA vaccines, could trigger inflammatory responses. This is characterized by activation of diverse inflammatory pathways, massive neutrophil infiltration, and production of various inflammatory

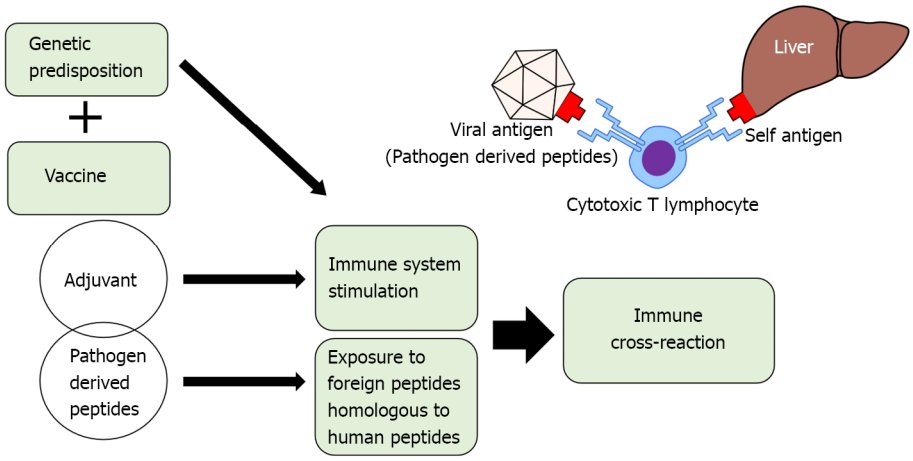

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Figure 1 Schema of the process leading to the development of immune cross-reaction after vaccination.

cytokines, including the secretion of interleukin (IL)-1β and IL-6[11,124].

Hence, although several hypotheses have been considered for the mechanism of vaccine-induced autoimmune disease, the exact mechanism remains to be elucidated. In any event, only a very small percentage of vaccinated subjects subsequently developed autoimmune phenomena, suggesting a genetic predisposition to vaccine-induced autoimmune disorders. Further research into the direct associations between vaccines and autoimmune diseases, as well as the biological mechanisms behind them, is warranted.

RECOMMENDATION

Since COVID-19 is an infectious disease with a high burden of morbidity and mortality, and that has resulted in a global pandemic, vaccination against COVID-19 is our best strategy for its control. For this, highly effective and safe vaccines are desperately needed. Although various COVID-19 vaccines have been proven to be highly effective and have good safety profiles in healthy populations, data regarding the efficacy and safety of vaccination in special population groups is limited. Thus, we believe it is worthwhile to summarize the efficacy and safety of the COVID-19 vaccines in patients with CLD. Based on the evidence from real-world studies, this review shows that vaccination in patients with CLD is effective and safe, and should be strongly recommended.

As shown in Table 3, although a number of case reports of acute liver injury after COVID-19 vaccination have been reported, their frequency is extremely low. Thus, given the serious health sequalae from COVID-19 in patients with liver disease, the potential benefits of vaccination appear to outweigh the risk of vaccine-related liver injury. However, it is important to remember that most of the studies referred to in this review were conducted in the era before the emergence of new viral variants. Since new SARS-CoV-2 variants are still emerging all over the world, COVID-19 remains a global public health problem. In addition, since vaccines against the mutant viruses are still being developed, it will be necessary to continue evaluating the efficacy and safety of these new vaccines.

CONCLUSION

Given the increased rates of severity and mortality of COVID-19 in patients with CLD, especially those with cirrhosis, the importance of aggressive vaccination in the effective management of SARS-CoV-2 infection should be emphasized. However, there is insufficient evidence about the immunogenicity and safety of COVID-19 vaccines in patients with CLD. According to the accumulated real-world data on each vaccine, the safety of COVID-19 vaccines in patients with CLD appears to be comparable to that in healthy individuals. Regarding efficacy, the disease behavior of COVID-19 is known to be attenuated in vaccinated compared with unvaccinated patients, including in those with CLD+ADs- however, vaccine-induced immunity seems lower in CLD patients as compared with the general population. Since a rapid decrease in acquired antibodies has also been observed in this patient population, an effective booster shot is desirable, particularly in patients with cirrhosis, LT recipients, and those with HCC.

On the other hand, acute liver injury following COVID-19 vaccination has also been frequently reported. However, recent large cohort studies found no increased risk of liver injury after COVID-19 vaccines. Since acute liver injury after SARS-CoV-2 infection is much more common than after COVID-

19 vaccination, the benefits of vaccination might outweigh the risk of liver injury during this pandemic. The reported rare immune-mediated liver injury after COVID-19 vaccination is clinically and pathologically similar to AIH. Although the involvement of abnormalities in the immune system, including molecular mimicry, bystander activation, and induction of ABCs in the pathogenesis of this condition have been pointed out, the relationship between vaccination and acute liver injury is an issue that remains to be clarified in the future. Finally, clinicians should consider the possibility of AIH-like liver injury in patients who present with elevated liver enzymes following COVID-19 vaccination, and treat it with corticosteroids.

FOOTNOTES

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Abstract

The global coronavirus disease 2019 (COVID-19) has become one of the biggest threats to the world since 2019. The respiratory and gastrointestinal tracts are the main targets for severe acute respiratory syndrome coronavirus 2 infection for they highly express angiotensin-converting enzyme-2 and transmembrane protease serine 2. In patients suffering from COVID-19, gastrointestinal symptoms have ranged from 12% to 61%. Anorexia, nausea and/or vomiting, diarrhea, and abdominal pain are considered to be the main gastrointestinal symptoms of COVID-19. It has been reported that the direct damage of intestinal mucosal epithelial cells, malnutrition, and intestinal flora disorders are involved in COVID-19. However, the underlying mechanisms remain unclear. Thus, in this study, we reviewed and discussed the correlated mechanisms that cause

gastrointestinal symptoms in order to help to develop the treatment strategy and build an appropriate guideline for medical workers.

Key Words: COVID-19; Angiotensin-converting enzyme-2; Transmembrane protease serine 2; Gastrointestinal symptom; Mechanism; Intestinal barrier permeability

Core Tip: Gastrointestinal symptoms in coronavirus disease 2019 patients have ranged from 12% to 61%, which include anorexia, nausea and/or vomiting, diarrhea, abdominal pain, and so on. However, the underlying mechanisms remain unclear. This study reviewed and discussed the correlated mechanisms that cause gastrointestinal symptoms in order to help to develop the treatment strategy and build an appropriate guideline for medical workers.

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INTRODUCTION

Since 2019, coronavirus disease 2019 (COVID-19) has become one of the world's most serious threats. In the past, much attention has been given to the respiratory symptoms of patients, but the occurrence of extrapulmonary symptoms has been ignored. The occurrence of gastrointestinal symptoms has ranged from 12% to 61% in patients suffering from COVID-19[1-5], which may result in a longer duration of illness but not increased mortality[2,3]. In a recent meta-analysis from China, the main gastrointestinal symptoms in COVID-19 patients were reported to include anorexia (21%), nausea and/or vomiting (7%), diarrhea (9%), and abdominal pain (3%)[2]. Moreover, gastrointestinal bleeding was rarely observed[6]. A study from the United States reported a higher prevalence of gastrointestinal symptoms (anorexia, 34.8%; diarrhea, 33.7%; and nausea, 26.4%)[4] (Figure 1). Thus, diarrhea, nausea and/or vomiting, abdominal pain, and anorexia are considered to be the main gastrointestinal symptoms.

Currently, after struggling with the Omicron variant, China has found that a large number of COVID-19 patients, especially elderly patients in critical condition, are more likely to suffer from gastrointestinal dysfunction. Over 85% of patients showed symptoms such as intestinal barrier dysfunction, digestive and absorption dysfunction, or gastrointestinal motility dysfunction due to the direct damage the virus caused to the intestinal mucosal epithelial cells. In addition, malnutrition and intestinal flora disorders occurred next. Imaging studies show that COVID-19 patients with gastrointestinal symptoms presented thickening of the bowel wall, sometimes with hyperemia and thickening of the mesentery, and large bowel fluid[7]. However, the underlying mechanisms remain unclear. Thus, in this study, we reviewed and discussed the correlated mechanisms of gastrointestinal symptoms and damage in order to help build an appropriate guideline for medical workers.

PATHOPHYSIOLOGY

The pathophysiology of gastrointestinal damage in COVID-19 is probably multifactorial. The most important factor is due to the direct infection of the virus. High titers of viral RNA from COVID-19 have been isolated from fecal samples[8,9]. Live viral shedding of infectious virions in fecal matter has been reported even after the resolution of symptoms, which may be a potential source of transmission[10]. Angiotensin-converting enzyme-2 (ACE2), as the entry receptor for the causative coronavirus of COVID-19, is expressed in multiple extrapulmonary tissues, including gastrointestinal tissue[11]. A study based on single-cell sequencing also showed that ACE2 and transmembrane protease serine 2 (TMPRS2) are expressed in cholangiocytes, colonocytes, esophageal keratinocytes, gastrointestinal epithelial cells, and so on[12-14]. The expression profile of ACE2 in the digestive system is shown in Figure 2. Histopathological studies also indicated that gastrointestinal tissue is the target organ of COVID-19[15]. This finding indicates that direct viral-induced tissue damage is a plausible mechanism of COVID-19 injury. Here, however, we hold the idea that the expression of ACE2 is related to the virus entering the body, but the expression level of ACE2 does not appear to be directly proportional to the severity of the disease[16].

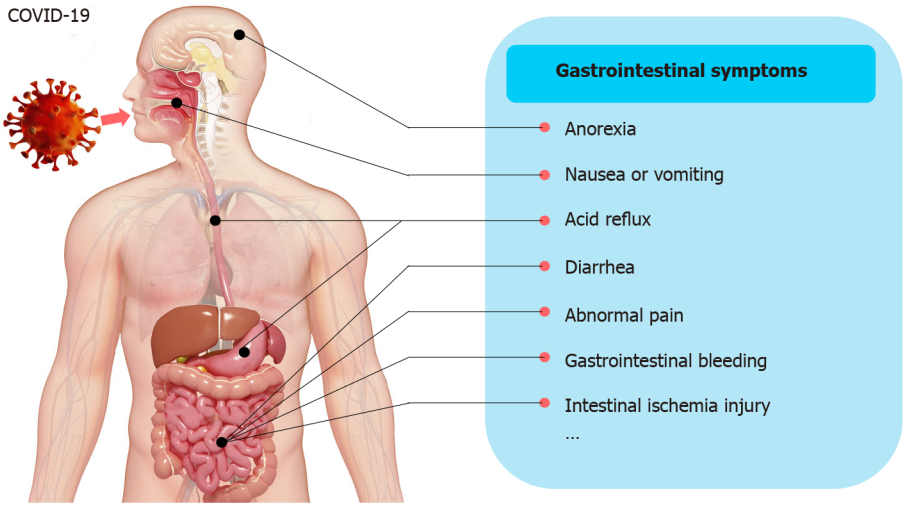

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Figure 1 Gastrointestinal symptoms involved in coronavirus disease 2019 infection. COVID-19: Coronavirus disease 2019.

In addition, responses following ACE2 activation are closely related to gastrointestinal side effects. ACE2 is also a key enzyme in the renin-angiotensin system (RAS)[17]. RAS dysregulation may exacerbate ion imbalance and inflammation, potentially affecting cellular metabolic status, microbial composition, and cell viability, leading to progressive bowel function and diarrhea[18]. Histopathological evidence also shows diffuse endothelial inflammation and mesenteric ischemia in the submucosal vessels of the small intestine in patients with COVID-19[19]. Furthermore, infiltrating plasma cells, lymphocytes, and interstitial edema have been found in the lamina propria of COVID-19 patients' stomachs, duodenums and rectums. Virus-induced cytokine storms, as well as inflammatory responses, may also contribute to enhanced permeability of the mucosal barrier, damaged enteric nervous system, altered intestinal flora[20], and gut-brain axis communication disorders and then form a vicious circle (Figure 3). The syndrome-correlated underlying mechanism is discussed in the following sections.

METHODOLOGY

A literature review was conducted using a keyword search in PubMed from 2019 to 2022. Keyword search items included "COVID-19", "severe acute respiratory syndrome coronavirus 2", "gastrointestinal disorders", "nausea", "vomiting", "diarrhea", "ACE2", "abdominal pain", "anorexia", and "combinations thereof". The inclusion criteria included articles with randomized or blinded studies, case-control studies, descriptive research, and studies with objective outcomes. Exclusion criteria included articles of primary opinion papers with no reference to available data and industry-sponsored publications.

GASTROINTESTINAL SYMPTOMS AND THE UNDERLYING MECHANISMS

Nausea and/or vomiting

Nausea and/or vomiting is an early alerting symptom of a challenge (toxic food and chemicals, bacterial toxin, and virus) to the upper digestive tract, which can also be the early presenting symptom in COVID-19 patients[21]. The reported incidences of vomiting and nausea were 1.0%-12.5% and 1.0%-27.5%, respectively[4,22]. Some of the patients may eject aerosolized, virally contaminated vomit, which also occurs in patients infected with norovirus[23]. Importantly, nausea and vomiting can be the early presenting symptoms of COVID-19[21].

The virus enters the digestive tract with the air during swallowing and binds to ACE2 receptors, which are highly expressed in the airways and digestive tract. COVID-19 could increase the release of neuroactive agents from enteroendocrine cells and inflammatory mediations, which act by stimulating/sensitizing abdominal vagal afferent terminals and/or act on the area postrema in the dorsal medulla where the blood-brain and blood-cerebrospinal fluid barriers are relatively permeable [21]. The consequences of vagal afferent and area postrema activation induce nausea and vomiting by the projection of information to higher brain regions (nausea and anorexia) and vomiting by motor pathways in the ventral brainstem and spinal cord[21]. Some researchers also indicated that the

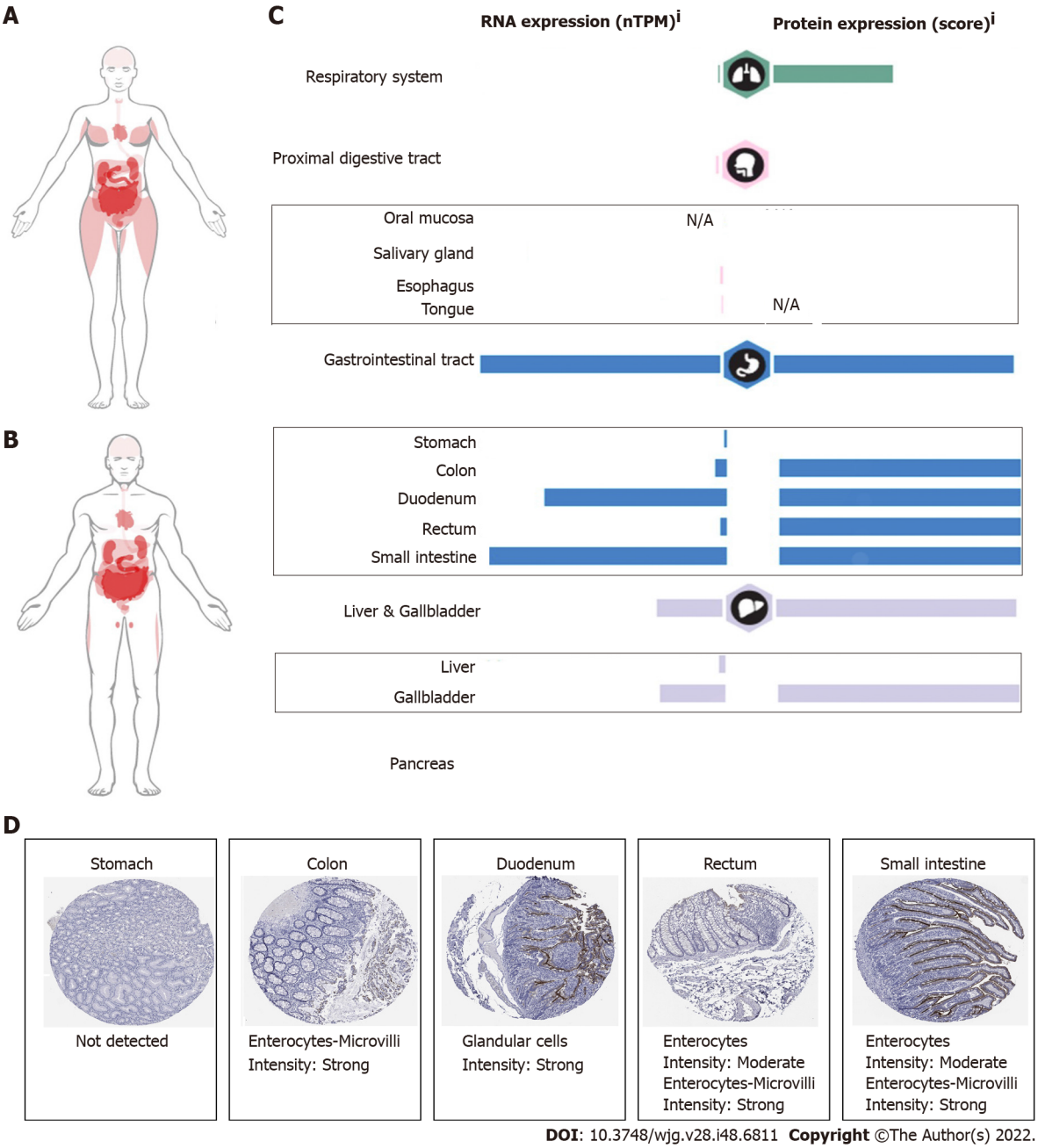

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Figure 2 Angiotensin-converting enzyme 2 expression in human gastrointestinal tissues. A: Angiotensin-converting enzyme 2 (ACE) expression in females; B: ACE expression in males, darker colors indicate higher expression levels; C: ACE2 mRNA and protein expression in the respiratory system and digestive system; D: ACE2 positive cell types in the stomach, colon, duodenum, rectum, and small intestine. These data are summarized from the human protein atlas https://www.proteinatlas.org/. N/A: Not applicable.

interaction between transient receptor potential (TRP) channels and food intake might be associated with anorexia due to COVID-19[24,25]. Some TRP channels are broadly expressed in the gastrointestinal tract and play important roles in noxious irritants[26]. TRPV1 expression in esophageal sensory neurons, stomach-labeled vagal nodose neurons and colon-labeled afferent neurons has been well described[27-29]. A TRPV1 agonist, capsaicin, can evoke nausea[30]. Moreover, TRPV1 and TRPA1 are co-expressed in the esophagus, stomach, intestine, and colon[31-33]. TRPV1 can participate in appetite regulation by affecting hormones and gastrointestinal vagal afferent nerves[25]. *In vitro* activation of TRPA1 by allyl isothiocyanates can increase serotonin release, leading to the stimulation of vomiting [34]. These results suggest that TRP channel activation is involved in COVID-19-induced nausea and vomiting. However, whether inhibiting TRP channels can alleviate the gastrointestinal symptoms caused by COVID-19 needs further investigation. The underlying mechanisms are summarized in Figure 4.

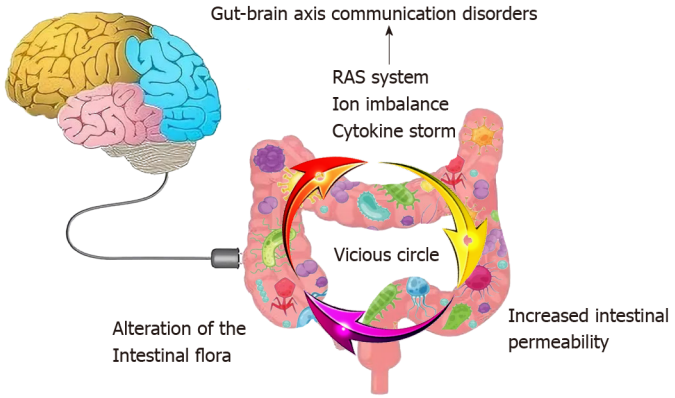

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Figure 3 Main mechanisms involved in coronavirus disease 2019-induced gastrointestinal syndromes. Gut infection of coronavirus disease 2019 (COVID-19) results in cytokine storm, increased intestinal permeability and alteration of the intestinal flora, and forming a vicious circle, extending the recovery time. Moreover, COVID-19 binding to gastrointestinal angiotensin-converting enzyme 2 also leads to ion imbalance and activation of renin-angiotensin system. Abnormal enteric neurotransmitters may further lead to gut-brain axis communication disorders. RAS: Renin-angiotensin system.

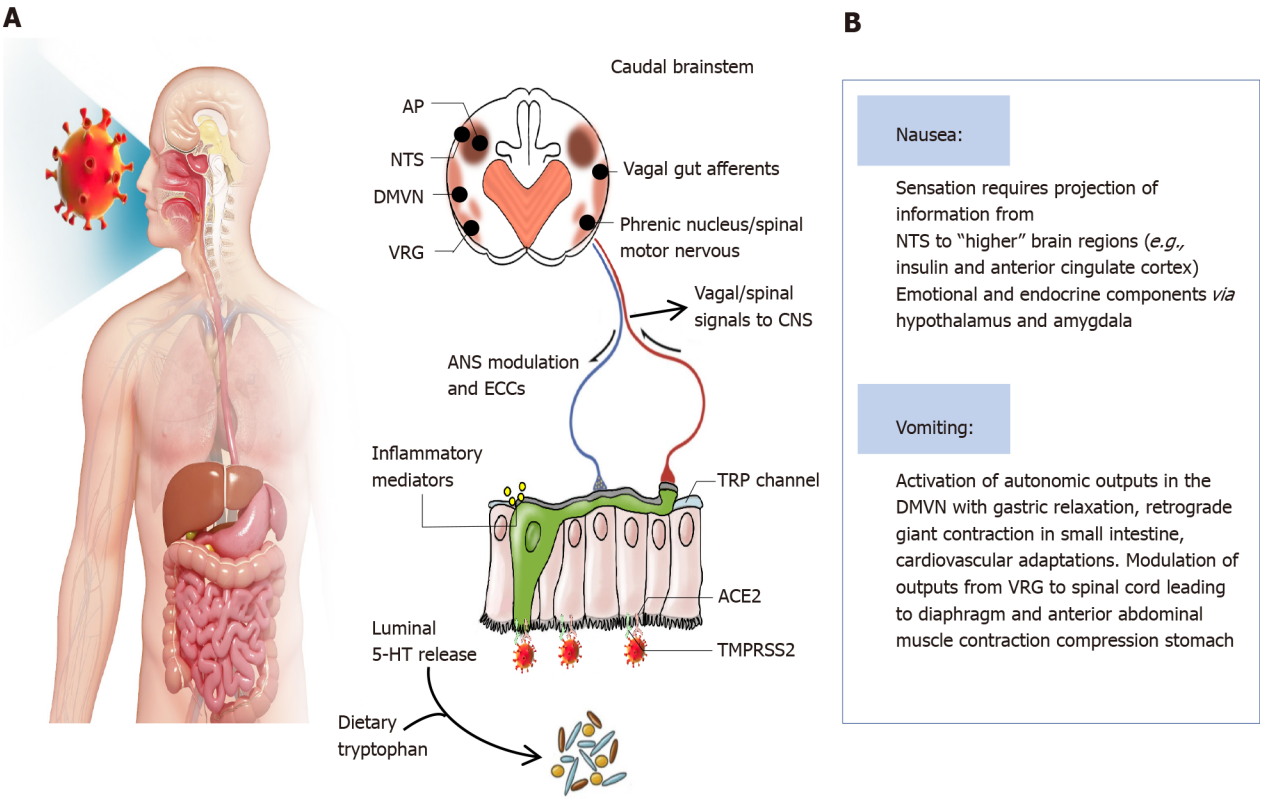

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Figure 4 Potential mechanisms for coronavirus disease 2019-induced nausea and vomiting. A: The virus enters the body through the airways and digestive tract during swallowing and the potential mechanisms for coronavirus disease 2019 to induce nausea and vomiting (based on the evidence discussed in the text). The virus interacts with digestive tract epithelium leading to the release of neuroactive agents from enteroendocrine cells and inflammatory mediations, and these can act by stimulating or sensitizing abdominal vagal afferent terminals; B: The consequences of vagal afferent and area postrema activation induce nausea and vomiting by projection of information to nausea and anorexia-related regions, and vomiting by motor pathways in the ventral brainstem and spinal cord. 5-HT: 5-hydroxytryptamine; ACE2: Angiotensin-converting enzyme-2; AP: Area postrema; DMVN: Dorsal motor vagal nucleus; NTS: Nucleus tractus solitarius; TMPRSS2: Transmembrane protease serine 2; VRG: Ventral respiratory group of neurons; TRP: Transient receptor potential; ANS: Autonomic nervous system; ECCs: Enteroendocrine cells.

Diarrhea

Diarrhea is a frequent presenting symptom in patients suffering from COVID-19. In the clinical case analysis, the incidence of diarrhea is between 2% and 50%[35]. It may precede or trail respiratory symptoms. Although the specific mechanisms involved in COVID-19-related diarrhea are not entirely known, we hold the idea that the targeting of intestinal ACE2 by the virus, virus infection-induced

cytokine storms, increased intestinal barrier permeability, and even changes in microbiome are all considered to be the main causes of gastrointestinal symptoms. Moreover, hepatic and pancreatic injuries may also cause diarrhea. Antibiotic-induced iatrogenic diarrhea caused by the activation of *Clostridium spp.* should also be taken into consideration.

The direct effect of binding ACE2: ACE2 mRNA appears to increase with age and to display higher levels in patients taking ACE inhibitors. This may be one of the causes of gastrointestinal symptoms such as diarrhea being more common in elderly patients or those with hypertension. ACE2 controls the production of antimicrobial peptides and participates in the uptake of dietary amino acids[36], which promote the homeostasis of the gut flora. Additionally, ACE2 expression is positively correlated with the severity of colitis, suggesting that virus activity may lead to changes in the way certain enzymes function, making people more susceptible to developing diarrhea and intestinal inflammation[37].

Altered serotonin metabolism: Altered serotonin metabolism has been found in COVID-19 patients [38]. Serotonin, known as the mood neurotransmitter, is important in certain bodily processes, such as sleep, libido, and body temperature. Studies have reported that enterohemorrhagic *Escherichia coli* O157 can significantly reduce the expression of a group of genes that cause infection after exposure to serotonin[39,40]. In addition, a study in mice showed that increased serotonin levels in the gastrointestinal tract could reduce the ability of murine *Citrobacter* to infect the host and cause disease. Intervention with fluoxetine, a selective serotonin reuptake inhibitor, produced similar results[40]. Activation of intestinal serotonin receptors may also lead to diarrhea by modulating the enteric nervous system and intestinal motility[41,42]. The authors believe that COVID-19-related diarrhea is correlated with increased serotonin levels and that elevated serotonin levels may be a protective regulatory mechanism that accelerates the excretion of enteroviruses.

Changes in fecal calprotectin: Increased levels of fecal calprotectin expression have also been detected in COVID-19 patients[43,44]. Fecal calprotectin is a reliable fecal marker for the detection of inflammatory bowel disease and infectious colitis[45]. The calprotectin value in stool is elevated in patients with acute or bloody diarrhea[46]. Calprotectin is a calcium-containing protein derived from neutrophils and macrophages that serves as a marker of inflammatory cell activation[47]. Therefore, diarrhea and increased fecal calprotectin levels could be related to the immune activation and inflammatory responses caused by COVID-19.

Cytokine storms and their induced inflammatory cascade: Cytokine release syndrome caused by a dysregulated immune response is also one of the important factors causing multiple organ dysfunction, especially diarrhea, in patients with COVID-19. Severe COVID-19 manifests as acute respiratory distress syndrome (ARDS) with elevated plasma proinflammatory cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor α (TNF-α), C-X-C motif chemokine ligand 10, macrophage inflammatory protein 1 α, and chemokine (C-C motif) ligand 2, with low levels of interferon type I (IFN-I) in the early stage and elevated levels of IFN-I during the advanced stage of COVID-19[48]. Changes in these proinflammatory cytokines can also be found in clinical and experimental colitis[49,50], accompanied by increased intestinal permeability *via* activation of the inflammatory-related cascade. High levels of circulating cytokines and mediators of the toxic response, including IL-6, TNF-α, nitric oxide, and activity modulation of the calcium channel, have been described[51]. Toll-like receptors (TLRs) are important sensors that interact with COVID-19. Previous studies indicated that COVID-19 interacts with TLRs in the host cell membrane and increases gene 88 of the primary response to myeloid differentiation (MyD88), following active nuclear transcription factor-κB (NF-κB), promoting an inflammatory cascade [52], which in turn aggravates the inflammatory response and increases intestinal permeability. Additionally, severe COVID-19 individuals have been found to have significant levels of indicators for tight junction permeability as well as the translocation of bacterial and fungal products into the blood [53,54]. Thus, virus infection-induced cytokine storms and their induced inflammatory response may be other factors that cause diarrhea.

Increased intestinal barrier permeability and microbiome change: Accumulating evidence shows that the intestinal microbiome is broadly altered in COVID-19 patients, which may be followed by increased intestinal permeability. The incidence of sepsis and ARDS, two high-mortality risks in COVID-19, may be minimized by the intestinal microbiota[55]. Intestinal ACE2 functions as a chaperone for the amino acid transporter B0AT1. The B0AT1/ACE2 complex within the intestinal epithelium acts as a regulator of gut microbiota composition and function[56], which can also be considered a marker of inflammation and disease severity[57]. Changes in ACE2 by COVID-19 can impair intestinal uptake of certain dietary amino acids, such as tryptophan, which is involved in enteritis[58-60]. ACE2 knockout mice also exhibit microbiome dysbiosis[61-63]. Through shotgun sequencing of total DNA extracted from stool, researchers found that the gut microbial ecological network was markedly weakened and became sparse, which combined with a decrease in gut microbiome diversity[64], could reflect the disease's severity[65]. The infection causes intestinal microbiome disturbance and reduction, which may activate immune cells and provoke the release of inflammatory cytokines that increase systemic inflammation [66,67]. In addition, probiotics may help enhance the host immune system, improve the gut microbiome

and gut barrier function, and reduce COVID-19-related diarrhea[68].

More significantly, B0AT1 substrates such as tryptophan and glutamine operate as signals to reduce lymphoid proinflammatory cytokines, activate antimicrobial release peptides, and control mucosal autophagy as a defensive mechanism[69]. Downregulated intestinal ACE2-B0AT1 on the cellular surface leads to a series of downstream sequelae to promote leaky gut and dysbiosis of gut flora[69]. Gut microbiomes also play important roles in gut inflammatory regulation. Butyric acid from gut flora was reported to inhibit cytokine storms[27]. This indicates the disrupted composition of intestinal microbiota and impaired gut permeability, followed by the creation of a destructive cycle. In summary, we propose the important role of intestinal microbiota in preventing and decreasing COVID-19 complications. The underlying mechanism involved in COVID-19-correlated diarrhea is summarized in Figure 5.

Abdominal pain

Abdominal pain is uncommon and extremely rare in patients with COVID-19. Clinical data indicate that abdominal pain is more common in intensive care unit (ICU) patients than in non-ICU patients[41]. The aggregative presence rate of RNA from COVID-19 in stool samples from COVID-19 patients was 54% [2]. In previous clinical research based on COVID-19 patients who underwent emergency abdominal surgery due to different conditions, the peritoneal samples from 5 patients were sufficient for reverse transcription polymerase chain reaction analysis, and no intraperitoneal viral RNA was observed in these 5 patients[70]. Although the number of cases is rare, we can speculate that abdominal pain is linked to gastrointestinal but not intraperitoneal viral infection. The possible mechanisms are summarized as follows.

Immune and inflammatory regulation: The most common cause of pain is the inflammation-induced release of many cytokines and chemokines. Cytokine storms are a potentially fatal immune disease characterized by high levels of activation of immune cells and excessive production of a large number of inflammatory cytokines and chemical mediators, and they have been reported to be associated with the exacerbation of a number of infectious diseases, including severe acute respiratory syndrome[71] and Middle East respiratory syndrome. They are also considered to be the main cause of disease severity and death in patients with COVID-19[72,73]. T cells play an important role in antiviral immunity. A rise in T cell activation and differentiation was found in early COVID-19-infected patients, resulting in immune rebalancing between IFN and NF-κB activity and restoration of cell homeostasis. Two major intracellular transduction antigen-activating signals, the phosphatidylinositol pathway and the MAP kinase-related pathway, are activated. However, the number of T cells is significantly decreased with increasing infective time in COVID-19 patients, accompanied by an increase in the T cell exhaustion marker programmed death 1[74]. The T cell count is negatively correlated with serum cytokine levels in patients with COVID-19[74]. Despite the lack of direct evidence for a relationship between T cell status and abdominal pain in COVID-19 patients, we hypothesized that abdominal pain is related to T cells. Moreover, COVID-19 can rapidly activate pathogenic Th1 cells to secrete proinflammatory cytokines, such as granulocyte-macrophage colony-stimulating factor and IL-6. Increased cytokines, chemokines, and other compounds can simultaneously cause a secondary pain response by activating pain-sensing neurons[75]. The COVID-19 cytokine storm is characterized by high expression of IL-6 and TNF-α[76]. Elevated IL-6 levels also increase mortality. Therefore, we hypothesized that the abdominal pain of COVID-19 patients may be associated with the high expression of IL-6 and TNF-α.

Eosinophils are circulating and tissue-resident white blood cells that have a powerful proinflammatory effect in many diseases. Recently, eosinophils have been shown to have a variety of other functions, including immunomodulatory and antiviral activity. Peripheral eosinophilia is hypothesized to play a protective role in COVID-19 patients[77]. In the United States, a review of administrative data compared the hospitalization rates, ventilator dependence, and death of patients with and without eosinophilic gastrointestinal disorders from an extensive central medical system. The results indicated that eosinophilic gastrointestinal disorder might provide a protective immune response[78]. The mechanism may be related to the upregulation of IL-13 in eosinophilic gastrointestinal disorder and the decreased expression of ACE2 and TMPRS2 on epithelial cells of eosinophilic gastrointestinal disorder patients. In addition, eosinophil-derived neurotoxins may exert direct antiviral effects[79]. Moreover, eosinophilia-related disorders such as eosinophilic gastroenteritis[80] and eosinophilic esophagitis[81] are often accompanied by symptoms of abdominal pain and vomiting, suggesting that abdominal pain in patients with COVID-19 may also be caused by increased eosinophils.

Regulation of the enteric and central nervous systems: Intestinal pain perception can be coregulated by the central and enteric nervous systems. A balance between neuronal excitatory and inhibitory signaling pathways maintains the physiological pain threshold in the intestine. Altered neurotransmitter levels may be closely linked to abdominal pain. A variety of receptors and their endogenous ligands are involved in pain signaling, including receptors responsible for nociceptive sensations [*e.g.*, 5-hydroxytryptamine (5-HT) receptors, TRP channels, IL receptors] and antinociceptive sensations (*e.g.*, opioid and cannabinoid receptors). In COVID-19 patients, intestinal inflammatory infiltration increases intestinal mucosal permeability, and the direct effect of viruses can aggravate dysbiosis and cause changes in tryptophan metabolism. Studies have shown that tryptophan is a precursor of 5-HT. 5-HT plays an

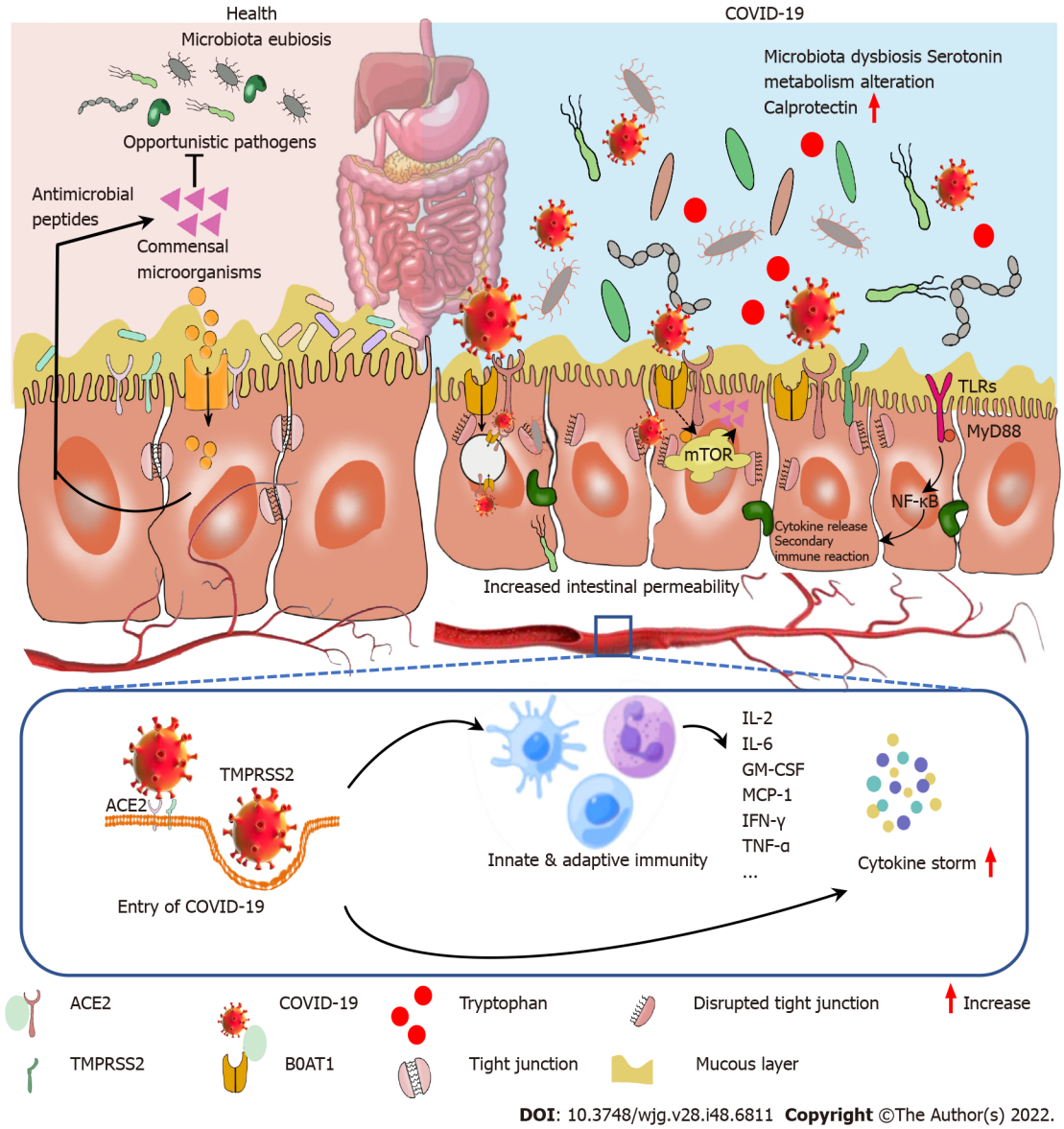

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Figure 5 Potential mechanism of cytokine storm and secondary pathogen infection resulting in diarrhea in patients with coronavirus disease 2019. After coronavirus disease 2019 (COVID-19) virus entry into the body, innate and adaptive immunity have been activated, followed by a cytokine storm. Gut microbiota is also disrupted by COVID-19 infection which potentially triggers cytokine storm and secondary pathogen infections. B0AT known as an angiotensin-converting enzyme-2 chaperone, mediates neutral amino acid uptake by luminal epithelial cells. B0AT substrates (tryptophan and glutamine) activate antimicrobial peptide release and promote tight junction formation, inhibit cytokine release and promote mucosal cell autophagy *via* mechanistic target of rapamycin signaling pathway. COVID-19 infection blocked this pathway, promoting opportunistic pathogen invasion, cytokine storm, activating toll-like receptors/nuclear factor-κB pathway and aggravated COVID-19. COVID-19: Coronavirus disease 2019; ACE2: Angiotensin-converting enzyme-2; TMPRSS2: Transmembrane protease serine 2; IL: Interleukin; GM-CSF: Granulocyte-macrophage colony-stimulating factor; MCP-1: Monocyte chemoattractant protein-1; IFN-γ: Interferon-γ; TNF: Tumor necrosis factor; mTOR: Mechanistic target of rapamycin; TLRs: Toll-like receptors; NF-κB: Nuclear factor-κB.

important role in gastrointestinal, nervous, and liver diseases. 5-HT acts on 5-HT receptors to initiate peristaltic and secretory reflexes in the viscera[82,83]. Research also indicated that 5-HT might be the key to exacerbating inflammatory bowel disease symptoms, including diarrhea and abdominal pain [84]. Intraperitoneal injection of 5-HT can significantly increase the expression of IL-1β and IL-6 and the activity of myeloperoxidase by activating 5-HT_3 and 5-HT_4 receptors in the colonic mucosa of mice with colitis and block the signal of pain relief[85]. Further studies have reported that elevated 5-HT can increase the expression of IL-6 and IL-8 and the production of monocyte chemoattractant protein-1, thereby leading to the initial event of intestinal inflammation[86]. However, the plasma 5-HT level is increased in hospitalized patients with COVID-19. The change in 5-HT in patients with COVID-19 may be an important cause of abdominal pain[87]. Therefore, we speculate that the abdominal pain in COVID-19 may be related to 5-HT. Moreover, regulating the level of 5-HT may be a therapeutic modality for the treatment of patients with abdominal pain due to COVID-19.

Ion channel: Pain signals are detected in response to harmful stimuli and release nerve impulses that encode pain. Many of these nociceptive neurons are equipped with a large number of specific ion channels that act as nociceptors. Stretching, inflammation, ischemia, pH, bacterial products, immune mediators, and neurotransmitters have all been implicated in visceral pain[88]. Studies have reported multiple electrolyte abnormalities in patients with COVID-19 infection[89]. COVID-19 infection is associated with decreased serum concentrations of sodium, potassium, magnesium, and calcium. Thus, we speculate that ion channels may play important roles in COVID-19-induced abdominal pain. As TRP channels are widely expressed in COVID-19-infected tissues, TRP channels and TRPML2 are also involved in the fusion of viral envelopes with endolysosomal membranes[90,91]. Thus, TRP channels may be valuable targets for disrupting the COVID-19 life cycle. A report indicated that TRP channels were involved in abdominal pain caused by COVID-19. TRPV1 and TRPA1 induce inflammation, increase sensory or vagal secretions, and cause pain[92]. It has also been suggested that afferent neuronal TRPV1 desensitization (*via* RTX) can reduce pain-related complications in COVID-19 patients [93]. Because TRP channels are widely expressed in the gastrointestinal tract, we speculate that the abdominal pain caused by COVID-19 is related to TRP channel activation.

Anorexia

Social pressure: Anorexia nervosa is an eating disorder characterized by restrictive eating and an intense fear of gaining weight. A study in 2020 evaluated the early effects of COVID-19 on patients with eating disorders and reported an increase in anxiety and alarming eating behaviors during the pandemic. This report shows that 69% of individuals had anorexia nervosa and experienced worries about their dietary schedules, while subjects with bulimia nervosa or binge eating disorders reported more episodes of binging[94]. This may be due to new living conditions, social distancing, self-isolation, changes in food access, daily habits, and so on; in addition, more difficult access to health care practitioners is also an essential factor leading to an increased incidence of anorexia[95].

Neuromodulation: Researchers have reported that neuroendocrine pathways are disrupted by miscommunication between brain-gut-adipose tissue in patients suffering from COVID-19[96]. Studies have shown that dopamine neurons in the ventral tegmental area of the midbrain and serotonin neurons in the dorsal raphe nucleus are involved in the regulation of motivational behaviors, including feeding[22,97], and increased serotonin levels have been observed in COVID-19 patients[38]. However, there is no direct evidence of whether it is related to anorexia nervosa. Changes in the microbiota-gut-brain axis from COVID-19 as well as gender differences may also be responsible for anorexia nervosa[98]. Moreover, changes in brain serotonin and tryptophan concentrations have been reported to be critical mechanisms in the regulation of eating behavior both in mice and humans[99]. Coincidentally, post-COVID-19 infection was also accompanied by changes in serotonin and tryptophan levels. We speculate that COVID-19-induced anorexia is at least partly correlated with increased serotonin and tryptophan levels. In addition, COVID-19 infection of nonneuronal cells can lead to anosmia and related odor perception impairment[100], which may be associated with the development and aggravation of anorexia[101,102].

Acid reflux

In a retrospective study of poor prognosis of gastrointestinal symptoms in patients with COVID-19, 12 (n = 1077, 1.1%) patients developed acid reflux[103], and the incidence of acid reflux was relatively low compared with other gastrointestinal symptoms. Generally, the main causes of gastric acid reflux include: (1) Relaxation of the lower esophageal sphincter; (2) Gastric and duodenal dysfunction leading to obstruction of gastric emptying; and (3) Esophageal mucosal barrier damage. In a clinical study, the prevalence of acid reflux was associated with poorer clinical outcomes in COVID-19, and the mechanism was related to damage to the upper esophageal sphincter[104]. We believe that increased serotonin levels and mucosal barrier damage caused by cytokine storms may also be risk factors for acid reflux. Antacid therapy is generally used, but some studies have noted that the use of proton pump inhibitors may increase the risk of achlorhydria-related intestinal infections in patients with COVID-19 [105], while histamine H_2 receptor antagonists do not increase this risk. Some studies have suggested that high-dose famotidine may be clinically beneficial to COVID-19 patients[106]. Therefore, the clinical use of histamine receptor antagonists may be more beneficial; however, due to low incidence and insufficient samples, there has been no systematic clinical evaluation of COVID-19 patients accompanied by acid reflux.

Gastrointestinal bleeding

A case-control study noted that the main causes of gastrointestinal bleeding in COVID-19 patients were peptic and rectal ulcers. Among the many potential predictors of upper gastrointestinal bleeding, a history of upper gastrointestinal bleeding was the only significant risk factor[107,108]. Gastrointestinal bleeding may be the direct impact of COVID-19 on gastrointestinal mucosa integrity. However, in a case of a COVID-19 patient who showed vomiting coffee crumbs and esophageal mucosal injury by esophagogastroscopy and duodenoscopy, there was no mucosal injury[107]. This may exist in other

mechanisms that have not been elucidated. Compared with patients in the initial presentation, most bleeding occurred during hospitalization. The results suggested that bleeding may be one of the treatment-related or secondary factors related to critical illness.

Intestinal ischemia injury

It has been reported in the literature that exposure to COVID-19 may lead to an increased risk of ischemia associated with extremity venous thrombosis, pulmonary embolism, and mesenteric ischemia [109,110]. The overall mortality in COVID-19 patients with gastrointestinal ischemia and imaging-diagnosed mesenteric ischemia was 38.7% and 40%, respectively[111]. Mesenteric ischemia can be a fatal clinical emergency with high mortality[112]. Existing studies also have shown that the incidence of intestinal obstruction and intestinal ischemia is positively correlated with elevated aminotransferase levels[113].

Other gastrointestinal symptoms

Abdominal distension and loss of taste also have been indicated in the previous investigation[114], however, the specific underlying mechanism needs to be further explored. Another important issue we should be concerned about is that some adverse gastrointestinal events in COVID-19 patients are related to medication[115]. An observation line cohort study among patients with moderate severity of COVID-19 pointed out that patients receiving hydroxychloroquine or chloroquine treatment may occur serious gastrointestinal adverse practices (diarrhea, nausea, abdominal pain, *etc*.) which is an important reason make the patients are forced to withdrawal[115].

CONCLUSION

Patients suffering from COVID-19 are often accompanied by various types of gastrointestinal symptoms. Gastrointestinal symptoms may be accompanied by or precede respiratory symptoms. This suggests that gastrointestinal symptoms may indicate the possibility of a new COVID-19 infection in the context of this unmanageable pandemic trend. During COVID-19 infection, the main mechanisms of gastrointestinal symptoms include the interaction between virus and gastrointestinal ACE2, inflammatory factor storm, intestinal mucosal barrier damage, and composition and metabolites of intestinal flora change. A vicious cycle is formed between the above factors in COVID-19 patients, which prolongs the recovery time. This also suggests that focusing on gut symptoms and alterations in gut microbes or their metabolites may be a beneficial option for coping with COVID-19. Adjunctive treatment with probiotics may help break this vicious cycle and help the patients recover. However, this review has some limitations. The assumptions in this review are mostly based on clinical observations. To clearly elucidate the mechanism of gastrointestinal symptoms caused by COVID-19 and find appropriate treatments still needs a lot of basic research.

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FOOTNOTES

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REVIEW

Clinical diagnosis and management of pancreatic cancer: Markers, molecular mechanisms, and treatment options

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Abstract

Pancreatic cancer (PC) is the third-leading cause of cancer deaths. The overall 5-year survival rate of PC is 9%, and this rate for metastatic PC is below 3%. However, the PC-induced death cases will increase about 2-fold by 2060. Many factors such as genetic and environmental factors and metabolic diseases can drive PC development and progression. The most common type of PC in the clinic is pancreatic ductal adenocarcinoma, comprising approximately 90% of PC cases. Multiple pathogenic processes including but not limited to inflammation, fibrosis, angiogenesis, epithelial-mesenchymal transition, and proliferation of cancer stem cells are involved in the initiation and progression of PC. Early diagnosis is essential for curable therapy, for which a combined panel of serum markers is very helpful. Although some mono or combined therapies have been approved by the United States Food and Drug Administration for PC treatment, current therapies have not shown promising outcomes. Fortunately, the development of novel immunotherapies, such as oncolytic viruses-mediated treatments and chimeric antigen receptor-T cells, combined with therapies such as neoadjuvant therapy plus surgery, and advanced delivery systems of immunotherapy will improve therapeutic outcomes and combat drug resistance in PC patients. Herein, the pathogenesis, molecular signaling pathways, diagnostic markers, prognosis, and potential treatments in completed, ongoing, and recruiting clinical trials for PC were reviewed.

Key Words: Pancreatic cancer; Pancreatic ductal adenocarcinoma; Molecular mechanisms; Diagnostic and prognostic markers; Treatment; Clinical trials

Core Tip: Pancreatic cancer (PC) is the third-leading cause of cancer deaths. Pancreatic ductal adenocarcinoma is the most common type of PC in the clinic. Multiple pathogenic processes including inflammation, fibrosis, angiogenesis, epithelial-mesenchymal transition, and proliferation of cancer stem cells are involved in PC initiation and progression. Although some therapies have been approved for PC treatment, the overall 5-year survival rate is still very low. A combined panel of serum markers is very helpful for PC diagnosis. New treatments and more clinical trials are required to search for new potent therapeutic agents and to evaluate their efficacy in PC treatment.

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INTRODUCTION

Pancreatic cancer (PC) accounts for 7% of all cancer-related deaths[1]. The overall 5-year survival rate of PC is 9%, with only 3% for metastatic PC[2]. However, the number of PC-induced death cases will increase about 2-fold by 2060[3,4]. Many factors are involved in the development of PC[5-7], including genetic mutations, environmental factors, and metabolic diseases such as obesity and diabetes. The most commonly diagnosed PC in the clinic is pancreatic ductal adenocarcinoma (PDAC), which accounts for more than 90% of all PC cases[4]. The rest of the PC cases are pancreatic neuroendocrine neoplasms. Pancreatic neuroendocrine neoplasms originate from precursor cells in the pancreatic ductal epithelium with neuroendocrine differentiation, which can be divided into well-differentiated pancreatic neuroendocrine tumors and poorly differentiated pancreatic neuroendocrine carcinomas[8].

PC is a heterogenous and desmoplastic cancer. Genetic variants of tumor cells, immunosuppressive tumor microenvironment (TME), high metastatic rate, and limited therapeutic outcomes cause challenges for current therapies[9-11]. Early diagnosis of PC is critically important for longer survival outcomes. Serum biomarkers can be applied for PC diagnosis, including microRNAs[12] and cancer antigens such as carbohydrate antigen 19-9 (CA 19-9)[13]. In addition, some of these markers such as CA 19-9 can be applied to predict tumor recurrence and survival of PC patients[14,15].

Herein, this review first summarized the pathogenic factors and their associated molecular signaling pathways that are involved in PC development and progression. Then, diagnostic and prognostic markers were reviewed, especially serum biomarkers. Based on the pathogenic factors, corresponding treatments were discussed, and currently completed, ongoing, and recruiting clinical trials for PC treatment were summarized.

PATHOGENESIS OF PANCREATIC CANCER AND RELATIVE MOLECULAR MECHANISMS

The initiation and progression of PC are impacted by many factors, including chronic inflammation or pancreatitis, fibrosis, immunosuppressive TME, epithelial-mesenchymal transition (EMT), proliferation and differentiation of cancer stem cells, and alteration of gut microbiota. In this section, we discussed each of these factors in PC pathogenesis.

Inflammation

Inflammation is a key mediator for the initiation and progression of PC[16]. In TME during PC development, tumor growth accompanies the infiltration of innate and adaptive immune cells. For example, tumor-associated macrophages are one of the major immune cells in the TME, which have been shown to play an essential role in the initiation, progression, and metastasis of PC as well as chemotherapeutic resistance[17]. Tumor necrosis factor alpha-expressing macrophages are recruited by monocyte chemoattractive protein-1 or CCL2, which can induce the reprogramming of classical neoplastic cells into an aggressive phenotype *via* the bromodomain-containing protein 4-mediated signaling pathway[18]. In addition, other infiltrating immune cells including monocytes, myeloid-derived suppressor cells, natural killer cells, neutrophils, and $CD4^+$ and $CD8^+$ T cells interplay with cancer cells by secreting cytokines. Molecular signaling pathways such as nuclear factor-κB, reactive oxygen species, and toll-like receptors (TLRs) are involved in the inflammatory condition in the TME of

PC[16].

Both local and systemic inflammation contributes to PC development and progression. A clinical study showed that PDAC patients with systemic inflammation characterized by a neutrophil/lymphocyte ratio > 3.1 have a lower median overall survival compared to patients with a neutrophil/lymphocyte ratio < 3.1 in response to the treatment of anti-CD40 monoclonal antibody in combination with gemcitabine[19]. Obesity can induce systemic inflammation and contribute to cancer progression, including PDAC[20]. The chronic low-grade inflammation in white adipose tissues of obese patients plays an important role in PDAC progression[21]. Some important adipokines such as lipocalin 2, proinflammatory cytokines [*e.g.*, tumor necrosis factor alpha and interleukin (IL)-6], and chemokines (*e.g.*, monocyte chemoattractive protein-1 or CCL2) drive the progression of PDACs[21,22].

Chronic pancreatitis characterized by redness and swelling inflammation in the pancreas can be induced by factors such as heavy alcohol consumption[23], smoking, and gallstones[24]. It is a risk factor for the initiation of the progression of PDAC[25] or PC[26]. In the United States, 1.04% of patients with chronic pancreatitis were diagnosed with PDAC, which was higher than the rate in the control group (0.2%)[27]. Therefore, targeting inflammation is a therapeutic strategy for PC treatments.

Desmoplastic stroma

Chronic pancreatitis and PC are commonly associated with desmoplastic tissue proliferation, which is mainly caused by activated pancreatic stellate cells[28]. Fibrotic stroma is formed by fibroblasts and their secreted extracellular matrix proteins that contribute to cancer cell proliferation and invasion, an immunosuppressive environment, and therapeutic resistance[29]. Some molecules including epithelium-specific E-twenty six factor 3[30], galectin-1[28], β-catenin[31], and transforming growth factor-β1 (TGF-β1)[32] are essential drivers for pancreatic stellate cell activation in PC. In addition, activated pancreatic stellate cells can secrete many molecules, such as IL-6, TGF-β1, stromal cell-derived factor-1, hepatocyte growth factor, and galectin-1, to induce PC cell proliferation, migration, and chemotherapeutic resistance[33].

Cancer-associated stromal fibroblasts (CAFs) are a heterogenous cell population, which can secrete many factors to regulate inflammation, cancer development, progression, metastasis, recurrence, and drug resistance[34]. For instance, CAFs and tumor cells can crosstalk through extracellular vesicles (EVs). Annexin A6-enriched EVs secreted by CAFs can increase the aggressiveness of PDACs[35,36]. CD9 is an important component of these EVs[36]. In addition, the uptake of $CD9^{+}ANXA6^{+}$ EVs secreted by CAFs can activate the mitogen-activated protein kinase signaling pathway to promote PC cell migration and EMT[36].

Immunosuppressive TME

Overexpression of immune checkpoints, such as programmed cell death protein 1 (PD-1), lymphocyte-activation gene 3, and cytotoxic T-lymphocyte-associated antigen 4, and their ligands programmed death-ligand 1 (PD-L1) and PD-L2, CD80 and CD86 (cytotoxic T-lymphocyte-associated antigen 4), and major histocompatibility complex molecule II or major histocompatibility complex-II (lymphocyte-activation gene 3) mediate the immunosuppression in TME[37]. Therefore, targeting these molecular signaling axes provide novel therapeutic options. For example, PD-L1 is overexpressed by tumor cells and some immunosuppressive cells in PDAC, which can be targeted by PD-1-expressing chimeric antigen receptor (CAR) T cells to mediate anti-tumor activity by PD-1/PD-L1 interaction[38].

Additionally, other proteins, such as fibroblast activation protein, CD73, and inhibitor of DNA binding 1[39], can mediate the immunosuppressive environment in the TME. In a mouse PDAC model, elevated expression of CD73, a cell surface-localized ecto-5′-nucleotidase, is positively associated with the infiltration of myeloid-derived suppressor cells and expression of granulocyte-macrophage colony-stimulating factor, which causes suppression of interferon-gamma production in intratumoral T cells. The CD73-mediated suppressive effect on T cells can be abolished by genetic knockdown in PDAC cells [40]. In human PDAC cells, the elevated expression of CD73 causes cancer cell resistance to gemcitabine by activating the protein kinase B (AKT) signaling pathway[41].

EMT

EMT plays a pivotal role in PC progression and metastasis, which is defined by cell phenotypic transition from an epithelial to a mesenchymal state[42]. Dermokine genes regulate the oncogenesis of PC. Overexpression of dermokine-α can promote PC cell proliferation, EMT, migration, and invasion by regulating the phosphorylation of signal transducer and activator of transcription 3 (STAT3)[43,44]. Methylsterol monooxygenase 1 as a tumor suppressor can inhibit PC progression by suppressing the phosphoinositide 3-kinase-AKT-mammalian target of rapamycin signaling pathway and EMT[45]. EMT of PC stem cells also plays a critical role in PC initiation and progression, which is discussed in the following section.

Angiogenesis

Angiogenesis plays a key role in PC development, progression, and metastasis, which is commonly associated with the activation of proangiogenic and angiogenic molecules. For example, the epidermal

growth factor receptor is overexpressed in PC cells, which is associated with angiogenesis and cancer cell metastasis[46]. A high density of macrovessels, impaired integrity of microvessels, and poorly perfused vessels are the characterizations of vascularization in TME of PC[29]. Many proteins are involved in the angiogenesis of PC, including vascular endothelial growth factor (VEGF), platelet-derived growth factor, fibroblast growth factor, and their receptors such as VEGF receptors (VEGFR1-3)[29]. In inflammatory conditions, the expression of fibroblast growth factor 1 on PC cells is stimulated by inflammatory product prostaglandin E2, resulting in the proliferation of CAFs and an increased VEGFA expression to maintain angiogenesis[47].

Cancer stem cells

Cancer stem cells (CSCs) are a small part of the TME, where they play a vital role in chemotherapy resistance. Pancreatic CSCs express several surface markers such as CD24, CD44, CD133, C-X-C chemokine receptor type 4, tyrosine-protein kinase Met, epithelial cell adhesion molecule, and double-cortin like kinase 1 as well as intracellular markers such as aldehyde dehydrogenase 1 and RNA polymerase II-associated factor 1[48]. One study showed that miR-497 can resensitize pancreatic CSCs to gemcitabine treatment by inhibiting nuclear factor-κB expression[49]. Exosomes can horizontally transfer drug-resistant traits from gemcitabine-resistant pancreatic CSCs to gemcitabine-sensitive PC cells by delivering miR-210[50]. Small nucleolar RNAs such as SNORD35A play an important role in the proliferation, migration, invasion, and EMT of pancreatic CSCs through regulating the hepatocyte growth factor/tyrosine-protein kinase Met signaling pathway, which is a prognostic biomarker and therapeutic target for PC treatment[51]. In addition, CSCs are involved in drug resistance. For instance, a hypoxic niche can further activate AKT/Notch1 signaling pathway to enhance gemcitabine-induced stemness to cause chemoresistance[52].

Alteration of gut microbiota

Gut microbiomes, including bacteria, viruses, archaea, and fungal species, play important roles in energy digestion, synthesis of secondary bile acids, vitamins and proteins, and immune regulation. Most of the microbes reside (> 95%) within the gut and maintain intestinal homeostasis[53]. Dysbiosis of gut microbiota can regulate inflammation and immune response in the TME to promote cancer progression[54,55], including PC[56,57]. A preclinical study showed that gut microbiota can promote PDAC progression by regulating the infiltration and anti-cancer activity of natural killer cells in the TME[58]. Cohort studies in three different countries from Asia (Japan) and Europe (Spanish and German) showed that *Streptococcus* and *Veillonella spp* were significantly enriched and *Faecalibacterium prausnitzii* was depleted in gut microbial profiles of PDAC patients[59]. A comprehensive analysis of microbiota in several tumors including PC showed that most intratumor bacteria were present intracellularly in both cancer and immune cells[60].

Recent studies also showed that intratumor bacteria were associated with the survival time of PDAC patients[61,62]. In addition, bacteria-mediated treatment (*e.g.*, a bacterium *Megasphaera sp. XA511*) can enhance the anti-tumor effect of anti-PD-1 treatment[61]. Another study also revealed that the alpha diversity of tumor microbiota was higher in long-term survival patients compared to that in short-term survival patients. Fecal microbiota transplantation from humans into mice can regulate tumor growth and anti-tumor immune response[62]. Moreover, pancreatic secretions (*e.g.*, antimicrobial peptides) influence the change of gut microbiota profiles, and the pancreas-bacteria interplay forms a gut-microbiota-pancreas axis[63].

Oral microbiota may also impact the development of PC. One study showed that the presence of oral *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* was positively associated with a high PC risk, whereas genus *Leptotrichia* (phylum Fusobacteria) was related to a low PC risk[64]. In addition, other oral bacterial species including *Clostridium difficile*, *Campylobacter jejuni*, *Escherichia coli*, *Enterococcus faecalis*, *Helicobacter pylori*, *Fusobacterium nucleatum*, *Vibrio cholera*, and *Porphyromonas gingivalis* have been reported to be associated with PC development, which may regulate anti-tumor immunity by signaling pathways such as the miR-21/phosphatase and tensin homolog axis[65].

Overall, multiple factors influence the initiation and progression of PC, which is summarized in Figure 1.

DIAGNOSTIC AND PROGNOSTIC MARKERS FOR PANCREATIC CANCER

The tumors in PC patients can be classified into resectable, borderline resectable, locally advanced, and metastatic tumors[66]. Early diagnosis ensures curable treatment by surgical resection[67]. An accurate diagnosis of PC can improve therapeutic outcomes. Although there is an advanced improvement in new diagnostic technologies, biopsy is still the gold standard for PC diagnosis[68]. Imaging methods including computed tomography, positron emission tomography, magnetic resonance imaging, and endoscopic ultrasound are commonly applied in PC diagnosis and staging[68,69]. In addition, several serum markers have promising diagnostic and prognostic values for PC.

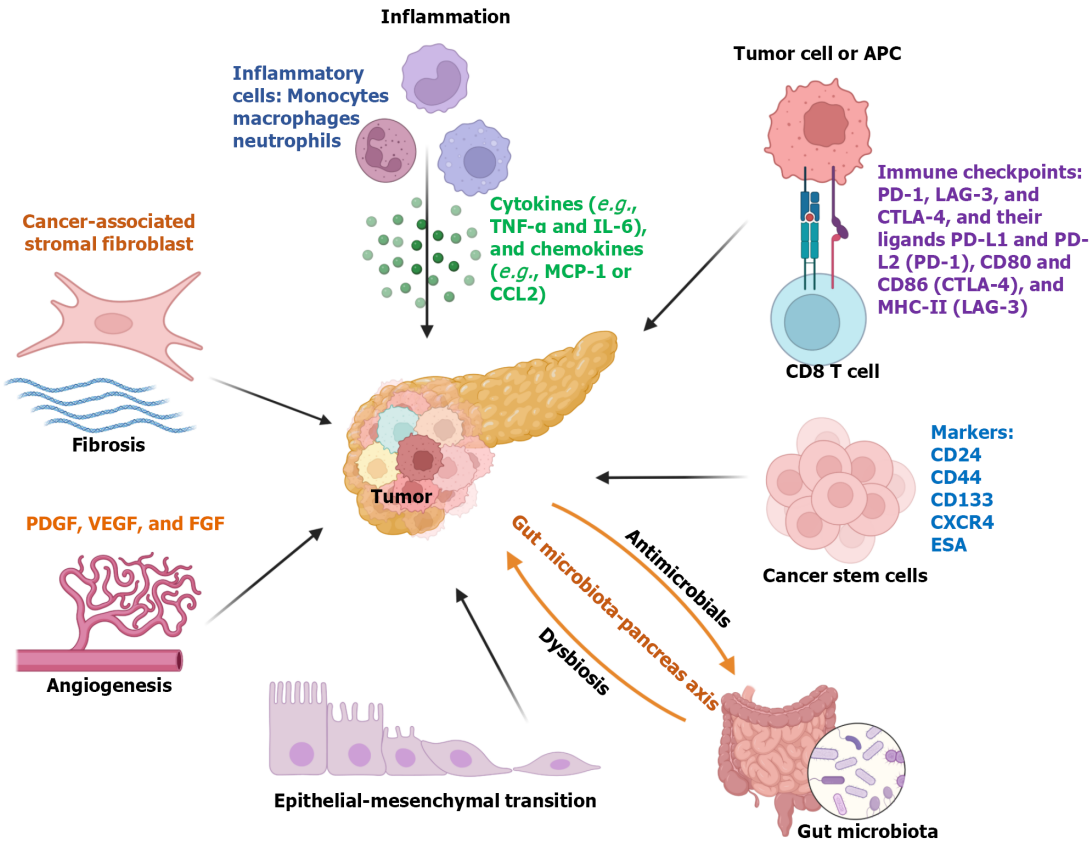

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Figure 1 Many factors are involved in pancreatic cancer initiation and progression. Many factors contribute to pancreatic cancer initiation and progression, including inflammation, fibrosis, angiogenesis, dysbiosis of gut microbiota, cancer stem cells, and immune suppressive tumor microenvironment. APC: Antigen-presenting cells; CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4; CXCR: C-X-C chemokine receptor; ESA: Epithelial-specific antigen; FGF: Fibroblast growth factor; IL: Interleukin; LAG-3: Lymphocyte-activation gene 3; MCP-1: Monocyte chemoattractive protein-1; MHC: Major histocompatibility complex; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; PDGF: Platelet-derived growth factor; TNF-α: Tumor necrosis factor alpha; VEGF: Vascular endothelial growth factor. All cartoons in this figure were prepared using Biorender (https://biorender.com).

Serum markers for PC diagnosis

Serum markers including carcinoembryonic antigen and CAs can be applied in PC diagnosis and prognosis[70,71]. Currently, CA 19-9 is the most broadly used serum biomarker for PC diagnosis. A meta-analysis showed that the average sensitivity and specificity of CA 19-9 in PC diagnosis were 72% [95% confidence interval (CI): 71%-73%] and 86% (95%CI: 85%-86%), respectively, with an area under the curve (AUC) of 0.8474 (95%CI: 0.8272-0.8676)[72]. It has been reported that CA 19-9 serum levels are significantly associated with positive lymph nodes and positive margin status in patients with resectable PDAC, which is important for the decision of neoadjuvant treatments[73]. In addition, the preoperative levels of CA 19-9 are negatively associated with the overall survival, nodal involvement, and margin status positivity in resectable PC. However, some limitations impair the role of CA 19-9 in PDAC preoperative staging and management[74], including up to 50% of PDAC patients without CA 19-9 secretion.

In combination with other markers, the diagnostic value of CA 19-9 can be amplified or increased. For example, a combination of CA19-9 with serum mucin 5AC, a heavily glycosylated protein of the mucin family, improves both sensitivity (73.8%) and specificity (88.6%) as well as the AUC (0.894; 95%CI: 0.844-0.943) for PC diagnosis in patients, better than the values of each individual marker[75]. There are many other serum markers that can be used for PC or PDAC diagnosis, including macrophage inhibitory cytokine-1[76], keratin 8[77], protein induced by vitamin K absence II[78], and gremlin 1 (GREM1)[79] (Table 1). Meanwhile, combining different markers in a panel could increase the values of sensitivity, specificity, and AUC. For example, a panel including CA 19-9, factor VIII, fibrinogen, albumin, and alkaline phosphatase increase the AUC value to 0.95 (95%CI: 0.89-0.99) when compared to 0.80 (0.71-0.88) for CA 19-9 alone in distinguishing PDAC from intraductal papillary mucinous neoplasm, a benign tumor[80]. Another study also showed that a panel of four biomarkers including S100 calcium-binding protein A2, S100 calcium-binding protein A4, CA 125, and CA 19-9 increased AUC to 0.913[81].

Table 1 Diagnostic markers of pancreatic cancer

Cancer type	Markers	Expression	Sensitivity	Specificity	AUC	Ref.
PC	CA 19-9	Serum	72.0%	86%.0	0.8474	[72]
PC	MUC5AC + CA19-9	Serum	73.8%	88.6%	0.8940	[75]
PC	MIC-1	Serum	80.0%	85.0%	0.8945	[76]
PDAC	Keratin 8	Serum	80.0%	85.0%	0.8945	[77]
PDAC	PIVKA-II	Serum	78.7%	90.7%	0.9000	[78]
PDAC	GREM1	Serum	Unknown	Unknown	0.7180	[79]

AUC: Area under the curve; CA19-9: Carbohydrate antigen 19-9; GREM1: Gremlin 1; MIC-1: Macrophage inhibitory cytokine-1; MUC5AC: Mucin 5AC; PC: Pancreatic cancer; PDAC: Pancreatic ductal adenocarcinoma; PIVKA-II: Protein induced by vitamin k absence II.

MicroRNAs are small non-coding RNAs with about 22 nucleotides, which regulate the expression of their target mRNAs through degradation or translational repression. Serum expression profiles of microRNAs in PC patients are significantly changed compared to healthy controls. Among them, a panel model including miR-125a-3p, miR-5100, and miR-642b-3p showed the most promising value for PC diagnosis with an AUC of 0.95, sensitivity of 0.98, and specificity of 0.97[82]. Another study also showed that serum microRNAs such as miR-25-3p, miR-19a/b-3p, miR-192-5p, miR-223-3p, and let-7b-5p were upregulated in PC patients and can be used as diagnostic markers as a panel[83].

Multi-omics profiling studies can provide new markers for PC diagnosis. Proteomic analysis of EVs derived from co-cultured epithelial and stromal cells in the condition mimicking TME showed that kinesin family member 5B and secreted frizzled related protein 2 had promising values as early PC biomarkers[84]. PancRISK score evaluated by three urine markers, including lymphatic vessel endothelial hyaluronan receptor 1, regenerating family member 1 beta, and trefoil factor 1, showed reasonable sensitivity and specificity for PDAC detection compared to CA 19-9[85]. Another study also showed that a panel of three urine markers with CA 19-9 had pre-diagnostic values before PDAC diagnosis, with a sensitivity of 72% at 90% specificity up to 1 year and 60% sensitivity with 80% specificity up to 2 years[86].

Bioinformatics analysis showed that gremlin 1, a bone morphogenetic protein signaling regulator, was overexpressed in PDAC and predicted a poorer prognosis for patients with PDAC[79]. In addition, serum gremlin 1 is increased in PDAC patients compared to healthy controls and has a diagnostic value. In combination with CA 19-9, the AUC value increases from 0.718 to 0.914[79].

Prognostic markers of pancreatic cancer

One study displayed that PC patients with low lymphocyte-C-reactive protein ratio have significantly low recurrence-free survival and overall survival values[87]. Another study showed that the systemic immune-inflammation index, which is calculated using the absolute platelet, neutrophil, and lymphocyte counts [systemic immune-inflammation index = platelet × (neutrophil/lymphocyte)], can be used as an independent negative prognostic marker of overall survival of PDAC patients receiving neoadjuvant therapy[88]. A meta-analysis showed that mucin 4 (hazard ratio = 2.04, 95%CI: 1.21-3.45) and mucin 16 (hazard ratio = 2.10, 95%CI: 1.31-3.37) had predictive values for the prognosis of PC patients[89]. The expression of aquaporin-5, a water channel protein, is increased in pancreatic adenocarcinoma (PAAD), which is positively associated with the infiltration of different immune cells (*e.g.*, macrophages) in tumors and poor prognosis of PAAD patients[90]. A bioinformatics study showed that overexpression of matrix metallopeptidase 14 and collagen XII alpha 1 is significantly related to the poor prognosis of PAAD patients[91]. Analysis of RNA sequencing data from the Gene Expression Omnibus and the Cancer Genome Atlas databases gives us some conclusion that some biomarkers such as transmembrane protein 170B (TMEM170B) can be applied to predict cancer progression[92]. Overall, improvement in bioinformatics and new technologies accelerates the development of new diagnostic and prognostic markers for PC, which will result in good outcomes for PC therapy.

TREATMENT OPTIONS

Surgical resection is a curable therapy for patients with early stages of PC and good health conditions [66,68]. There are some Food and Drug Administration (FDA)-approved drugs for PC treatment (Table 2), including belzutifan[93,94], erlotinib hydrochloride[95,96], everolimus[97,98], fluorouracil, also known as 5-fluorouracil[99,100], gemcitabine hydrochloride[101,102], irinotecan hydrochloride liposome[103,104], mitomycin[105,106], olaparib[107,108], paclitaxel albumin-stabilized nanoparticle

Table 2 Food and Drug Administration-approved treatments for pancreatic cancer patients

Drug names	Conditions	Targets	Ref.
Belzutifan	Pancreatic neuroendocrine tumors	An inhibitor of hypoxia-inducible factor-2α	[93, 94]
Erlotinib hydrochloride	Gemcitabine hydrochloride-treated PC, not removable with surgery, with metastasis or local progression	An EGFR inhibitor	[95, 96]
Everolimus	Progressive pancreatic neuroendocrine tumors, not removable with surgery, with metastasis or local advance	A mammalian target of rapamycin inhibitor	[97, 98]
Fluorouracil, also called 5-FU	Pancreatic cancer	An anti-metabolite drug with multiple functions such as inhibition of cellular thymidylate synthase to prevent DNA replication and inhibit RNA synthesis	[99, 100]
Gemcitabine hydrochloride	PC with metastasis, local advance, or fluorouracil treatment	An antimetabolite drug and an inhibitor of DNA synthesis	[101, 102]
Irinotecan hydrochloride liposome	Metastatic PC or gemcitabine hydrochloride-treated PC with precision	An inhibitor of topoisomerase I	[103, 104]
Mitomycin	Pancreatic adenocarcinoma with local advance or metastasis to other parts of the body, which has no approvement with other types of treatment	An inhibitor of DNA synthesis and thioredoxin reductase	[105, 106]
Olaparib	Metastatic PC after first-line therapy with platinum chemotherapy and with certain germline mutations in the breast cancer 1 or BRCA2 gene	A poly ADP-ribose polymerase inhibitor	[107, 108]
Paclitaxel albumin-stabilized nanoparticle formulation	PC with metastasis	It prevents cell mitosis and inhibits the growth of cancer cells	[109, 110]
Sunitinib malate	Progressive neuroendocrine tumors that are not removable with surgery, with metastasis to other parts of the body or local advance	An antiangiogenic tyrosine kinase inhibitor	[111, 112]

EGFR: Epidermal growth factor receptor; PC: Pancreatic cancer.

formulation[109,110], and sunitinib malate[111,112]. In addition, combined treatments including leucovorin calcium (folinic acid) + fluorouracil + irinotecan hydrochloride + oxaliplatin, gemcitabine hydrochloride + cisplatin, gemcitabine hydrochloride + oxaliplatin, and oxaliplatin + fluorouracil + leucovorin calcium (folinic acid) have been also approved by the United States FDA for PC treatment [113-115].

In this section, we discussed some treatments targeting the driving factors of PC, including immune checkpoint inhibitors, antifibrosis, anti-inflammation, anti-angiogenesis, growth factor inhibitors, anti-cancer peptides, alteration of gut microbiota, T cell therapy, and oncolytic viruses as well as combined therapies.

Immune checkpoint inhibitors

Immunotherapy by targeting immune checkpoints such as PD-1 and PD-L1 has achieved big success in the treatment of many different tumors[116,117]. Currently, pembrolizumab (anti-PD-1) is the only FDA-approved immune checkpoint inhibitor for the treatment of patients who have advanced PDACs with mismatch repair deficiency or microsatellite instability high[118]. There are many ongoing clinical trials for the evaluation of synergistic effects of ipilimumab or tremelimumab (anti-cytotoxic T-lymphocyte-associated antigen 4 antibody), nivolumab (anti-PD-1 antibody), and durvalumab (anti-PD-L1 antibody) with other chemotherapy, vaccines, or radiotherapy[119].

Antifibrotic treatments

CAFs are one of the most abundant stromal cells in PDAC and contribute to cancer progression and chemoresistance[120]. Therefore, reshaping the fibrotic stroma is a strategy to treat PC. The integrin-mediated signaling pathway plays a critical role in remodeling and induction of pancreatic tissue stiffness during PC development and progression, promoting chemoresistance. The phosphorylation of tyrosine397 in focal adhesion kinase (FAK) of CAFs is significantly increased compared to that in fibroblasts of the normal pancreas. Therefore, inhibiting FAK activity can dramatically suppress CAF migration and extracellular matrix deposition[120]. Meanwhile, FAK inhibition can also resensitize PDAC cells to chemotherapy[121]. Some tyrosine kinase inhibitors such as cabozantinib, pazopanib, lenvatinib, and surufatinib are under clinical evaluation for the treatment of pancreatic neuroendocrine tumors[122]. A study in a murine PC model also showed that stromal hyaluronan degradation by PEGylated recombinant human hyaluronidase in combination with FAK inhibitor could improve anti-

PD-1 antibody efficacy on the survival of PDAC-bearing mice by increasing T cell infiltration and efficacy[123].

Anti-inflammatory treatments

The anti-inflammatory drug aspirin can inhibit cell proliferation of PC cell lines by suppressing cyclin D1 expression to induce G_0/G_1 cell cycle arrest. Aspirin can also inactivate the glycogen synthase kinase-3β signaling pathway and regulate the expression of microRNAs in PC cells[124]. A phase I trial (https://clinicaltrials.gov, registration number NCT03207724) showed that treatment with bermekimab (anti-IL-1α antibody) can decrease inflammatory cytokines and endothelial growth factor, which is associated with an increase in healthy gut microbiota *Akkermansia* compared to the baseline[125]. A phase 3 clinical trial (NCT02923921) showed that adding pegilodecakin (PEG, a pegylated recombinant human IL-10) to folinic acid, fluorouracil, and oxaliplatin increased the expression of total IL-18, interferon-gamma, and granzyme B and decreased TGF-β in patients with post-gemcitabine metastatic PDACs[126].

Anti-angiogenesis treatments

The expression of mitofusin-2 in PC tissues is significantly decreased, which is negatively associated with VEGFA expression. A molecular study showed that overexpression of mitofusin-2 could inhibit the expression of VEGFA, VEGFR2, angiopoietin-1 gene, and tissue inhibitor of metalloproteinase 1 in human umbilical vein endothelial cells[127]. Another study showed that escin, a pentacyclic triterpenoid isolated from the horse chestnut, can inhibit angiogenesis by suppressing the expression of IL-8 and VEGF in PC cells through the blockade of nuclear factor-κB[128]. Treatment with apatinib, a small molecule targeting VEGFR2, can inhibit the proliferation, migration, and invasion of PC cells (ASPC-1 and PANC-1 cells) and the growth of their xenografted tumors by inhibiting cancer cell growth and angiogenesis *via* suppression of the phosphorylation of VEGFR2, AKT, and ERK1/2[129].

Anti-growth factors and growth factor receptor inhibitors

Growth factors play essential roles in PC cell survival, progression, and drug resistance[130]. For example, TGF-β contributes to PDAC progression by inducing N-glycomic changes in SMA-related and MAD-related protein 4-deficient PDAC cell line PaTu-8955S cells[131].

Anti-cancer peptides

Anti-cancer peptides are short peptides with direct and indirect anti-cancer properties. Anti-cancer peptides can be classified into natural and synthetic peptides. For example, human cathelicidin peptide LL-37 can suppress PC growth *in vitro* and *in vivo* by activating the mammalian target of rapamycin signaling pathway to suppress autophagy of PC cells and induce reactive oxygen species production to cause DNA damage and cell cycle arrest[132]. KS-58, a derivative of KRpep-2d that is an artificial cyclic peptide that can selectively inhibit K-Ras (G12D), can suppress the human PC cell line PANC-1 proliferation *in vitro*. In addition, KS-58 also displays anti-tumor activity in subcutaneous and orthotropic PANC-1 cell xenografted tumors, which shows a synergistic effect with gemcitabine[133].

Alteration of gut microbiome

Patients with high levels of antibodies against a pathogenic periodontal bacterium *Porphyomonas gigivalis* have a double risk of developing PC compared to subjects with low levels of antibodies. In contrast, individuals who have high levels of antibodies against commensal oral bacteria reduce the risk of PC development[134]. The microbiome in the cancerous pancreas can induce immune tolerance by causing macrophage-mediated suppression of T cell functions through TLR signaling pathways (*e.g.*, TLR2 and TLR5)[135]. Depletion of gut microbiota by antibiotics can inhibit tumor progression and metastasis *via* upregulation of interferon-gamma-producing T cells and downregulation of IL-10 and IL-17A-producing T cells[136]. Therefore, the alteration of microbial components in the gut and mouth, as well as the TME, can effectively inhibit PC progression.

CAR-engineered T cell therapy

CAR-engineered T cell (CAR-T) therapy shows potential for many tumors. To date, six CAR-T products have been approved by the United States FDA for the treatment of hematopoietic cancers, such as B-cell acute lymphoblastic leukemia (tisagenlecleucel), mantle cell lymphoma (brexucabtagene autoleucel), and multiple myeloma (idecabtagene vicleucel)[137]. However, current clinical trials of CAR-T therapy in PC patients have not shown significance in the improvement of survival and other outcomes[138]. CAF-derived extracellular matrix proteins, enzymes, and growth factors impact the infiltration and efficacy of CAR-T. Enhancing the expression of chemokine ligands such as chemokine (C-C motif) ligand 19 (CCL19) can increase the infiltration of the CAR-T to promote their anti-PDAC efficacy[139].

Oncolytic viruses

Virus-mediated delivery of cytokines and shRNAs shows promising effects in PC treatment. Oncolytic viruses are designed to directly target tumor cells or to activate anti-tumor immune responses.

However, treatment of oncolytic viruses alone is not sufficient to eliminate PC to date[140]. For example, oncolytic adenoviruses loading CD55-ST13 (suppression of tumorigenicity 13)-tumor necrosis factor-related apoptosis-inducing ligand can significantly inhibit but not delete tumor development in a murine xenografted PDAC tumor model by inducing cancer cell apoptosis[141].

Combined therapies

The combined therapy of MEK inhibitor (trametinib) and STAT3 inhibitor (ruxolitinib) inhibits the phenotype of proinflammatory and myofibroblastic IL6$^+$CXCL1$^+$LRRC15$^+$CAFs and increases mesenchymal stem cell-like Ly6a$^+$Cd34$^+$CAFs in a murine model detected by single-cell RNA sequencing[142]. The CAF phenotype change is associated with M2-to-M1 reprogramming of tumor-associated macrophages s and effector CD8$^+$ T cell infiltration. In addition, treatment of MEK and STAT3 inhibitors together with a PD-1 inhibitor (nivolumab) shows clinical benefit in patients with chemotherapy-refractory metastatic PDACs[142]. In contrast, a pharmacodynamic separation treatment for erlotinib plus gemcitabine can improve their treatment efficacy, especially for patients with detected plasma Kirsten rat sarcoma virus mutation[143].

Four-week intraperitoneal injection of gemcitabine together with oral administration of probiotic cocktails (*Lactobacillus paracasei* GMNL-133 and *Lactobacillus reuteri* GMNL-89) can inhibit pancreatic intraepithelial neoplasia formation and suppress serum levels of aspartate aminotransferase and alanine aminotransferase and the expression of vimentin and Ki-67 in pancreatic sections[144].

CLINICAL TRIALS

Some treatments are under clinical investigation, see Table 3 (https://clinicaltrials.gov, accessed on September 6, 2022). These treatments include galunisertib plus gemcitabine[145], Janus kinase 1/2 inhibitor ruxolitinib[146], adoptive transfer of T cells[147], gemcitabine and trastuzumab plus erlotinib [148], erlotinib plus gemcitabine[149], pegilodecakin plus folinic acid, fluorouracil, and oxaliplatin[126], liposomal irinotecan plus 5-fluorouracil/leucovorin[150], bevacizumab[151], and sunitinib[152].

In addition, more than 900 studies in the world are recruiting or enrolling by invitation, and some have a 'not yet recruiting' status for evaluating PC treatments (https://clinicaltrials.gov, accessed on September 17, 2022). A graphic map shows the number of studies at different locations (Figure 2), and some examples are listed in Table 4.

FUTURE PERSPECTIVES

A synergistic treatment is a good option for killing drug-resistant PC cells. For example, astaxanthin can resensitize human PC cells to gemcitabine by activating the hypoxia-inducible factor 1α/STAT3 signaling pathway to promote gemcitabine-induced cell apoptosis and inhibit gemcitabine-induced EMT of PC cells[153]. Guadecitabine, an effective inhibitor of DNA methyltransferase 1, has the potential to resensitize PDAC cells to chemotherapy and immune checkpoint blockade therapy (*e.g.*, anti-PD-L1)[154,155].

Neoadjuvant therapy has been applied in clinical trials for patients with resectable PDACs, which include neoadjuvant chemotherapy, neoadjuvant radiotherapy, and neoadjuvant chemoradiotherapy [156]. The results from three randomized controlled trials with a total of 130 patients (56 receiving neoadjuvant therapy and 74 in the control group) indicated that neoadjuvant therapy (chemotherapy or chemoradiation + surgery followed by adjuvant therapy) increased the disease-free survival time compared to upfront surgery followed by adjuvant therapy[157]. Another single-center long-term study also showed that PDAC patients with treatment of neoadjuvant therapy, consisting of folinic acid + fluorouracil + irinotecan + oxaliplatin, single gemcitabine, or combined with cisplatin, nab-paclitaxel, or capecitabine with or without radiation had longer median disease-specific survival and disease-free survival than those receiving treatment with upfront surgery[158]. The benefit of neoadjuvant therapy could be a stage-dependent manner. A retrospective cohort study showed that neoadjuvant therapy was positively associated with an improved survival benefit compared with conventional upfront surgery, especially in clinical stage III PC after propensity score matching within each stage[159]. Overall, it can benefit surgical treatment.

Delivery systems can be applied to enhance the efficacy of anti-cancer treatments. For example, arginine glycine peptide-human serum albumin-mediated drug nanoparticles show tumor-targeting effects and increase the cytotoxicity of gemcitabine and curcumin[160]. Administration of VG161, the first recombinant oncolytic herpes simplex virus type 1 that delivers multiple synergistic antitumor immunomodulatory factors, can systematically activate both innate and adaptive immunity and improve the anti-tumor function of the tumor immune microenvironment[161]. Another study showed that using gold nanoparticles could enhance the intracellular delivery of oncolytic adenoviruses into PC cell lines[162]. Radiofrequency hyperthermia also can enhance the local delivery of oncolytic immuno-

Table 3 Completed clinical trials with results for pancreatic cancer treatment

Trial number	Phase	Treatment	Condition
NCT01373164	1b/2	Galunisertib, a TGF-β receptor inhibitor, or placebo plus gemcitabine	Unresectable PC
NCT01423604	2	Ruxolitinib, a Janus kinase 1/2 inhibitor or placebo plus capecitabine	PC
NCT00965718	2	Activated T lymphocyte (*ex vivo*-expanded, CIK cells cultured with anti-CD3 monoclonal antibody and IL-2)	PC
NCT01204372	2	Gemcitabine, trastuzumab plus erlotinib	Metastatic PC
CONKO-005	3	Erlotinib (inhibits the intracellular phosphorylation of tyrosine kinase associated with the EGFR) or placebo plus gemcitabine	Primarily resectable PDAC after R0 resection
NCT02923921	3	Pegilodecakin (a pegylated recombinant human IL-10) plus folinic acid, fluorouracil, and oxaliplatin	Metastatic PDAC
NCT01494506	3	Liposomal irinotecan (it prevents the religation of the DNA strand by binding to the topoisomerase I-DNA complex.) or placebo plus 5-FU/LV	Metastatic PC
NCT01214720	3	Bevacizumab that acts by selectively binding circulating VEGF, thereby inhibiting the binding of VEGF to its cell surface receptors plus chemotherapy	Metastatic PC
NCT00428597	3	Sunitinib, an inhibitor of multiple receptor tyrosine kinases	Metastatic pancreatic neuroendocrine tumors
NCT01525550	4		

5-FU/LV: 5-Fluorouracil/leucovorin; CIK: Cytokine-induced killer; EGFR: Epidermal growth factor receptor; IL: Interleukin; PC: Pancreatic cancer; PDAC: Pancreatic ductal adenocarcinoma; TGF-β: Transforming growth factor beta; VEGF: Vascular endothelial growth factor.

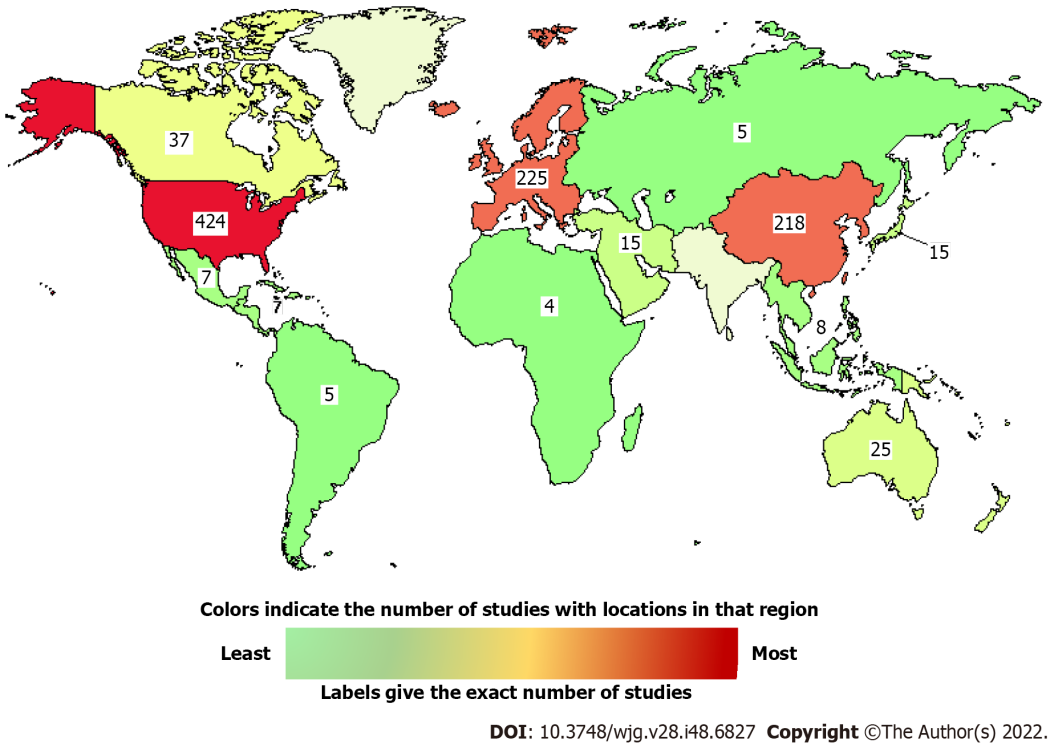

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Figure 2 Recruiting studies for pancreatic cancer at different locations in the world. The studies are in Africa (4), South America (5), Europe (225), the Middle East (15), the United States (424), Canada (37), Mexico (7), Pacifica (25), Japan (15), East Asia (218), North Asia (5), and Southeast Asia (8).

virotherapy for PAAD *in vitro* and *in vivo*[163].

Furthermore, radiofrequency ablation may be applied to treat patients with PC who are unfit for surgery and includes endoscopic ultrasound-guided radiofrequency ablation[164] and endoluminal biliary radiofrequency ablation[165].

Table 4 Ongoing or recruiting clinical trials for pancreatic cancer treatment

Trial number	Phase	Treatments	Conditions
NCT03192462	1 or 2	Intravenous infusions of TAA-specific cytotoxic T lymphocytes	Pancreas cancer with metastatic, locally advanced unresectable, or resectable disease
NCT04637698	1 or 2	Oncolytic viral therapy (Type 2 Herpes simplex virus) expressing GM-CSF	Pancreatic cancer
NCT04247165	1 or 2	Dual checkpoint inhibition (nivolumab and ipilimumab) in combination with gemcitabine and nab-paclitaxel followed by immune-chemoradiation	Borderline resectable, locally advanced, or metastatic pancreatic cancer
NCT05141149	1 or 2	Anti-PAUF monoclonal antibody PBP1510 or in combination with gemcitabine	Advanced/metastatic pancreatic cancer
NCT04825288	1 or 2	Anti-IL-1α true human antibody XB2001 or in combination with ONIVYDE + leucovorin + 5-FU chemotherapy	Advanced pancreatic cancer
NCT03662412	1 or 2	Sirolimus, a selective inhibitor of mTOR	Advanced pancreatic cancer
NCT05131776	2 or 3	Concurrent EUS-guided intratumor injection of P-32 microparticles (OncoSil)	Locally advanced pancreatic carcinoma
NCT03941093	3	Neoadjuvant treatment with pamrevlumab or placebo in combination with either gemcitabine plus nab-paclitaxel or FOLFIRINOX	Locally advanced pancreatic cancer
NCT05529940	3	FOLFIRINOX	Resectable pancreatic cancer
NCT04969731	3	Adjuvant Immuncell-LC (Cytokine-induced killer cells) therapy combined with gemcitabine	Resectable pancreatic cancer
NCT04025840	4	Perioperative epidural block and/or dexamethasone	Resectable pancreatic cancer
NCT04217096	4	Paclitaxel liposome plus S-1, an oral anticancer drug that consists of tegafur, gimeracil, and potassium oteracil in a molar ratio of 1.0:0.4:1.0	Advanced metastatic pancreatic cancer as the first-line therapy

5-FU: 5-Fluorouracil; EUS; Endoscopic ultrasound; FOLFIRINOX: Folinic acid + fluorouracil + irinotecan + oxaliplatin; IL: Interleukin; GM-CSF: Granulocyte macrophage colony-stimulating factor; mTOR: Mammalian target of rapamycin; PAUF: Pancreatic adenocarcinoma up-regulated factor; TAA: Tumor-associated antigen.

CONCLUSION

PDAC is the most common type of PC in the clinic. Multiple factors induce PC development and progression, including but not limited to inflammation, fibrosis, angiogenesis, EMT, and proliferation of CSCs. A combined panel of serum markers is very helpful for PC diagnosis, which is essential for curable therapy. Although several mono or combined therapies have been approved by the FDA for PC treatment, the overall 5-year survival rate is still not promising. The development of novel immunotherapies such as oncolytic viruses-mediated treatments and CAR-T, combined therapies (neoadjuvant therapy plus surgery), and advanced delivery systems of immunotherapy will improve therapeutic outcomes and combat drug resistance in PC patients. More clinical trials are required to evaluate the efficacy of existing treatments and to find new potent therapies.

FOOTNOTES

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REVIEW

Bile acids and microbes in metabolic disease

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Abstract

Bile acids (BAs) serve as physiological detergents that enable the intestinal absorption and transportation of nutrients, lipids and vitamins. BAs are primarily produced by humans to catabolize cholesterol and play crucial roles in gut metabolism, microbiota habitat regulation and cell signaling. BA-activated nuclear receptors regulate the enterohepatic circulation of BAs which play a role in energy, lipid, glucose, and drug metabolism. The gut microbiota plays an essential role in the biotransformation of BAs and regulates BAs composition and metabolism. Therefore, altered gut microbial and BAs activity can affect human metabolism and thus result in the alteration of metabolic pathways and the occurrence of metabolic diseases/syndromes, such as diabetes mellitus, obesity/hypercholesterolemia, and cardiovascular diseases. BAs and their metabolites are used to treat altered gut microbiota and metabolic diseases. This review explores the increasing body of evidence that links alterations of gut microbial activity and BAs with the pathogenesis of metabolic diseases. Moreover, we summarize existing research on gut microbes and BAs in relation to intracellular pathways pertinent to metabolic disorders. Finally, we discuss how therapeutic interventions using BAs can facilitate microbiome functioning and ease metabolic diseases.

Key Words: Bile acids; Metabolic diseases; Gut microbe; Diabetic mellitus; Obesity; Hypercholesterolemia

Core Tip: Bile acids (BAs) in enterohepatic circulation regulate metabolism through interorgan communication between the gut and liver microbiota. BAs secreted from the liver contribute to glucose and lipid metabolism. Disruption of the BA-gut microbiome link contributes to the occurrence of metabolic diseases, such as obesity, type 2 diabetic mellitus, and dyslipidemia. BAs and their metabolites can be used as potential therapeutics for treating metabolic diseases.

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INTRODUCTION

Bile acids (BAs) are unique amphipathic molecules that are primarily produced in the liver. They function as physiological detergents to facilitate bile flow and promote the transportation of nutrients, vitamins, and lipids *via* intestinal absorption[1]. Hepatic BA production accounts for a significant portion of the total cholesterol turnover in humans[2]. The principal constituents of bile are BAs, bilirubin, cholesterol, and phospholipids. BAs are mainly classified into primary and secondary types. Primary BAs (PBAs) include cholic acid (CA) and chenodeoxycholic acid (CDCA). Their corresponding secondary BAs are deoxycholic acid (DCA) and lithocholic acid (LCA), which are produced by microbial enzymes in the colon *via* deconjugation and 7α-dehydroxylation and are the most ubiquitous BAs in humans[3]. PBAs are formed by cholesterol in pericentral hepatocytes through a series of staged processes that are catalyzed by metabolic enzymes, particularly cytochrome P450 enzymes[4].

BAs synthesis is predominantly mediated by classic and alternative pathways in the liver. In the classic pathway, the rate-limiting enzyme CYP7A1 in the endoplasmic reticulum converts cholesterol into 7α-hydroxycholesterol (HOC). The intermediate 7α-hydroxy-4 cholesterin-3-one (C4) is converted by the sterol 12′α-hydroxylase (CYP8B1) to 7′α, 12′α-dihydroxy-4-cholesterin-3-one, which results in the production of CA. Without the 12α-hydroxylation of CYP8B1, C4 is ultimately transformed into CDCA. Both CA and CDCA syntheses use the mitochondrial enzyme CYP27A1 to catalyze the oxidation of the steroid side chains. In the alternative pathway, cholesterol is transformed by CYP27A1 into 27-HOC, which is in turn transformed into CDCA. Bacterial 7-dehydroxylase eliminates a hydroxyl group at C-7 in the large intestine, which converts CA into DCA and CDCA into LCA. The secondary BAs hyocholic acid, murideoxycholic acid, α-muricholic acid (ω-MCA), hyodeoxycholic acid (HDCA), and ursodeoxycholic acid (UDCA) are produced by CYP3A1 and epimerases from CDCA. The majority of LCA and ω-MCA are eliminated *via* feces[5].

In addition to their involvement in the absorption of dietary lipids and cholesterol homeostasis, BAs play a versatile signaling role. Many signaling pathways can be activated by BAs. These include a wide range of metabolic pathways, such as those involved in glucose, lipid, drug, and energy metabolism[6]. During BAs metabolism, cholesterol is converted into BAs in the liver and is further metabolized by the gut microbiota. Moreover, dense populations of microorganisms inhabit the gut, making it one of the most complex ecosystems for health. For the past two decades, research has focused on the influence of the gut microbiome on health. BAs deconjugation occurs in the small intestine and is mediated by bile salt hydrolase (BSH)-active bacteria, resulting in the maintenance of normal circulating levels of deconjugated BAs and cholesterol. Through these bioconversions, BAs modulate diverse metabolic pathways in the host through signaling mediated by nuclear farnesoid X receptors (FXRs) and G-protein-coupled membrane receptors (GPCRs). Furthermore, BAs can influence the gut microbial composition both directly and indirectly by activating innate immune responses. Consequently, the host metabolism is affected by altered signaling *via* BA receptors (BARs) induced by microbial modification and by altered microbiota composition[7]. Therefore, the gut microbiota must be maintained for normal metabolic function and homeostasis. Altered gut microbiota composition may be related to metabolic diseases, such as diabetes mellitus (DM) and obesity[8]. Altered BAs synthesis and function are also associated with metabolic diseases. This review mainly focuses on the relationship between gut microbiota and BAs in metabolic diseases, emphasizing on the BA-mediated reversal of metabolic diseases (Figure 1).

ROLE OF BILE ACIDS-GUT MICROBIOME INTERACTION IN METABOLIC REGULATION

The term microbiome refers to the entire genome of the gut microbiota, which is two orders of magnitude larger than the human nuclear genome. Humans inherit the vaginal microbiome of their mothers at the time of birth. Eventually, a mutualistic relationship between this microbiota and the host

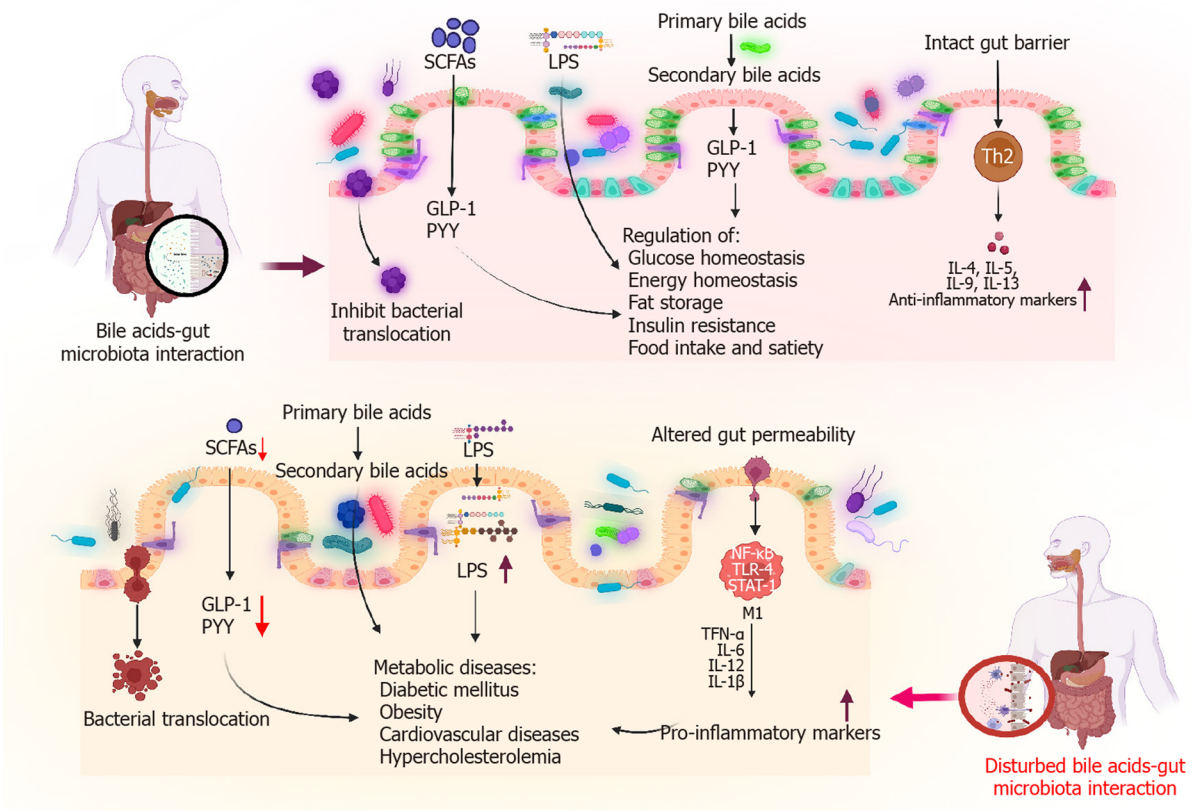

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Figure 1 Graphical abstract. GLP-1: Glucagon-like peptide-1; LPS: Lipopolysaccharide; PYY: Peptide YY; SCFAs: Short-chain fatty acids.

is developed[9]. The human gastrointestinal system is colonized by numerous microorganisms, collectively known as the gut microbiota; these include bacteria, viruses, archaea, fungi, and protozoa. The human gut microbiota contains up to 100 trillion microorganisms[10]. It plays an integral role in maintaining host health as it not only helps derive nutrients from food but also builds various metabolites that can regulate host metabolism[11]. One of these metabolites, BA, is produced in the liver through cholesterol and is additionally metabolized by the gut microbiota into secondary BAs[12]. A key physiological function of the gut microbiota is the modification of BAs composition. In addition to secondary BAs, different BAs species are produced in humans by the gut microbiota[13]. In the gut, branched-chain amino acids, short-chain fatty acids (SCFAs), indole, succinate, and imidazole are metabolites produced by gut microbes during anaerobic fermentation. These metabolites serve as key signaling components in the BA-gut microbe signaling pathways[14]. Various microbial genera produce these metabolites; these include *Akkermansia*, *Bacteroides*, *Clostridium*, *Coprococcus*, *Eubacterium*, *Faecalibacterium*, *Fusobacterium*, *Lactobacillus*, *Prevotella*, *Propionibacterium*, *Ruminococcus*, *Roseburia,* and *Streptococcus*[15]. The BAs composition is shaped by gut microbes that exhibit certain enzymatic activities, *e.g.*, BSH or 7-dehydroxylation activity mediated by BA-inducible enzymatic reactions. BAs exert their effects by activating a class of receptors known as BARs. This receptor family comprises nuclear receptors, such as FXRs, vitamin D receptors, pregnane X receptor, and GPCRs (including GPBAR1)[13]. The gut microbiota modulates fibroblast growth factor 15 (FGF15) signaling through an FXR-dependent mechanism[16]. Recent research has linked gut microbe metabolism to the size of the BAs pool.

In germ-free (GF) and conventionally raised mice, the gut microbiota could not only regulate secondary BAs metabolism but also inhibit hepatic BA synthesis by suppressing FXR inhibition in the ileum[16]. Moreover, BAs can affect the gut bacterial composition by directly and indirectly activating genes associated with innate immunity in the small intestine[7]. Therefore, bacteria-induced changes in BAs may result in altered signaling of BARs and affect host metabolism. BAs and the gut microbiota can interact in various ways, and interruptions in these physiological interactions can cause several diseases. The composition of the intestinal microbiota and/or the intraluminal metabolome may be the cause or consequence of various disorders; however, their association remains unknown. Various recent studies have reported the association of dyslipidemia, insulin resistance (IR), and DM with the dysregulation of BAs metabolism and alteration of the gut microbiota. This review mainly focuses on altered BA-gut microbiome interactions in metabolic diseases.

METABOLIC DISEASES CAUSED BY DISRUPTION OF BILE ACIDS-GUT MICROBIOME INTERACTIONS

Research on BA has significantly enriched our understanding of BAs synthesis and metabolic syndrome over the last two decades. BAs play a crucial role in regulating glucose, lipid, and energy metabolism. Several metabolic diseases, including type 2 DM (T2DM), obesity, and nonalcoholic fatty liver disease (NAFLD), result from disrupted BA homeostasis[17]. The gut microbiota is a "metabolic organ" that regulates host metabolism[18]. Gut microbes, including *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterobacter*, and *Lactobacillus* species, play an important role in the synthesis, modification, and signaling of BAs[15]. The gut microbiota has recently been reported to play a role in obesity, in addition to other widely acknowledged major causes, which include an increased caloric intake and decreased energy expenditure. These factors are also linked to T2DM, metabolic syndrome, and CADs[19]. Diverse mechanisms have been proposed by which gut microbes can modulate metabolic diseases. Disrupted BA-gut microbiome interactions can cause metabolic disease (Table 1 and Figure 2).

OBESITY

The global prevalence of various chronic diseases is increasing; obesity is the main cause and has been a serious concern for decades[20]. Obesity is linked to T2DM, NAFLD, hypertension, CAD, and cancer [21]. The prevalence of obesity is influenced by genetic and environmental factors, such as diet, culture, and socioeconomic status[22]. There is mounting evidence that the intestinal microbiota is inextricably related to general health, including obesity risk. Obesity-related metabolic diseases are defined by unique changes in the diversity and function of the human gut microbiome[23]. The human gut is home to trillions of microbes, which break down otherwise indigestible foods[24]. A study revealed that transferring the gut microbiota from healthy mice to GF recipients could increase body fat without a significant increase in food consumption and suggested that the composition of gut microbial communities could affect how much energy is derived from food[25]. In particular, the gut–brain axis indirectly affects commensal organisms, intestinal permeability, motility, and secretion and modifies the levels of plasma peptides, particularly glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), by releasing signaling molecules into the gut lumen[26].

In humans, the gut microbiota has been linked to body weight and weight loss following a lifestyle change. Gram-negative opportunistic pathogens in the gut may play a significant role in obesity[27]. The gut microbiota of ectomorphs has more *Bacteroidetes* species, whereas that of obese individuals has more *Firmicutes* species, particularly *Clostridium* clusters[23]. Thus, the bacterial composition could enhance the capacity of the host to absorb energy from their diet and retain it in adipose tissues[28]. In lean as well as obese pregnant subjects, an increase in *Bacteroides*, *Staphylococcus*[29], and *Bifidobacterium* species was found to increase high-density lipoprotein cholesterol (HDL-C) and folic acid levels and reduce triglyceride (TG) levels[22]. On the other hand, the abundance of *Akkermansia muciniphila* (*A. muciniphila*), a mucoprotein-degrading bacterium present in the mucus layer[30], was negatively correlated with body weight[22]. A decrease in the abundance of *A. muciniphila* was noted in obese and diabetic mice[31]. Feeding high-fat diets with viable *A. muciniphila* can hinder the development of metabolic disorders, such as obesity, low-grade inflammation, and metabolic endotoxemia[32]. A metatranscriptomic analysis revealed that mice receiving the microbiome of obese twins had higher expression levels of microbial genes associated with detoxification and oxidative stress, amino acid metabolism, cobalamin biosynthesis, and the pentose phosphate pathway[13].

BA metabolism is altered in obese and diabetic individuals[33]. Patti *et al*[34] reported that patients who underwent Roux-en-Y gastric bypass (RYGB) had improved glucose and fat metabolism. This finding was attributed to the activation of GPCRs and subsequent stimulation of GPBAR1 (TGR5, a membrane-bound BAR) and increase in deiodinase (a type II thyroid hormone) levels. Although recent research has revealed a link between the gut microbiota and obesity, the precise molecular pathways remain unknown. In particular, the role of distinct gut microbial species and their metabolites in the regulation of obesity-related lipid metabolism and formation of the obese phenotype remains unknown. Mechanisms linking the gut microbiota to obesity are being revealed through a collaborative approach of translation-focused human and animal studies. Increasing evidence indicates that the gut microbiota mediates the effects of diet on host metabolism[35]. In BA metabolism, TGR5 signaling is regulated by the microbiota by generating agonists[36], whereas FXR signaling is regulated by metabolizing antagonists[3]. Both TGR5 and FXR have a significant influence on metabolism, and an altered microbiota may impact host physiology by modifying the signals transmitted through these receptors. The ability to metabolize TauroMCA, a naturally occurring FXR antagonist, is required for the microbiota to induce obesity, steatosis, and impaired glucose and insulin tolerance. An altered microbiota is responsible for these effects[37]. Taken together, these results indicate that targeting BAs, which function as microbiome-produced molecular regulators of energy homeostasis, can offer a substantial opportunity for treating obesity.

Table 1 Metabolic diseases caused by altered bile acid–gut microbiome interactions

No.	Model	Findings	Ref.
1	A T1DM clinical study	The abundance of *Alistipes shahii*, *Asaccharobacter celatus*, *Blautia obeum*, *Coprococcus eutectic*, *Coprobacillus cateniforms*, *Clostridium symbiosum*, and *Eggerthella lenta* significantly increased in adolescents with T1DM. Compared with healthy adolescents, the biosynthesis of vitamins, amino acids, electron carriers, and enzyme cofactors was downregulated, whereas fermentation pathways were upregulated in adolescents with T1D	[150]
2	An HFD-fed obese mouse model	Non-12-OH BA levels were higher in HF-OR mice. The levels of non-12-OH BASs, such as UDCA, CDCA, and LCA, decreased in HF-OP mice and were linked to changed gut flora. The abundance of *C. scindens* were reduced in HF-OP mice and positively correlated with UDCA and LCA. The administration of *C. scindens* to animals increased the levels of hepatic non-12-OH BAs and serum 7-hydroxy-4-cholesterin-3-one (C4). Changes in BA composition in HF-OP mice were associated with considerably lower GLP-1 expression levels in the ileum and PGC1 and UCP1 expression levels in brown adipose tissues	[18]
3	Patients with GDM and germ-free mice	The abundance of *Bacteroides and Akkermansia* decreased and that of *Faecalibacterium* increased with hyperglycemia	[151]
4	Women with GDM: A clinical study	The relative abundance of *Streptococcus*, *Faecalibacterium*, *Veillonella*, *Prevotella*, *Haemophilus*, and *Actinomyces* significantly increased with an increase in FBG levels and hyperlipidemia	[51]
5	A combination of BAs with dietary lard feeding in C57BL/6N mice	Impaired glucose tolerance; lower fasting insulin levels; lower counts of enteroendocrine cells; fatty liver; and elevated levels of hepatic TGs, cholesteryl esters, and monounsaturated fatty acids were noted. The relative abundance of *Lachnospiraceae* decreased and that of *Desulfovibrionaceae*, *Clostridium lactatifermentans*, and *Flintibacter butyricus* increased	[152]
6	A T2DM clinical study	Postprandial total BAs levels increased with an increase in the meal fat content and peaked after 1-2 h. Unconjugated and glycine-conjugated forms of DCA, CA, and UDCA were altered and FGF-19 levels were reduced in participants with T2DM	[153]
7	HFD-fed C57BL/6J mice with *Enterobacter cloacae* B29	Obesity and IR were induced	[45]
8	A T2DM clinical study	BAs increased twofold, and more hydrophobicity and higher 12α-hydroxy/non-12α-hydroxy BAs ratios were linked with lower insulin sensitivity and higher plasma TG levels	[154]
9	C57BL/6J ob/ob mice, lean ob/$^{+}$, and HFD-fed mice	The abundance of *A. muciniphila* decreased in mice who were obese and had T2DM	[32]
10	A clinical study	The postprandial total bile acid response decreased in obese participants	[155]
11	Pregnancy with obesity: A clinical study	The abundance of *Bifidobacterium* and *Bacteroides* decreased and that of *Staphylococcus,* Enterobacteriaceae, and *E. coli* increased in overweight pregnant women compared with that in normal-weight pregnant women. The abundance of *E. coli* was higher in women with excessive weight gain than in those with normal weight gain during pregnancy. *Bifidobacterium* and *A. muciniphila* showed an opposite trend. The abundance of total bacteria, *Staphylococcus*, *Bacteroides*, *Bifidobacterium*, Enterobacteriaceae, and *E. coli* increased and that of *Bifidobacterium* decreased	[22]
12	ApoA-1-knockout mice, HFD-fed mice, and wild-type mice	Gut barrier-protecting *Bifidobacterium* species were absent, and impaired glucose tolerance was significantly increased	[27]
13	Zucker rats (obese/lean)	Increased numbers of *Halomonas* and *Sphingomonas* species, plasma LDL and VLDL levels, and reduced urinary hippurate and creatinine levels were noted in obese rats	[156]
14	Overweight pregnant women: A clinical study	Increased numbers of *Bacteroides* and *Staphylococcus* species were noted in obese pregnant women	[29]
15	HFD-fed mice	The abundance of intestinal gram-negative and gram-positive bacteria and *Bifidobacterium* species significantly decreased and endotoxemia significantly increased	[146]
16	C57BL/6J ob/ob mice, lean ob/$^{+}$, and wild-type mice	A 50% reduction was noted in the abundance of *Bacteroidetes*, and an increase was noted in the abundance of *Firmicutes*	[157]

ApoA-1: Apolipoprotein A-1; *A. muciniphila*: *Akkermansia muciniphila*; BAs: Bile acids; BASs: Bile acid sequestrants; C4: 7α-hydroxy-4-cholesten-3-one; CA: Cholic acid; CDCA: Chenodeoxycholic acid; *C. scindens*: *Clostridium scindens*; DCA: Deoxycholic acid; *E. coli*: *Escherichia coli*; FGF-19: Fibroblast growth factor 19; FBG: Fasting blood glucose; GDM: Gestational diabetes mellitus; GLP-1: Glucagon-like peptide-1; HFD: High-fat diet; LCA: Lithocholic acid; LDL: Low-density lipoprotein; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; TGs: Triglycerides; UDCA: Ursodeoxycholic acid; VLDL: Very-low-density lipoprotein.

DM

The prevalence of T2DM is increasing worldwide. By 2030, the incidence of T2DM is expected to be 360 million worldwide, with the estimated population being 8.5 billion[38]. BAs are involved in the alteration of glucose metabolism associated with obesity and T2DM. By stimulating GLP1 synthesis in

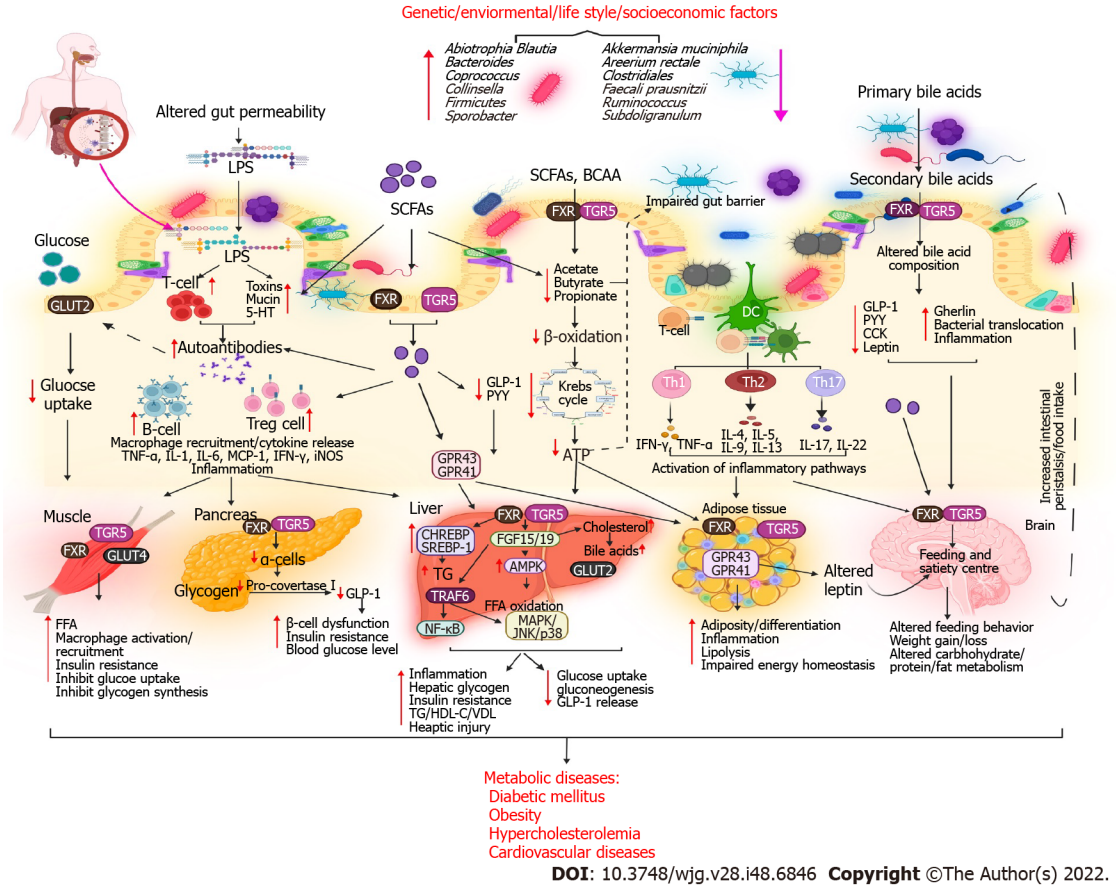

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Figure 2 Metabolic diseases resulting from altered bile acid–gut microbiome interactions. The health and gut microbiota of individuals are affected by genetic, environmental, lifestyle, and socioeconomic factors. Due to altered gut microbial composition, the permeability of the gut barrier is impaired, facilitating pathogen invasion in the intestinal lumen *via* receptor-mediated pathways. Increased levels of lipopolysaccharide and short-chain fatty acids trigger the immune system, result in the production of autoantibodies (B cells and Treg cells), and cause the infiltration of macrophages, which release toxins and lead to inflammation and metabolic endotoxemia. Conversely, the release of glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and ghrelin is decreased, altering carbohydrate, protein, and fat metabolism. When glucose uptake is decreased in the intestine, free fatty acid synthesis is increased, macrophages are activated, and muscles become insulin resistant. When circulating autoantibodies enter the pancreas, they destroy α and β cells, thereby reducing insulin secretion and enhancing insulin sensitivity. A decrease in FGF15/19, CHREBP, SREBP-1, and TRAF6 expression levels in the liver activates NF-κB/MAPK inflammatory pathways, resulting in liver dysfunction and altered fat metabolism in the liver. Proinflammatory markers and reduced GLP-1, PYY, and ghrelin levels affect adipose tissue function, leading to increased adiposity and impaired energy metabolism. In the brain, altered levels of GLP-1, PYY, ghrelin, and leptin affect feeding and satiety centers. These changes lead to the development of metabolic diseases. FFA: Free fatty acid; GLP-1: Glucagon-like peptide-1; SCFAs: Short-chain fatty acids; LPS: Lipopolysaccharide; PYY: Peptide YY; CCK: Cholecystokinin; FXR: Farnesoid X receptor; CCK: Cholecystokinin; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; VLDL: Very-low-density lipoprotein.

the small bowel and colon, BAs contribute to carbohydrate and fat metabolism. In addition to inducing IR and T2DM, BAs exhibit an insulin-sensitizing effect[33]. They control glucose homeostasis by directly acting on FXR and TGR5 in the liver, intestine, and pancreas and by indirectly stimulating FXR-dependent intestinal FGF15/19 production[39]. Both FXR and TGR5 are abundant in pancreatic b cells, where they favorably control insulin production and glucose-induced insulin secretion. TGR5 activation in pancreatic α cells promotes the expression of proconvertase-1, which shifts the synthesis of glucagon to GLP-1, thereby enhancing β-cell density and functioning in a paracrine manner. In patients with DM, BAs may promote FXR activation in L cells in the ileum. In an animal model of obesity, Cipriani *et al*[40] found that 6E-CDCA activated FXR, reversed IR, and restored lipid metabolism. Moreover, the microbiota could downregulate FXR with the maximum efficiency by converting PBAs into secondary BAs[16].

External factors, such as diet, can alter the gut microbiota and cause dysregulation and secretory changes in intestinal microbial metabolites, triggering a series of possible mechanisms that lead to DM and insulin sensitivity[41]. In a metagenome-wide association study involving 345 Chinese participants with DM, gut microbial dysbiosis caused by opportunistic pathogens was moderate. Moreover, the reduction of butyrate-producing bacteria was associated with sulfate reduction and oxidative stress resistance[42]. Complex interactions between the immune system and gut microbiome have also been linked to both T1DM and T2DM. Aggarwal *et al*[43] reported that a combination of antidiabetic and antibiotic treatments reversed IR, hyperglycemia, and dyslipidemia and normalized blood glucose utilization in iNOS$^{-/-}$ mice. Duodenal-jejunal bypass liner (DJBL) replacement in obese patients with

T2DM was found to increase unconjugated BA levels in a clinical study[44]. Fei *et al*[45] reported a high percentage of endobacteria (35%), pathogenic bacteria that produce endotoxin, in the gut microbiome of obese participants with hypertension, DM, and other severe metabolic complications. Patients with T2DM are particularly deficient in butyrate-producing microbes, such as *Clostridiales* species, *Ruminococcus* species, *Subdoligranulum* species, *Areerium rectangle*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *R. inulinivorans*, and exhibit a high abundance of specific genera, such as *Abiotrophia*, *Blautia*, *Coprococcus*, *Collinsella*, *Parasutterella*, *Peptostreptococcus*, and *Sporobacter*[15].

Moreover, Lambeth *et al*[46] demonstrated that *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Pseudonocardiaceae*, *Verrucomicrobia*, and *Colorado* species were significantly more prevalent in the pre-DM stage, whereas *Enterobacteriaceae* and *Collinsella* species were significantly more prevalent in patients with T2DM. Similarly, Larsen *et al*[47] reported a significant decrease in the prevalence of phylum *Firmicutes*, class Betaproteobacteria, and genus *Clostridium* in patients with T2DM. The *Bacteroidetes*: *Firmicutes* ratio and the *Bacteroides-Prevotella* group: *C. coccoides-E. rectale* group ratio showed a positive correlation and significantly increased the plasma glucose levels. During pregnancy, the abundance of the beneficial species *R. intestinalis* and *F. prausnitzii* decreases, whereas that of Proteobacteria and Actinobacteria phyla increases[48]. If these compositions are altered, pregnant women may experience an increase in adipose mass, blood sugar levels, IR, and circulating proinflammatory cytokines, resulting in gestational DM (GDM)[49]. In patients with GDM, obesity, and T2DM, the relative abundance of SCFA-producing bacteria belonging to the genera *Faecalibacterium*, *Rubinococcus*, *Roseburia*, *Coprococcus*, *Akkermansia*, *Phascolarctobacterium*, and *Eubacterium* was found to decrease[50]. Moreover, Liu *et al*[51] demonstrated increased hyperlipidemia and fasting blood glucose (FBG) levels and increased relative abundance of *Streptococcus*, *Faecalibacterium*, *Veillonella*, *Prevotella*, *Haemophilus*, and *Actinomyces* species in patients with GDM.

THERAPEUTIC INTERVENTIONS TARGETING BILE ACIDS-GUT MICROBIOME INTERACTIONS TO ALLEVIATE METABOLIC DISEASES

BAs play an important role in signaling and metabolism, reigniting interest in these molecules as potential therapeutic targets. Studies have revealed that drugs used to treat metabolic diseases can alter the gut microbial environment. Similarly, antidiabetic medications may alter the composition of the gut microbiota, plasma, and fecal BAs, which may improve metabolic health. Notably, patients with T2DM had better glycemic control when taking medications for preventing BAs absorption from the small intestine or limiting enterohepatic circulation. Hence, experimental and clinical studies have focused on the therapeutic applications of BAs in metabolic diseases. Furthermore, microbiota targeting could open novel research avenues. Table 2 and Figure 3 depict BAs and their metabolites used for treating metabolic diseases.

BAS METABOLITES ALLEVIATE METABOLIC DISEASES

BA sequestrants

For several years, BA sequestrants (BASs) have been utilized as therapeutics for patients with dyslipidemia and T2DM[52]. The BA-binding properties of BASs reduce the amount of BAs in enterohepatic circulation, thereby accelerating the conversion of cholesterol to BAs[53]. The effects of BASs on hyperglycemia have been demonstrated in both animal and clinical models of T2DM[54]. Furthermore, animal studies have revealed that BAs and BASs influence energy expenditure. A BAS molecule, also known as a resin, is a large, nonabsorbable polymeric molecule that binds negatively charged bile salts to the intestinal lining[55]. This promotes BA excretion through the feces by diverting the acids from the enterohepatic cycle. Consequently, BA synthesis increases and low-density lipoprotein (LDLR) receptors are upregulated. BASs can lower blood glucose as well as cholesterol levels, which may be beneficial for treating T2DM[54].

BASs can exert their metabolic effects beyond cholesterol-lowering effects through several mechanisms, including GLP-1, the FXR-small heterodimer partner-liver X receptor pathway, and TGR5 [56]. BAS reduces intestinal FXR activity by trapping BAs in the lumen, resulting in decreased expression levels of ileal *Shp* and *Fgf15*[57]. The resulting decrease in the hepatic accessibility of BA and FGF15/19 leads to the deactivation of hepatic FXR and the CYP7A1-mediated conversion of cholesterol to BA, reducing LDL cholesterol (LDL-C) levels. Consequently, lipogenesis is attenuated by FXR. BAS raises plasma TG levels and accumulates hepatic lipids, while lowering LDL-C levels[58]. However, the exact mechanism by which BASs exert their metabolic effects beyond cholesterol-lowering effects remains unknown. Rectal administration of taurocholic acid (TCA) was found to increase GLP-1 and PYY production in obese participants and those with T2DM[59]. Similarly, CDCA and colesevelam increased glycogen and GLP-1 levels and delayed stomach emptying in patients with T2DM[60].

Table 2 Drugs that target bile acids and gut microbes to alleviate metabolic diseases

No.	Drugs	Model	Findings	Ref.
1	A combination of GLP-1 and DMR	An insulin-dependent T2DM clinical study	Combined treatment allowed the discontinuation of insulin treatment in 69% of patients, increased postprandial unconjugated bile acid responses, induced an overall increase in the secondary bile acid response, induced an increase in the 12α-hydroxy: non-12α-hydroxy BA ratio, and improved the microbiome response	[158]
2	Colesevelam	Germ-free C57BL/6 mice with obesity, NAFLD, and NASH	Reduced body and liver weight gain were noted in microbiome-humanized mice, in addition to the amelioration of hepatic inflammation, steatosis, fibrosis, and IR. Colesevelam increased *de novo* bile acid synthesis and reduced the hepatic cholesterol content in microbiome-humanized mice, induced the expression of the antimicrobial genes *Reg3g* and *Reg3b* in the distal small intestine, and reduced plasma LPS levels	[159]
3	Vancomycin	iNOS$^{-/-}$ mice	Metabolic disturbances, dyslipidemia, and insulin resistance in iNOS$^{-/-}$ mice were improved by the vancomycin-mediated reduction of gram-positive bacteria	[160]
4	Sevelamer	Western diet-fed C57BL/6J mice with NASH	Interruption of intestinal reabsorption and reduction of circulating bile acid levels were noted. Microbiota complexity in the cecum was reversed by increasing the abundance of *Lactobacillus* and decreasing the abundance of *Desulfovibrio*. Hepatic injury was reversed, and the progression of NASH, including steatosis, inflammation, and fibrosis, was inhibited	[161]
5	Sevelamer	CDHF-fed C57BL/6J mice	Hepatic steatosis, macrophage infiltration, and pericellular fibrosis were prevented in CDHF-fed mice. The portal levels of total bile acid were reduced, and hepatic and intestinal FXR activation was inhibited. The α-diversity was decreased, and decreases in *Lactobacillaceae* and *Clostridiaceae* populations and increases in *Desulfovibrionaceae* and *Enterobacteriaceae* populations were prevented in CDHF-fed mice. Intestinal tight junction proteins were restored and portal LPS levels were reduced, resulting in the suppression of the hepatic toll-like receptor 4 signaling pathway	[162]
6	*B. animalis* 01	A T2DM rat model	Treatment with *B. animalis* 01 improved OGTT, HOMA-IR, and lipid profiles; reduced hepatic tissue injury; increased glycogen levels; improved antioxidant levels; and modulated the expression of genes involved in hepatic glucose metabolism and the IRS/PI3K/AKT pathway. Moreover, it positively regulated the hepatic Keap1/Nrf2 pathway	[141]
7	*A. muciniphila*	Overweight/obese insulin-resistant volunteers	*A. muciniphila* improved insulin sensitivity; reduced insulinemia and plasma total cholesterol levels; and slightly reduced body weight, fat mass, and hip circumference. Three months after supplementation with *A. muciniphila*, liver dysfunction and inflammatory blood marker levels decreased without affecting the gut microbiome structure	[132]
8	*Bacteroides* transplantation	A clinical study on children with diabetes/germ-free NOD mice	Compared with germ-free NOD mice, the onset of diabetes was markedly delayed in all bacteriome-humanized participants	[163]
9	*A. muciniphila*	C57BL/6 mice/HFD-fed mice	*A. muciniphila* treatment reversed HFD-induced fat mass gain, metabolic endotoxemia, adipose tissue inflammation, and IR. *A. muciniphila* supplementation increased the intestinal levels of endocannabinoids that control inflammation, the gut barrier, and gut peptide secretion	[32]
10	Acarbose	A clinical study	The ratio of primary: secondary BAs and plasma levels of unconjugated BAs were increased. The relative abundance of *Lactobacillus* and *Bifidobacterium* species in the gut microbiota was increased and *Bacteroides* species were depleted in participants with T2DM	[164]
11	Metformin	A clinical study	Metformin-altered microbiota improved glucose tolerance, and a significant negative correlation was noted between unconjugated BAs and HbA1c levels	[165]
12	*Lactobacillus acidophilus*, *L. casei*, and *Bifidobacterium bifidum* for 6 wk	GDM: A clinical study	Significant reductions were noted in fasting plasma glucose, serum insulin, serum triglyceride, and VLDL cholesterol levels and a significant increase was noted in the quantitative insulin sensitivity check index in women with GDM	[119]
13	Probiotics	Cherry Valley Pekin ducks	The LXRα and CYP7α1 enzymatic activity increased and TG and TC concentrations decreased	[123]
14	Probiotics (*Lactobacillus salivarius* UCC118)	GDM: A clinical study	The body weight, FBG, and IR index significantly decreased and insulin sensitivity index increased in women with GDM	[166]
15	Probiotics (*Lactobacillus salivarius* UCC118)	Obese pregnant women: A clinical study	Significant alteration was noted in the BMI	[167]
16	Probiotic *Lactobacillus sporogenes*	Third-trimester pregnancy: a Clinical study	A significant decrease was noted in serum insulin levels and HOMA-IR, and a significant difference was noted in HOMA-B	[168]
17	Probiotics (VSL#3)	C57J/B6 male mice/HFD-fed mice	Probiotic supplementation reduced the body weight IR; modulated the gut microbe composition; and increased GLP-1 release, glucose tolerance, SCFA levels, and butyrate levels	[121]
18	Probiotics	Pregnant women: A	A significant reduction was noted in serum total LDL, HDL cholesterol, serum TG, and	[122]

		clinical study	serum TC levels	
19	Fecal microbiota transplantation	Male Caucasian obese participants	Improvement in peripheral and hepatic insulin sensitivity was noted, along with an increase in butyrate-producing intestinal microbiota	[126]
20	Probiotics	Obese (ob/ob) mice	An increase in the abundance of *Bifidobacterium* species reduced metabolic endotoxemia and inflammation. Intestinal permeability was lowered by altering GLP-2 levels	[147]
21	Probiotics (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12; diet/probiotics)	First-trimester pregnancy: A clinical study	Reduced blood glucose and insulin levels, improved glucose tolerance, and the highest quantitative insulin sensitivity check index were noted	[169]

A. muciniphila: *Akkermansia muciniphila*; BAs: Bile acids; *B. animalis*: *Bifidobacterium animalis*; BMI: Body mass index; CDHF: Choline-deficient high-fat diet; DMR: Duodenal mucosal resurfacing; FBG: Fasting blood glucose; FXR: Farnesoid X receptor; GDM: Gestational diabetes mellitus; GLP-1: Glucagon-like peptide-1; HFD: High-fat diet; HbA1c: Hemoglobin A1C; HDL: High-density lipoprotein; HOMA: Homeostatic model assessment; IR: Insulin resistance; *L. casei*: *Lactobacillus casei*; LDL: Low-density lipoprotein; LPS: Lipopolysaccharide; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; SCFAs: Short-chain fatty acids; T2DM: Type 2 diabetes mellitus; TC: Total cholesterol; TGs: Triglycerides; VLDL: Very-low-density lipoprotein.

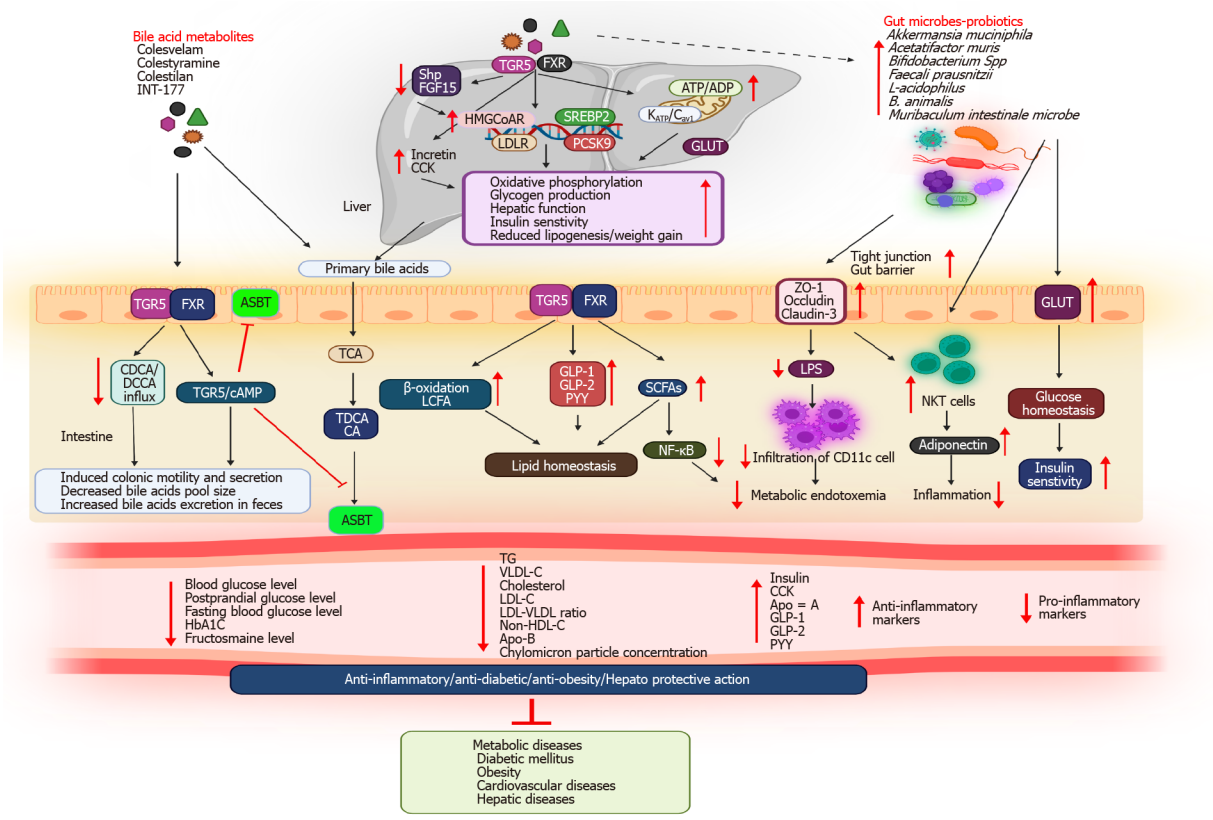

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Figure 3 Mechanism by which bile acid metabolites and gut microbes alleviate metabolic diseases. Bile acid metabolites, *e.g.*, bile acid sequestrants (BASs), enter the liver *via* receptor-mediated pathways. As a result of their effects, mitochondrial oxidative phosphorylation is stimulated; *HMGCoAR*, *LDLR*, and *SREBP2* gene expression is induced; incretin and cholecystokinin levels are increased; and the abundance of intestinal bacteria is increased, reducing the levels of triglyceride (TG), very-low-density lipoprotein (VLDL), and cholesterol in the blood. By increasing the expression of ZO-1, occludin, and claudin-3 in the gut lumen, probiotics facilitate tight junction proteins, preventing macrophage infiltration and metabolic endotoxemia. In contrast, BAS improves colonic motility; increases glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and ghrelin secretion; and regulates carbohydrate, fat, and energy metabolism, thereby reducing blood glucose, TG, cholesterol, and VLDL levels and improving insulin sensitivity and liver function. Because of their anti-inflammatory, antidiabetic, and antiobesity properties, BASs and gut microbes alleviate metabolic diseases. CDCA: Chenodeoxycholic acid; TDCA: Taurodeoxycholic acid; GLP-1: Glucagon-like peptide-1; SCFAs: Short-chain fatty acids; LPS: Lipopolysaccharide; PYY: Peptide YY; FXR: Farnesoid X receptor; CCK: Cholecystokinin; TG: Triglycerides; LDLR: Low-density lipoprotein; NKT: natural killer T.

Colestyramine

Cholestyramine is a polystyrene-based polymer that has been crosslinked with divinylbenzene and functionalized to quaternary ammonium units to produce a robust anion exchange resin and increase the secretion of the pancreatic exocrine hormone cholecystokinin (CCK)[61]. A study revealed that cholestyramine administration increases the expression levels of genes encoding HMGCoAR, LDLR,

PCSK9, and SREBP2[62]. Cholestyramine stimulates hepatic BA synthesis from cholesterol, which activates SREBP2 by inhibiting BA absorption from the intestine. LDLR increases the transport of cholesterol from the plasma when SREBP2 is expressed. The upregulation of HMGCoAR compensates for the reduction in LDL-C levels in the plasma. In addition to activating SREBP2, PCSK9 gets upregulated, thereby degrading LDLR. By modulating PCSK9, cholestyramine-induced increases in LDLR expression can be modulated[58]. In addition to treatments using cholestyramine and inhibitors of ileal BA uptake, treatments aimed at reducing PCSK9 expression would be beneficial for reducing the enterohepatic circulation of BAs[63].

Similarly, several clinical and experimental models have revealed that cholestyramine improves BA-gut microbiome interactions, thereby facilitating glucose and fat metabolism. In clinical models of primary biliary cholangitis (PBC), two SCFA-producing *Lachnospiraceae* species were found to be enriched in the microbiome of the superior remission group after cholestyramine treatment. SCFAs derived from dietary fibers are produced by the gut microbiota, and SCFA signaling has anti-inflammatory, antiobesity, and antidiabetic properties[52]. This denotes the favorable effects of cholestyramine in treating PBC by enhancing BA-gut microbiome interactions[64]. Newman *et al*[57] reported that cholestyramine reduced hyperglycemia by increasing the ileal expression of glucagon through an increase in the prevalence of *Acetatifactor Muris* and *Muribaculum intestinal*. In another study, cholestyramine-treated ZDF rats showed reduced glycosylated hemoglobin A1c (HbA1c) levels, serum glucose levels, and FXR activation and increased PYY levels, GLP-1 Levels, and insulin release[65].

Colesevelam

Colesevelam hydrochloride (HCl) is a polyallylamine that has been crosslinked with epichlorohydrin and alkylated with (6-bromohexyl)-trimethylammonium bromide and 1-bromodecane[66]. In clinical and animal studies on T2DM, obesity, and hyperlipidemia, colesevelam reduced blood glucose[67], FBG [68], mediator complex subunit 1, miR-182[69], HbA1c[70], hepatic TG, total LDL[71], very-low-density lipoprotein (VLDL), chylomicron particle[72], LDL-C[73], non-HDL-C, ApoB, TGR5/GLP-1-dependent glycogenolysis, FXR-dependent cholesterol, cytochrome P450, Cyp7a1[74], FGF-19[75], BA reabsorption [76], high-sensitivity C-reactive protein[77], and fructosamine levels[78-80] and increased glycolysis, postmeal glucose tolerance, insulin levels[81], splanchnic sequestration of meal-derived glucose[82], GLP-1/GIP levels[83], total HDL particle levels, miR-96/182/183 expression levels, β-cell function [as revealed by homeostatic model assessment (HOMA)][56], BA synthesis, ApoA-1 levels[54], and CCK levels[84]. As a molecularly engineered, second-generation BA sequestrant, colesevelam has been recommended for reducing LDL-C in patients with primary hypercholesterolemia by inhibiting b-hydroxymethylglutaryl coenzyme A reductase[85]. Colesevelam enhances glycemic control in patients with T2DM[86]. When metformin-based, sulfonylurea-based therapy fails to completely control T2DM, colesevelam can improve glycemic and lipid indices[54]. Moreover, colesevelam significantly alters BA metabolism. A non-absorbable complex of colesevelam in the gastrointestinal tract can stimulate the excretion of BAs through feces and their removal from enterohepatic circulation. Therefore, colesevelam treatment may reduce the total BA pool size[75]. Colesevelam reduces the influx of CDCA and DCA, two of the most potent FXR ligands, into ileal enterocytes. Therefore, plasma levels of FGF19 are likely to decrease when FXR is less activated[75].

Colestimide

Colestimide, an anion exchange resin, lowers serum cholesterol levels by binding to BAs in the intestinal tract[87]. Although colestimide is used to treat hyperlipidemia in Japanese patients, the mechanism by which it lowers blood glucose levels remains poorly understood[88]. CA reduces blood glucose levels and facilitates energy metabolism through the type 2 iodothyronine deiodinase (D2) enzyme. Various clinical and experimental studies have revealed that colestimide treatment reduced blood glucose, FBG, postprandial blood glucose, HbA1c, IR, and serum LDL-C levels and increased serum 1,5-AG and postprandial plasma GLP-1 Levels in patients with T2DM[89-91]. Another study revealed that colestimide altered BA composition and CA ratios, thereby reducing blood glucose levels *via* the TGR5-Camp-D2 pathway[92]. Similarly, elobixibat induced colonic motility and secretion by inhibiting an ileal BA transporter in a highly selective manner[93], reduced the LDL-C levels and LDL-C: HDL-C ratio, and increased the circulating GLP1 levels in a clinical study on dyslipidemia[94]. Colestilan is also a BAS that could reduce body weight and HbA1c, FBG, LDL-C, and total cholesterol levels and increase fecal lipid excretion in patients with T2DM[95].

Receptor-mediated therapeutics

Since BAs were initially considered lipid solubilizers, they have evolved into complex metabolic integrators. BAs can modulate their energy expenditure through the stimulation of TGR5- and FXR-mediated signaling pathways[36]. The metabolism-related protein TGR5 may be a novel promising target for treating metabolic disorders associated with obesity. Recently, TGR5 expression has been reported in enteroendocrine L cells, including STC-1 cells, which secrete GLP-1 upon calorie intake[96]. In preclinical studies, INT-177 (a semisynthetic BA derivative) and nonsteroidal TGR5 agonists promoted glucose homeostasis[97]. The activation of TGR5 by BAs reduced diet-induced obesity by

increasing energy expenditure in brown adipose tissues and muscles[97]. Moreover, TGR5-mediated release of GLP-1 modulated the ATP/ADP ratio and oxidative phosphorylation in the mitochondria by activating the K_{ATP}/C_{av} channels. Thus, the TGR-5-mediated pathway is therapeutically beneficial, considering that incretin-based therapies are effective in treating DM[98]. Moreover, FXR activation has not yet been associated with significant weight loss[99]. FXR activation reduces hepatic glucose and fatty acid outputs by increasing glycogen production and decreasing lipogenesis and VLDL production, thereby increasing insulin sensitivity[99]. Similarly, 6E-CDCA was found to reduce blood glucose, insulin, TG, and plasma cholesterol levels and fatty acid synthesis and facilitate FXR activation in Zucker (fa/fa) obese rats with liver steatosis[40]. Moreover, tauroUDCA increased muscular and hepatic insulin signaling by phosphorylating the insulin receptor substrate Tyr and Akt at Ser473 in obese participants[100]. In summary, TGFR5 agonists activate the TGR5 signaling pathway by increasing mitochondrial function and enteroendocrine cell function, ultimately leading to increased incretin release. This has various metabolic effects, including reduction of weight gain and hepatic steatosis, improvement of liver function, and maintenance of insulin sensitivity and glucose homeostasis.

BAs metabolites and bariatric surgery

CDCA (3α,7α-dihydroxy-5β-cholan-24-oic acid) is a PBAs produced in the liver from cholesterol. CDCA is a potent inhibitor of CYP7A1, the enzyme responsible for BA synthesis. In addition to suppressing cholesterol synthesis, CDCA may inhibit HMGCoA reductase[101]. Mantovani *et al*[102] reported decreased plasma levels of CA and TCA but significantly increased plasma levels of TCDCA, TDCA, HDCA, GDCA, GLCA, and DCA in patients with T2DM. Moreover, Cariou *et al*[103] reported that plasma levels of CDCA, CA, and DCA were negatively correlated with insulin sensitivity in patients with T2DM. CDCA may inhibit high-fat diet (HFD)induced obesity and hyperglycemia through the activation of TGR5 and inhibition of PPARγ transcriptional activity[104]. Another study revealed that CDCA increased GLP-1 and glucagon secretion and delayed gastric emptying by activating GPBAR1 in patients with T2DM[60]. The activation of FXR and TGR5 through CDCA and DCA mimicked and suppressed SPX promoter activity induced by CDCA and DCA. SPX promoter activity was significantly increased by adenylate cyclase (AC)/cAMP activators and reduced by CDCA, DCA, and PKA pathway inhibitors. Through FXR- and TGR5-mediated AC/cAMP/PKA and MAPK cascades, CDCA and DCA could promote SPX expression at the hepatic level[105].

Obeticholic acid (OCA, 6E-CDCA) is a semisynthetic BAs with a 30-fold higher potency than that of CDCA for activating FXR. OCA-mediated inhibition of BAs synthesis increased the abundance of *Firmicutes* species and reduced nonalcoholic steatohepatitis in humans[106]. UDCA is commonly used for treating liver dysfunction. UDCA treatment reduced hyperinsulinemia and fasting hyperglycemia in a mouse model of T2DM with hepatic steatosis[107]. Moreover, Osorio *et al*[108] reported that UDCA inhibited sodium–glucose co-transporter overexpression, thereby reducing oxidative stress in mice with streptozotocin (STZ)-induced DM. A recent meta-analysis revealed that UDCA significantly reduced fasting plasma glucose, HbA1c, and insulin levels, indicating a positive impact on glucose homeostasis [109]. Another clinical trial demonstrated that UDCA treatment reduced HbA1c levels and increased early-phase GLP-1 secretion[110].

Bariatric surgery is effective in treating obesity, DM, and related complications. However, this surgery is not the only factor responsible for treating obesity. Bariatric surgery alters gut microbiota profiles and induces gut microbes to synthesize SCFAs. Gut microbes are crucial for improving the outcomes of bariatric surgery. Gut microbes are also important for reducing weight and lowering adverse events after bariatric surgery. Therefore, prebiotics, probiotics, and postbiotics are recommended for patients who have undergone bariatric surgery in order to improve their clinical outcomes[111]. Bariatric surgery causes changes in the gut microbiota because of a malabsorptive status and changes in BA metabolism, gastric pH, and hormone metabolism[112]. It may also change the levels of hormones, such as leptin and ghrelin. Changes in hormones have been reported as a result of energy metabolism and the microbiota. Prebiotics modulate the intestinal microbiota and reduce the levels of ghrelin in the blood; however, the relationship between the two is not fully understood[113]. Similarly, postsurgical microbiomes were more different from lean microbiomes than obese microbiomes, whereas postsurgical microbiomes were less different from lean microbiomes than obese microbiomes. Body mass index loss following bariatric surgery could be predicted based on the presurgical microbiome. After surgery, the relative abundance of Proteobacteria and Fusobacteria increased, whereas that of *Firmicutes* decreased[114]. On the other hand, in patients with mild obesity, RYGB is an effective treatment option. It can also improve the metabolic and inflammatory status. Lau *et al*[115] reported that RYGB altered 29 rich bacterial genera in the gut microbiota of patients with T2DM. To better understand the weight-independent antidiabetic mechanisms of RYGB, researchers have developed DJB surgery. Han *et al*[116] demonstrated that DJB increased intraduodenal BAs levels and upregulated duodenal SIRT1 expression in rats with HFD- and STZ-induced DM. Patients with T2DM reported significant and long-lasting glycemic improvements after undergoing duodenal mucosal resurfacing, an endoscopic technique that involves circumferential hydrothermal ablation and subsequent regeneration of the duodenal mucosa[117].

Gut microbe-mediated alleviation of metabolic diseases

Studies have revealed that obesity alters microbial composition and nature[23,28]. The development of metabolic illnesses, such as obesity and T2DM, has recently been linked to the gut microbiota. Increasing attention has been paid to altering the gut microbiota for treating metabolic diseases. Numerous microbial compositions (probiotics, symbiotics, and antibiotics) have been used to treat illnesses. Probiotics are live microorganisms that have positive effects on host health when administered in adequate concentrations[118].

PROBIOTICS

The use of probiotic bacteria as prophylactics and therapeutics is receiving attention because of the potential effects of gut microbes in lowering IR and lipid levels. Increasing evidence suggests using probiotics to prevent metabolic diseases[119]. Probiotics are live microbes that provide the host with health benefits when administered in optimal concentrations[120]. Probiotic administration may lower TG and VLDL cholesterol (VLDL-C) levels by inhibiting the NF-κB pathway and the gut microbiota-SCFA-hormone axis[121]. Moreover, a substantial decrease in lipid levels was noted in healthy pregnant women without GDM after the administration of a two-strain probiotic containing *L. acidophilus* LA5 and *B. animalis* BB12 for 9 wk[122]. Probiotics can also increase the β-oxidation of long-chain fatty acids in muscle and liver tissues, modifying the energy pathways for fatty acid oxidation, lowering the formation of new TGs, and eventually reducing serum TG and VLDL-C levels[123]. Furthermore, probiotic consumption can increase the number of natural killer T cells in the liver[124], reduce inflammatory signaling, increase adiponectin levels, reduce inflammation, and prevent GLUT4 inhibition to improve glucose homeostasis. Probiotic dosages can also trigger enteroendocrine L cells to release GLP-1, thereby improving glucose metabolism, reducing glucotoxicity, and improving insulin sensitivity in the target tissue[125].

Recently, there have been many discussions on fecal microbiota transplantation. Vrieze *et al*[126] reported that participants with metabolic syndrome showed enhanced peripheral and hepatic insulin sensitivity in response to modest intestinal transfusions of fecal microbiota from allogenic lean donors, together with an upsurge of the gut microbiome. Various microbes are used as probiotics. Our review mainly focuses on *A. muciniphila* and *Bifidobacterium* species, which are closely associated with metabolic diseases.

A. muciniphila

In recent years, *A. muciniphila*, a commensal bacterium found in the intestine, has attracted increasing interest because of its health-promoting effects[127]. Interestingly, various clinical disorders in humans, including obesity, T2DM[128], inflammatory bowel disorder, hypertension, and liver disease, decrease the abundance of *A. muciniphila*[129]. Animal studies have demonstrated that *A. muciniphila* can alleviate obesity and related illnesses, such as steatosis, gut permeability, glucose intolerance, and IR[130,131]. Moreover, in one study, animals treated with live *A. muciniphila* did not exhibit IR or inflammatory cell (CD11c) infiltration in adipose tissues, which are crucial for the development of obesity, because of low inflammation[32]. *A. muciniphila* and *F. prausnitzii* can protect against the development of T2DM[132]. By activating tight junction proteins (occludin, claudin-3, and ZO-1) and preventing the accumulation of lipopolysaccharides (LPSs) and occurrence of metabolic endotoxemia, *A. muciniphila* can restore the thickness of the mucus layer[127]. Furthermore, *A. muciniphila* exhibits antibacterial and anti-inflammatory effects when administered endogenously and influences the endogenous synthesis of GLP-1 and GLP-2[133]. Notably, all these findings have now received backing from different firms and have been used for treating various disorders, including metabolic diseases, such as DM[134], obesity[135], atherosclerosis, hepatic inflammation, and hypercholesterolemia[136].

Bifidobacterium species

Probiotics, which are a component of the gut microbiome, successfully regulate the intestinal microbiota and have potential antidiabetic applications[137]. *Bifidobacterium*, one of the most significant probiotics found in the mammalian gut, exhibits positive effects on health[138]. Numerous studies have demonstrated that *Bifidobacterium* species improved insulin sensitivity in patients with T2DM[139,140]. In HFD-fed rats with T2DM, the administration of *B. animalis* 01 reduced food and water intake, blood glucose levels, HbA1c levels, and hepatic injuries and increased the antioxidant status, HOMA-IR, and lipid levels by affecting the IRS/PI3K/AKT and Keap1/Nrf2 signaling pathways[141]. Similarly, Le *et al*[142] reported that STZ-induced C57BL/6J mice treated with *Bifidobacterium* species exhibited significantly reduced blood glucose levels and significantly increased IR, IRS1, Akt/PKB, IKKα, and IκB α levels. Moreover, increased extracellular signal-regulated kinase 2 and adiponectin expression levels and decreased macrophage chemoattractant protein-1 and interleukin-6 expression levels were noted following the administration of *Bifidobacterium* species. Furthermore, in obese and DM models, treatment with *B. animalis* subsp. *lactis* GCL2505 reduced visceral fat accumulation, increased GLP-1 and acetate levels, and enhanced glucose tolerance[143].

Bifidobacterium, one of the most significant gut bacteria, diminishes intestinal endotoxin concentrations and enhances mucosal barrier function[144]. Recently, HFD-induced models verified an increase in intestinal inflammation. By lowering the levels of metabolic endotoxins and reducing intestinal inflammation, *Bifidobacterium* species may benefit patients with metabolic syndrome[145]. In an HFD-fed mouse model, *Bifidobacterium* species dramatically improved glucose-induced insulin secretion, increased glucose tolerance, and reduced endotoxemia and proinflammatory cytokine levels [146] by altering GLP-2 levels[147]. Thus, by lowering metabolic endotoxin levels and intestinal inflammation and increasing the expression level of intestinal Reg I, a growth factor regulator[148], *Bifidobacterium* supplementation could alleviate HFD-induced metabolic syndrome. Specific strategies for altering the gut microbiota in favor of *Bifidobacterium* species may be beneficial for mitigating the effect of HFD on the occurrence of metabolic syndrome.

In summary, *A. muciniphila* and *Bifidobacterium* species are highly viable and proliferative probiotics that can alleviate metabolic syndrome through increased glucose tolerance and reduced visceral fat accumulation by altering the overall bacterial composition of the gut microbiota. Moreover, they can increase the levels of SCFAs, which can activate several signaling pathways, including the AKT/PKB/IRS/ERK/Nrf2 pathways.

LIMITATIONS AND FUTURE PERSPECTIVES

Research on the synthesis of BAs and the pathogenesis of liver diseases and metabolic diseases has made significant progress in the last two decades. BAs exert several metabolic functions, and their physicochemical properties can affect their metabolic activities. Gut microbes can be modified by various factors, such as age, diseases, diet, and drugs. BAs play a significant role in regulating gut microbes. Moreover, the size of the BA pool has been shown to be affected by microbial metabolism in the intestines; however, most of these studies have been conducted on experimental animals. Therefore, further research is warranted to identify novel therapeutic targets for maintaining human intestinal health. Importantly, while increasing experimental evidence is available, clinical research on the importance of the human microbiota in relation to rodent metabolic functions is still in its inception. For example, BAS is not recommended for individuals who have a bowel obstruction or are pregnant. Cholestyramine and colestipol are classified as pregnancy category C, while colesevelam HCl is classified as pregnancy category B[149].

Clinical research has mainly been epidemiological in nature and has therefore failed to determine whether modifications in the intestinal microbiota play a molecular role in metabolic diseases. A better understanding of these aspects is required to determine whether BA-gut microbiota axes can promote human health and how these pathways can be used to design novel therapeutic interventions for metabolic diseases, such as obesity, DM, and hyperlipidemia, and CADs using BAs and its metabolites, probiotics, and microbial transplantation.

CONCLUSION

The major objective of this review was to assess the functional implications of gut microbes and BAs for metabolic diseases. In the past, the gut microbiota was considered a bystander in the intestinal tract. The role of these microbes in supporting intestinal function has become more widely recognized in recent years. BAs and the gut microbiota interact in a mutually beneficial manner. When the gut microbiota is disturbed in metabolic illnesses, inflammation occurs and the gut barrier is compromised. Modulating receptor-mediated transport, energy balance, gut permeability, and serum LPSs can impact BAs metabolism. The gut microbiota composition and the specific mechanisms in which the gut microbiota and BAs interact to alter the metabolism and functioning of the host-gut barrier remain somewhat unclear. Understanding the significance of the BAs-gut microbiota relationship in metabolic health could lead to revolutionary advances in the treatment of metabolic illnesses in the future.

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MINIREVIEWS

Recent advances in the management of autoimmune pancreatitis in the era of artificial intelligence

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Abstract

Autoimmune pancreatitis (AIP) is a type of immune-mediated pancreatitis subdivided into two subtypes, type 1 and type 2 AIP. Furthermore, type 1 AIP is considered to be the pancreatic manifestation of the immunoglobulin G4 (IgG4)-related disease. Nowadays, AIP is increasingly researched and recognized, although its diagnosis represents a challenge for several reasons: False positive ultrasound-guided cytological samples for a neoplastic process, difficult to interpret levels of IgG4, the absence of biological markers to diagnose type 2 AIP, and the challenging clinical identification of atypical forms. Furthermore, 60% and 78% of type 1 and type 2 AIP, respectively, are retrospectively diagnosed on surgical specimens of resected pancreas for suspected cancer. As distinguishing AIP from pancreatic ductal adenocarcinoma can be challenging, obtaining a definitive diagnosis can therefore prove difficult, since endoscopic ultrasound fine-needle aspiration or biopsy of the pancreas are suboptimal. This paper focuses on recent innovations in the management of AIP with regard to the use of artificial intelligence, new serum markers, and new therapeutic approaches, while it also outlines the current management recommendations. A better knowledge of AIP can reduce the recourse to surgery and avoid its overuse, although such an approach requires close collaboration between gastroenterologists, surgeons and radiologists. Better knowledge on AIP and IgG4-related disease remains necessary to diagnose and manage patients.

Key Words: Autoimmune pancreatitis; Pancreatic ductal adenocarcinoma; Immunoglobulin G4-related disease; Prednisone; Rituximab; Artificial intelligence; Plasmablasts

Core Tip: The diagnosis of autoimmune pancreatitis (AIP) is challenging. Indeed, 60% and 78% of type 1 and type 2 AIP, respectively, are retrospectively evaluated on surgical specimens of resected pancreas for suspected cancer. Obtaining a definitive diagnosis can thus prove difficult, since endoscopic ultrasound fine-needle aspiration or biopsy of the pancreas are suboptimal. This paper focuses on recent innovations in the management of AIP using artificial intelligence, new serum markers, and new therapeutic approaches and outlines the current recommendations. Improved knowledge of AIP can reduce the recourse to surgery, although this requires collaboration between gastroenterologists, surgeons and radiologists.

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a type of chronic fibro-inflammatory response in immune-mediated pancreatitis[1,2]. Histological examination reveals diffuse lymphoplasmacytic infiltration associated with extensive storiform fibrosis, acinar atrophy, and obliterative venulitis[2,3]. Radiological imaging shows ductal stenosis and an enlarged pancreas or pancreatic mass resembling pancreatic ductal adenocarcinoma (PDAC)[3]. The distinction between these two entities is sometimes difficult and has clear therapeutic implications. Indeed, AIP has a good response to steroids, which constitutes an important diagnostic criterion[4,5].

In the last decade, the prevalence of AIP has increased worldwide due to the better description and recognition of the disease[6-8]. In the majority of the studies conducted in Asian countries, its prevalence more than doubled between 2011 and 2016. In Japan, for example, the prevalence was estimated at 10.1 per 100000 inhabitants in 2016 with an annual incidence of 3.1 per 100000 inhabitants [7]. The prevalence in Europe seems to be less than 1 per 100000 inhabitants (0.29/100000), or 9% of patients with non-alcoholic acute pancreatitis[8], although these numbers are most certainly underestimated due to the lack of diagnoses and the occurrence of paucisymptomatic cases that do not require treatment[8].

Two AIP subtypes, AIP-1 and AIP-2, present different clinical profiles such as mean age at disease onset, male/female ratio, geographical distribution, as well as histological and immunological features (Table 1)[9,10]. AIP-1, the most prevalent type in Asia, is a systemic disease with the possible involvement of other organs, higher immunoglobulin G4 (IgG4) in blood, IgG4 positive infiltrates, as well as increased autoantibody levels in blood. AIP-1 primarily affects men aged over 50 years and is currently considered the pancreatic manifestation of the IgG4-related disease[11]. AIP-2 corresponds to the idiopathic duct-centric pancreatitis, which can be identified by pathognomonic histological features known as granulocyte epithelial lesions[9,12]. This subgroup is more common in Europe and affects younger patients with an equivalent male/female ratio. AIP-2 often occurs with isolated cases of pancreatitis without other organ involvement, although it is associated with chronic inflammatory bowel disease in 20%-30% of cases[6,13]. The physiopathological mechanisms of AIP are poorly understood and multiple immunological pathways have been proposed. The aim of this paper is not to describe these different mechanisms.

ESTABLISHING A DIAGNOSIS

Several diagnostic criteria have been proposed for AIP based on its clinical, biological, radiological and histological presentation in addition to treatment response: Diagnostic criteria of the Japanese (2002, 2006)[14], Korean (2007), Asian (2008) and Italian Societies of Gastroenterology (2003, 2009), as well as the Mannheim (2009) and HISORt criteria (2009)[15]. With the improved detection of AIP-2 and IgG4-related disease, a group of international experts published new reference criteria known as the International Consensus Diagnostic Criteria (ICDC) in 2011 with five main diagnostic criteria categorized according to two levels of evidence (Tables 2 and 3)[10]. New Japanese diagnostic criteria (JPS2011 followed by JPS2018) were subsequently published[16,17]. Unlike the ICDC criteria, the JPS2011 criteria provided the following clarifications: (1) Differentiation between diffuse, segmental and focal types in the classification; (2) Blood IgG4 used as the only biological marker; (3) Sclerosing cholangitis, sclerosing sialadenitis and retroperitoneal fibrosis classified as other organ involvement; (4) No level of evidence given for other organ involvement or serological criteria (IgG4); and (5) The optional use of steroids only after excluding pancreatic cancer by fine-needle aspiration (FNA)[16]. In 2018, the JPS2018 added

Table 1 Characteristics of the two subtypes of autoimmune pancreatitis

Characteristic	AIP-1	AIP-2
Male/female ratio	3/1	1/1
Mean age	65 yr	40 yr
Geographical distribution	Asia > Europe and United States	Europe and United States > Asia
Clinical presentation	Jaundice 60%-80%. Acute pancreatitis 15%. Weight loss 65%	Acute pancreatitis 80%. Jaundice < 30%
Biological presentation	IgG4 > 1.35 g/L (sensitivity 70%, specificity 93%). IgG4 > 2.7 g/L (sensitivity 53%, specificity 99%). Lipase < 3xN. Cholestasis: > 80% of cases. Diabetes: 65% of cases. Insulin-dependent diabetes: 20% of cases. Exocrine pancreatic insufficiency: 40% of cases	Unspecific. Lipase > 3xN. Rare endocrine and exocrine pancreatic insufficiency
Histological criteria	Lymphoplasmacytic infiltration without neutrophils. Storiform fibrosis. Obliterative venulitis. IgG4 plasma cells > 10 in a high-power field	Destruction of inter- and intralobular ducts by neutrophils (granulocytic epithelial lesions). Few or no IgG4 plasma cells
Relapse rate after corticosteroid therapy	> 30%	< 15%

AIP: Autoimmune pancreatitis; IgG: Immunoglobulin G.

Table 2 Summary table of the International Consensus Diagnostic Criteria for autoimmune pancreatitis-1[10]

ICDC	Level 1	Level 2
P: Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement (rim-like enhancement)	Indeterminate: Segmental or focal enlargement with delayed enhancement
D: Ductal imaging	Single long stricture (> 1/3 length of MPD) or multiple stricture without marked upstream dilatation	Segmental or focal narrowing without marked upstream dilatation (< 5 mm)
S: Serology	IgG4 > 2x upper limit of normal value (> 2.70 g/L)	IgG4 rate: 1-2x upper limit of normal value
OOI: Other organ involvement	Histology of extra-pancreatic organ (3/4)	Histology of extra-pancreatic organ must show both: (1) Periductal lympho-plasmacytic infiltration without granulocyte epithelial lesions; and (2) > 10 cells/HPF of IgG4 positive cells
	Typical radiological evidence: (1) Stenosis of intrahepatic bile duct or proximal and distal common bile duct; and (2) Retroperitoneal fibrosis	Physical or radiological evidence (1/2): (1) Symmetrically enlarged salivary/lachrymal glands; and (2) Radiological renal involvement
H: Pancreatic histology	3/4 criteria	2/4 criteria
Periductal lymphoplasmacytic infiltration without granulocyte epithelial lesions		
Obliterative phlebitis		
Storiform fibrosis		
> 10 cells/HPF of IgG4 positive cells		
Rt: Corticosteroid response	Rapid (≤ 2 wk) radiologically demonstrable resolution or marked improvement in pancreatic/extrapancreatic manifestation	

HPF: High power field; ICDC: International Consensus Diagnostic Criteria; IgG: Immunoglobulin G; MPD: Main pancreatic duct.

two new factors to its diagnostic criteria: The use of magnetic resonance imaging (MRI) for radiological diagnosis, primarily using magnetic resonance cholangiopancreatography, and the use of endoscopic ultrasound (EUS)-FNA in order to exclude a neoplastic process by histology[17].

The main limitation of this diagnostic algorithm concerns AIP-2 patients with normal IgG4 levels and disease limited to the pancreas[18]. Indeed, 50% of AIP-1 patients present with other organ involvement [19], which facilitates the diagnosis. If specific clinical, morphological, or biological evidence confirms the AIP diagnosis, no further investigation is necessary. Nevertheless, in the presence of a focal mass or

Table 3 Summary table of the International Consensus Diagnostic Criteria for autoimmune pancreatitis-2[10]

ICDC	Level 1	Level 2
P: Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement (rim-like enhancement)	Indeterminate: Segmental or focal enlargement with delayed enhancement
D: Ductal imaging	Single long stricture (> 1/3 length of MPD) or multiple stricture without marked upstream dilatation	Segmental or focal narrowing without marked upstream dilatation (< 5 mm)
OOI: Other organ involvement		Clinically diagnosed inflammatory bowel disease
H: Pancreatic histology	Both of the following: (1) Granulocytic infiltration of duct wall with or without granulocytic acinar inflammation; and (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells	Both of the following: (1) Granulocytic and lymphoplasmacytic acinar infiltration; and (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells
Rt: Corticosteroid response	Rapid (≤ 2 wk) radiologically demonstrable resolution or marked improvement in manifestations	

HPF: High power field; ICDC: International Consensus Diagnostic Criteria; IgG: Immunoglobulin G; MPD: Main pancreatic duct.

diffuse pancreatic enlargement without associated autoimmune disease or specific biological and morphological features, a biopsy is necessary for histological analysis. The effectiveness and feasibility of obtaining pancreatic samples by EUS-FNA or biopsy (EUS-FNB) are still the subject of debate. Indeed, the primary aim of EUS-FNA and EUS-FNB is to collect pancreatic tissue so as to exclude a malignant process and thus contribute to the AIP diagnosis. The ICDC therefore recommends the use of biopsy tissue (trucut biopsy). However, given that this procedure is not feasible in all healthcare establishments, it is not compulsory in the diagnostic algorithm, although it is an important diagnostic criteria of the JPS2018 classification[17]. In the last decade, the proportion of pancreatic samples obtained by EUS has significantly increased from 48% in 2007 to 86% in 2016 in Japan[7]. Several studies nevertheless report the difficulty in diagnosing AIP with EUS-FNA (sensitivity of 43%-60%) and obtaining a sufficient amount of fibrotic tissue[11-16], which explains the shift toward EUS-FNB[20,21]. A Japanese study on 44 AIP patients obtained an adequate histological sample in 93% of cases, leading to a confirmed diagnosis of AIP in 43% of cases, a diagnosis of idiopathic chronic pancreatitis (CP) in 50% of cases, and no false positives for pancreatic cancer[22].

Laboratory tests

The serological diagnostic criteria corresponds to IgG4 levels at the upper limit of normal between 135 and 140 mg/dL[23]. It is generally accepted that IgG4 levels twice the normal limit are a valid criteria for AIP, although this can also occur in 10% of PDAC. Moreover, elevated IgG4 levels that are more than twice the normal value are associated with recurrence and exocrine pancreatic insufficiency in IgG4-related disease[24]. Nonetheless, some AIP-1 cases do not present elevated blood IgG4 or IgG4-positive cells on histology[25].

The efficacy of monoclonal anti-CD20 antibodies in AIP highlights the possible involvement of B cells in the pathogenesis of this disease[26,27]. Two types of B cells have been investigated in IgG4-related disease: Regulatory B cells and plasmablasts. Derived from the B cell lineage, plasmablasts are characterized as $CD27^{+}CD38^{+}$, which situates them between B cells and plasmocytes. Diagnostic tools such as the quantification of circulating plasmablasts in serum have already been shown to contribute to the diagnosis of AIP in patients with autoimmune disease[10]. In a retrospective study on 37 patients with IgG4-related disease, all patients showed high levels of plasmablasts, while only 64% had high IgG4 serum[28].

Imaging

When investigating pancreatic lesions, several types of imaging are necessary, as no single imaging technique can provide a definitive diagnosis of AIP. The most typical feature is a global enlargement of the pancreatic gland associated with the loss of lobulations, giving it a sausage-like appearance[29]. The capsule-like rim sign, which can also be seen with other procedures, is a relatively distinctive feature of AIP in computed tomography (CT). This sign is defined by a band-like structure around all or part of the pancreas. It is characterized by a lower absorption than the pancreatic parenchyma of the lesion during the pancreatic parenchymal phase and shows a delayed enhancement pattern with dynamic CT. Other elements have been described such as decreased peripheral enhancement causing a peripheral halo or ring, involution of the pancreatic tail, enhancement of the thickened bile duct wall resembling a cocoon, stenosis of the Wirsung duct without upstream dilation, and focal hyperdense pseudotumors. MRI shows a loss of T1 signal intensity and the T2 hyperintensity of the parenchyma correlated with an inflammation of the gland. In terms of ducts, stenosis of the Wirsung duct without upstream dilation can be observed, even in the focal pseudotumors[29]. A capsule-like rim reflecting the strong fibrosis of the peripancreatic lesions can be observed on T2-weighted images as a low signal and is highly specific

to AIP. EUS findings in AIP can be hypoechoic with scattered high-echo spots in the enlarged area in some cases show a diffuse or localized lesion of the parenchyma and irregularities in the main pancreatic duct such as bile duct wall thickening, or produce a duct-penetrating sign[30]. Further use of Positron-Emission-Tomography-Fluorodeoxyglucose can be useful in detecting other organs involved in AIP.

Artificial intelligence

The use of artificial intelligence in the medical domain has expanded rapidly in recent years. Artificial intelligence is a mathematical technique that automates the learning and recognition of data patterns. Diagnostic techniques such as digestive EUC (DEUS) can interact with this interface. A database was developed in Rochester using DEUS images of normal pancreas (NP) and pancreas of patients with AIP, PDAC, and CP with the aim to develop a convolutional neural network, a type of network with artificial neurons that recognize and classify images [convolutional neural network (CNN)] able to distinguish between these entities. For every patient in each cohort, all available still images and recorded video assets were identified and extracted. Images and videos obtained from both the radial and curvilinear echoendoscopes were included. Potentially confounding image features and patient identifying information were removed during image processing. Liver images, images with marks or annotations, and images in which calcification was visible were excluded. Using data from the training and validation subsets, various candidate CNN architectures, optimizers, and configurations were implemented, trained, and evaluated to determine an effective design for the EUS-CNN. Occlusion heatmaps were then generated and used to assess the features identified by the CNN model to differentiate all conditions (AIP, PDAC, CP, and NP). In a cohort of 585 patients (146 AIP, 292 PDAC, 72 CP, and 73 NP) with 1174461 extracted images, the CNN was 99% sensitive and 98% specific to differentiate AIP from NP, 95% sensitive and 71% specific to differentiate AIP from CP, 90% sensitive and 93% specific to differentiate AIP from PDAC, and 90% sensitive and 85% specific to differentiate AIP from all other pancreatic diseases[31]. Other groups have used this technology to discriminate portal venous CT images with the aim to differentiate between AIP and PDAC[32].

PANCREATIC CANCER AS A DIFFERENTIAL DIAGNOSIS

As the main important differential diagnosis of AIP is pancreatic cancer, it is important to recognize any differences in the clinical, radiological, and histological features[3,6]. Clinically, AIP patients present with mild abdominal pain such as discomfort, rarely with weight loss, and fluctuant jaundice that tends to respond positively to steroid therapy. On the other hand, PDAC patients present severe, persistent, and progressive abdominal pain with weight loss and progressive jaundice. Extrapancreatic manifestations are more frequent in AIP, whereas PDAC is more localized in the pancreatic gland and induces lower bile duct stenosis, presenting metastatic lesions and direct invasion in some cases. Biologically, IgG4 is elevated in AIP patients, although elevated levels have also been reported in a few cases of PDAC[33]. By contrast, elevated carbohydrate antigen 19-9 is rarely seen in AIP. Radiologically, smooth margins and capsule-like rims in the body and tail region that represent severe fibrotic changes are seen in the CT and MRI of patients with AIP. Amelioration of swelling after steroid treatment is a characteristic of AIP, whereas PDAC patients do not or rarely present an improvement. Duct dilatation should raise the suspicion of PDAC. Using contrast-enhanced CT, AIP is characterized by homogenous delayed enhancement of the gland that indicates the diffuse loss of parenchymal volume and severe fibrosis, whereas heterogenous enhancement that represents necrosis or bleeding in the tumor can be seen in PDAC. Using EUS, AIP is characterized by a duct penetrating sign as well as a diffuse homogenous hypoechoic pattern and linear or reticular hyperechoic inclusions that reflect interlobular fibrosis. In PDAC, EUS findings show a localized hypoechoic mass and a double duct sign, often accompanied by lymph node swelling or vascular invasion. Histological patterns of AIP are characterized by periductal lymphoplasmacytic infiltration, storiform fibrosis, and obstructive phlebitis. Immunohistological identification of carcinoma cells is observed in PDAC, and inflammatory reactions can be commonly observed.

TREATMENT

Approximately 10%-25% of patients spontaneously improve and do not require specific treatment or intervention. Since no triggers for AIP have been identified to date, no lifestyle modifications have been proposed. Nevertheless, according to the 2017 recommendations, untreated patients with active AIP should receive treatment with the exception of those with a steroid contraindication[34]. The treatment of choice and the standard treatment at present is corticosteroid therapy. There are currently no standard therapeutic protocols regarding the indications for corticosteroid therapy, its duration, posology, monitoring measures, and maintenance therapy. In Asia, the initial dose of prescribed oral

prednisone is 0.6 mg/kg/d for 2 to 4 wk, followed by a single maintenance dose of 7.5 mg/d for 6 mo to 3 years. In the United States and Europe, the dose is 40 mg/d for 4 wk followed by a recommended reduction of 5 mg per week following symptom improvement; a single maintenance dose of 5-7.5 mg/d is recommended for 12 wk to 6 mo. A smaller dose of 30 mg/d can be given to diabetic patients[35]. An alternative administration with two courses of methylprednisolone 500 mg for 3 d with a 4-d interval can be useful to induce remission in refractory cases[34].

The aim of treatment is to improve symptoms, prevent fibrosis development within the affected organs, and improve endocrine and exocrine pancreatic insufficiency. Corticosteroid therapy has an effectiveness of around 90%, with a recurrence rate of 30%-50% after reducing treatment. The rate of recurrence is higher in AIP-1 (31%-37.5%) than in AIP-2 (9%-15.9%)[36,37]. Treatment evaluation by imaging and biological analysis is recommended within 1-2 wk of induction.

Three treatment options exist in the case of recurrence. The first approach is to maintain long-term low-dose corticosteroids (7.5 mg/d for 1-3 years), while the second is to use immunomodulator therapy such as azathioprine (2 mg/kg/d for 1-3 years)[38], methotrexate, or mycophenolate mofetil. A new therapeutic approach was proposed with rituximab, a monoclonal anti-CD20 antibody, and it seems to be a promising treatment, notably in IgG4-related disease[34,39].

Diverse studies comparing immunomodulators with corticosteroids alone did not show its superiority in terms of efficacy[34,40]. For patients who are resistant or intolerant of steroids and immunomodulators, rituximab is the only possible therapeutic alternative to induce remission. Rituximab can be used as first-line treatment for patients with a high risk of recurrence. Proximal duct involvement, young age, and higher alkaline phosphatase at initial presentation are high risk factors of recurrence after first-line treatment[41]. Moreover, for these patients with a significantly higher chance of recurrence, an induction and maintenance phase (375 mg/m^2 1x/wk every 2-3 mo for 2 years) would be significantly more effective than an induction phase alone (375 mg/m^2 per week for 4 wk or two injections of 1000 mg at 15 d interval)[41].

CONCLUSION

To conclude, the diagnosis of AIP remains challenging for clinicians as it must rapidly be distinguished from PDAC. The available diagnostic tools such as EUC are currently evolving, and the use of artificial intelligence could lead to the development of new approaches, allowing for a more precise diagnosis of AIP and a better differentiation of the disease from pancreatic cancer. The use of rituximab in the treatment algorithm in case of recurrence has already been proven, and it should be proposed as first-line treatment for patients with risk factors for recurrence. The optimal dose and treatment duration are yet to be defined.

FOOTNOTES

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MINIREVIEWS

Molecular mechanisms implicated in SARS-CoV-2 liver tropism

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Hepatic involvement is common in SARS-CoV-2-infected individuals. It is currently accepted that the direct and indirect hepatic effects of SARS-CoV-2 infection play a significant role in COVID-19. In individuals with pre-existing infectious and non-infectious liver disease, who are at a remarkably higher risk of developing severe COVID-19 and death, this pathology is most medically relevant. This review emphasizes the current pathways regarded as contributing to the gastrointestinal and hepatic ailments linked to COVID-19-infected patients due to an imbalanced interaction among the liver, systemic inflammation, disrupted coagulation, and the lung.

Key Words: SARS-CoV-2; Viral hepatitis; Non-infectious liver disease; Hyperinflammation; Coronavirus disease 2019

Core Tip: Clinical manifestations of coronavirus disease 2019 (COVID-19) may be triggered by the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in the liver. SARS-CoV-2 genomic RNA and its replicative intermediates were found in liver tissues. SARS-CoV-2 causes direct cholangiocyte damage. Systemic inflammation due to COVID-19 correlated with the degree of acute liver injury as revealed by the rise in aspartate aminotransferase levels. SARS-CoV-2 infection increased the risk of morbidity and mortality in patients with a history of advanced liver disease.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Globally, as of 5 August 2022, there have been 579119505 confirmed cases of COVID-19, including 6457101 deaths, as reported by the World Health Organization[1].

Coronaviruses are enveloped with crown-shaped spike glycoprotein, positive-sense single-stranded RNA viruses that include three genera: alphacoronavirus, betacoronavirus, and gammacoronavirus, mainly related to respiratory infections. SARS-CoV-2 employs receptor recognition mechanisms comparable to those used by preceding virulent coronaviruses such as SARS-CoV, responsible for the SARS epidemic of 2003[2-5].

In addition to common respiratory symptoms, COVID-19 patients also present with gastrointestinal symptoms comprising diarrhea, nausea, and vomiting[6]. Anal swabs from COVID-19 patients test positive for genomic SARS-CoV-2 RNA, and the virus can be isolated from stool samples[7,8]. However, the possibility of fecal-oral transmission cannot be completely ruled out[9,10].

The entry of the virus into target cells is mediated by the coronavirus spike protein (S) that engages angiotensin-converting enzyme 2 (ACE2) expressed in multiple cell types allowing SARS-CoV-2 to infect different organs such as the nasopharynx, lungs, lymph nodes, kidney, stomach, small intestine, spleen, brain, and liver leading to multiple organ damage[11]. Cell entry also requires the transmembrane serine protease 2 or other proteases[12]. The binding efficiency of the virus to ACE2 is a key determinant of transmissibility[13]. Different reports have revealed that SARS-CoV-2 has higher binding affinity to ACE2 than the previous SARS-CoV. This finding may in part explain the increased transmissibility of SARS-CoV-2, organ tropism, and ultimately multi-organ damage and mortality[14-16]. The mechanisms that could be involved in the multi-organ injury due to infection with SARS-CoV-2 comprise the damage of endothelial cells, dysregulation of the immune response, and an imbalance in the renin-angiotensin-aldosterone system (RAAS). The relative significance of these mechanisms in the pathophysiology of COVID-19 is at present not completely known. Although some of these mechanisms comprising ACE2-mediated viral entry and tissue injury with misbalance of the RAAS may be specific to COVID-19, immune pathogenesis produced by systemic delivery of cytokines and impaired microcirculatory function can also take place due to viral sepsis[17].

Hepatic involvement is common in SARS-CoV-2-infected patients. In cases with severe COVID-19 and to a lesser extent in mild/moderate COVID-19, some authors have reported a significant increase in alanine aminotransferase and aspartate aminotransferase (AST) indicating abnormal liver function[18]. Likewise, albumin decreased while alkaline phosphatase, gamma-glutamyl transferase (GGT), C-reactive protein (CRP), procalcitonin, ferritin, lactate dehydrogenase (LDH) as well as bilirubin levels were also significantly higher in severe than in other cases[19-21]. Patients without a history of liver illness were found to have abnormal liver test results[20]. These data suggests a direct connection between SARS-CoV-2 infection and digestive tract impairment. This review emphasizes the pathways currently regarded as contributing to the gastrointestinal and hepatic ailments linked to COVID-19.

To recognize the relevant literature, we employed a search and screening strategy. This process consisted of an extensive search of the online scientific database on the PubMed webpage using the most frequent synonyms to detect all possibly pertinent studies. In the following steps, references were analyzed to eliminate papers that were not relevant, and the remaining references were organized into categories for additional evaluation.

MOLECULAR MECHANISMS IMPLICATED IN SARS-COV-2 LIVER TROPISM DETECTION OF SARS-COV-2 IN LIVER TISSUE

The liver coordinates an essential role in the host-microbe defense by assembling the portal and systemic circulation. Changes in the liver such as ductular fibrosis, hepatic steatosis, cholestasis, acute liver necrosis, and central vein thrombosis with concomitant lymphocytic infiltrate were detected in autopsies from COVID-19 deceased patients[22,23].

In line with previous reports, ACE2 is mostly expressed in cholangiocytes and to a lesser extent in hepatocytes. Accordingly, formalin-embedded liver tissues contain the SARS-CoV-2 RNA genome[24]. Other studies have stated that 2 of 3 autopsy livers carry the infectious virus, and 31 of 45 postmortem liver tissues contain the SARS-CoV-2 RNA genome[25]. Such studies that demonstrate the presence of SARS-CoV-2 RNA and proteins in liver cells are significant, as these may be found in the vascular

lumen of blood vessels feeding the liver but also in portal vein endothelial cells, suggesting that the virus can also invade the liver through the circulation[26]. Postmortem liver biopsies have shown the presence of typical coronavirus particles in the cytoplasm of hepatocytes by electron microscopy[27], while hepatic parenchymal cells have also shown the presence of SARS-CoV-2 RNA[25]. Furthermore, the viral nucleocapsid protein has been detected in hepatic stem cells, hepatocytes, and cholangiocytes [28]. The presence of the SARS-CoV-2 RNA genome and its replicative intermediates in liver tissues has also been reported[28]. Most importantly, the nucleocapsid and the spike protein have been found in the liver 6 mo after recovery from COVID-19[29].

There is some proof that COVID-19 may be triggered by the presence of SARS-CoV-2 infection in the liver. However, other histological examinations revealed non-detectable viral particles in the liver and signs of significant hepatic damage[23,30]. This supports the notion that both direct and indirect pathways contribute to liver damage in the context of SARS-CoV-2 infection; more investigation is required to assess the importance of each pathway.

VIRAL EFFECT ON THE LIVER

The concept that SARS-CoV-2 can reach the liver cells through the ACE2 receptor is supported by the fact that ACE2 is present in liver and bile duct cells[11]. Furthermore, recent research found that 59.7% of cholangiocytes and 2.6% of hepatocytes express ACE2. Likely, SARS-CoV-2 might infect cholangiocytes and cause liver damage since the amount of ACE2 found in cholangiocytes is similar to that reported in type 2 alveolar cells of the lung[31]. SARS-CoV-2-associated liver damage may be related to the presence of ACE2 receptors in cholangiocytes rather than hepatocytes[32]. Moreover, given the rich supply of blood to the liver from the small bowel, the SARS-CoV-2 virus can use the gut-liver way across the liver reticular system to reach the liver[33,34]. Taking into account that ACE2 was regarded as an interferon-inducible gene in human epithelial cells from respiratory tissues, the hepatocyte permissiveness for SARS-CoV-2 might be also modified when the viral receptor expression is increased after liver injury[35], but it would be because of the shortened isoform of ACE2, identified as deltaACE2, instead of the functional viral receptor[36].

In homeostasis, the bile acid released by hepatocytes into bile ducts is transported by cholangiocytes. The tight junction between cholangiocytes conserves the barrier function of the bile ductal epithelial cells, allowing bile acid collection and excretion. Besides, cholangiocytes play an important function in liver renewal and immune response[37]. Thus, the disruption of the cholangiocyte function can induce hepatobiliary injury. Previous reports have demonstrated that SARS-CoV-2 infection impairs the barrier through the modulation of tight junctions[38]. This effect could be attributed to the direct viral cytopathic effect on cholangiocytes. Likewise, SARS-CoV-2 infection induces the expression of apoptotic factors, including cluster of differentiation 40 (CD40), caspase recruitment domain family member 8, and serine/threonine kinase 4 in cholangiocytes[38]. Thus, SARS-CoV-2 causes direct cholangiocyte damage with subsequent liver homeostasis disruption in COVID-19 patients.

Liver biopsies from SARS-CoV-2 infected patients have revealed fatty degeneration, cellular infiltration, hepatocellular necrosis, increased ballooned hepatocytes, and mitotic cells. These findings are in line with the idea that liver damage in COVID-19 patients is caused by an indirect effect as a result of direct viral cholangiocyte damage and subsequent bile acid accumulation. However, it remains uncertain whether hepatic involvement during SARS-CoV-2 infection reveals direct cytopathic effects of the virus, ischemia and hypoxic reperfusion-related injury, exacerbated immune response responsible for the systemic inflammatory response syndrome, or a combination of mechanisms that have not been completely elucidated until now[39].

EFFECT OF SYSTEMIC HYPERINFLAMMATION IN THE LIVER

SARS-CoV-2 infection is related with an acute phase response characterized by the secretion of very high levels of proinflammatory cytokines in conjunction with CRP and ferritin[40]. The mechanisms involved in this "cytokine storm" are not completely elucidated. The proinflammatory response appears to be associated with the ability of the SARS-CoV-2 spike protein to activate C-type lectins and 20 family member 2 on innate immune cells[41]. A transcriptome analysis of 284 samples from 196 SARS-CoV-2-infected patients revealed that diverse peripheral immune alterations are associated with clinical characteristics comprising severity and disease phase of COVID-19. The increased expression and release of S100A8/A9 during inflammation exerts a critical role in controlling the inflammatory reaction by inducing leukocyte recruitment and stimulating cytokine release. These are calcium-binding proteins constitutively expressed as a heterodimer in neutrophils and monocytes, and they are the most important platelet-derived activators of endothelial cells. Additionally, SARS-CoV-2 RNA was detected in different epithelial and immune cells, followed by transcription alterations within virus-positive cells [42].

One of the mechanisms that encourage platelet adhesion to inflamed vascular endothelium is the early adhesive events that stimulate platelets to "roll" along endothelium. Among them is the interaction between the main platelet membrane receptor, GP1b (CD42)-1X-V, and von Willebrand factor secreted by endothelial cells. This phenomenon is strengthened by interactions between upregulated CD62P expression, which is present in both cell types and its counter receptor, P selectin glycoprotein ligand-1, which is also present in both cell types, although weakly in platelets. In a similar manner, the SARS-CoV-2 spike protein bound to the CD42b receptor to activate platelets *via* two different signaling pathways and stimulated platelet-monocyte interaction by engaging P selectin glycoprotein ligand-1 (PGSL-1) and CD40 ligand (CD40L)/CD40, which causes monocytes to produce proinflammatory cytokines. These findings demonstrate the correlation between hypercoagulation, monocyte activation, and a cytokine storm in patients severely affected.

The contact between the spike protein of SARS-CoV-2 and CD42 activates platelets and stimulates platelet-monocyte interactions through CD40L/CD40 and P-selectin/PGSL-1, contributing to hypercytokine secretion by monocytes[43]. Additionally, systemic inflammation is evidenced by a complement activation induced by the interferon (IFN)-Janus Kinase 1/2 (JAK1/2)-signal transducer and activator of transcription 1 (STAT1) signaling pathway[44].

The significance of the IFN pathways has been revealed in postmortem analyses of deceased patients due to severe COVID-19, where the livers displayed a significant induction of type I and II IFN responses and their related-JAK-STAT signaling pathways[25].

Moreover, the inflammation and cytokine stimulation observed in SARS-CoV-2 disease can contribute to endothelial injury and vascular damage, revealed by hypercoagulation, and arterial and venous embolism with the activation of immune cells and platelets, leading to clot formation[45,46]. High levels of CRP, which are indicative of acute liver injury, are correlated with the degree of systemic inflammation[47,48]. Increased levels of high-sensitive CRP, interleukin 6 (IL-6), and ferritin in COVID-19 patients are indicative of systemic inflammation, which correlates with the degree of acute liver injury as determined by the rise in AST levels[49]. Through the downregulation of hepatobiliary uptake and deficiencies in the excretory systems, the massive systemic inflammation contributes to hepatocellular cholestasis in the late stages of the disease[50]. Thus, it can be conclusively stated that SARS-CoV-2 infection is accompanied by a "cytokine storm" with a high proinflammatory cytokine profile that causes hepatic injury. It is obvious that systemic inflammation may contribute to acute liver damage, but SARS-CoV-2 infection cannot be ruled out directly. This theory is supported by the observation that liver damage appears early in the course of infection, as indicated by an increase in AST levels.

SARS-COV-2 INFECTION IN PATIENTS WITH PREVIOUS LIVER DISEASE

Since the onset of the COVID-19 pandemic, an increased risk of morbidity and mortality was observed among SARS-CoV-2-infected patients with a history of advanced liver disease[51]. Data obtained from multicenter studies have revealed a higher possibility of hospitalization and risk of death compared to patients without chronic liver disease[52]. To date, the mechanisms linking both pathologies are unknown. However, it has been proposed that the prothrombotic alteration caused by COVID-19 upsets the delicate homeostatic balance of cirrhotic patients, leading to venous microthrombosis and parenchymal dysfunction along with subsequent respiratory failures[53] (Figure 1).

VIRAL HEPATITIS

Patients with viral hepatitis are more likely to experience liver damage, according to data from previous SARS-CoV infections. The severity of liver disease and a worse prognosis are associated with SARS-CoV-2 superinfection in patients with chronic hepatitis B virus (HBV) infection. According to a study of 105 patients with both chronic HBV infection and SARS-CoV-2, the risk of complications such as acute chronic liver failure, acute cardiac injury, and shock was higher in co-infected patients than in patients who only had the SARS-CoV-2 virus[54]. Consequently, patients with liver damage had a higher mortality rate (28.6%) than patients without liver damage (3.3%), and the treatment of COVID-19 in co-infected individuals had a significant negative impact on liver function[54]. A higher risk of abnormal liver function was found in inactive HBV carriers in a retrospective study that included 133 hospitalized patients with confirmed COVID-19, 116 of whom tested negative for serum hepatitis B antigen, and 17 were HBV inactive carriers. Hepatocytes and the immune response, as shown by the production of IL-6, D-dimer, and LDH are involved in the liver damage observed in SARS-CoV-2/HBV[55]. Moreover, chronic HBV infection-induced immune dysfunction may be a key factor in the development of COVID-19. Due to persistent viral antigens, studies have shown that chronic HBV infection is linked to the depletion of virus-specific $CD4^+$ and $CD8^+$ T cells[56]. Interleukin (IL-2) and tumor necrosis factor alpha (TNF-α) release are particularly hampered by HBV-specific exhausted T lymphocytes, resulting in progressively diminished antiviral function[57]. IL-2, IL-6, and TNF-α are among the proinflammatory

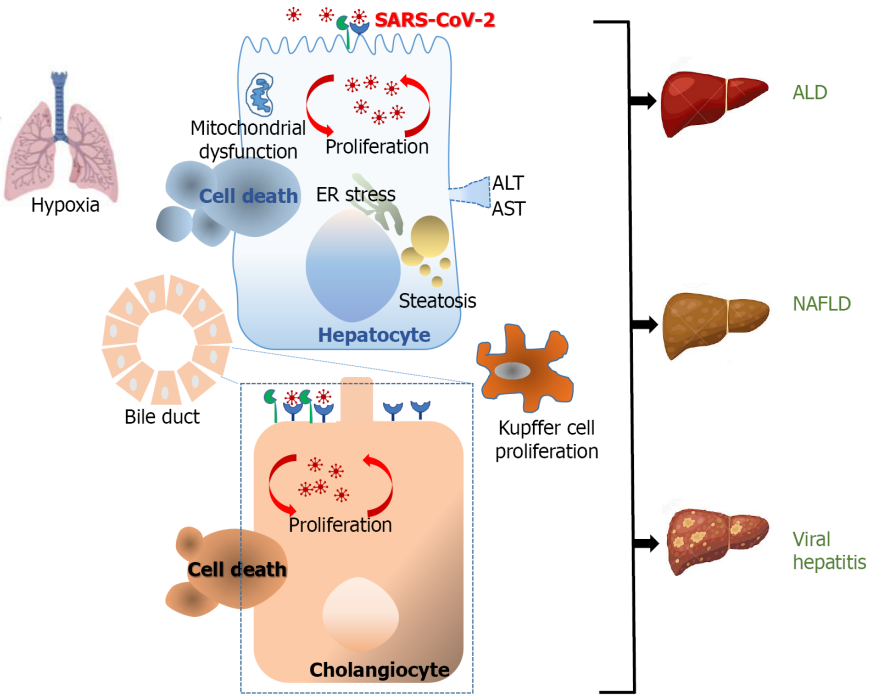

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Figure 1 Mechanisms of pathological injury upon severe acute respiratory syndrome coronavirus 2 infection. The major cause of mortality in coronavirus disease 2019 (COVID-19) is largely caused by lung damage with the increase of acute respiratory distress syndrome (ARDS). Liver damage or liver dysfunction has been linked with the general severity of COVID-19 infection and serves as a prognostic factor for ARDS progress. The scale of liver injury may range from direct severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral antigens, inflammatory progressions, hypoxemia, the antiviral treatments that induced hepatic damage and the existence of previous liver diseases. Angiotensin-converting enzyme 2 was mostly expressed in cholangiocytes and to a lesser extent in hepatocytes. These findings are in line with the viral presence reported in cholangiocytes and hepatocytes and the direct cytopathic effects observed. SARS-CoV-2 infection of hepatocytes and the indirect effects of "cytokine storm" induce a significant increase in alanine aminotransferase and aspartate aminotransferase indicating abnormal liver function. This leads to endoplasmic reticulum stress, steatosis, and finally to hepatocyte cell death. Consequently, Kupffer cell activation appears to be commonly funded in livers. The synergism between SARS-CoV-2 and chronic viral hepatitis B and C has also been suggested. Additionally, the superimposed cytokine storm caused by SARS-CoV-2 in patients with alcohol-associated liver disease, and non-alcoholic fatty liver disease (NALDF) displayed a higher risk of severe COVID-19. ALD: Alcohol-associated liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

cytokines overproduced as a result of the excessive immunological response to SARS-CoV-2 infection (cytokine storm), which is to our knowledge a significant factor associated with disease severity and mortality[58]. In this situation, it is conceivable that immunosuppression and depletion of HBV-specific T lymphocytes might prevent an excessive immunological response to SARS-CoV-2 and lessen the cytokine storm, resulting in milder illness. Although HBV reactivation is a potential side result of SARS-CoV-2 infection, the overall risk is minimal. Reactivation is primarily described as an abrupt and quick rise in HBV DNA levels in individuals with a history of detectable HBV DNA or recurrence of HBV DNA viremia in those with previous undetectable viral DNA[59]. Immunosuppressive therapy such as IL-6 receptor antagonists, IL-1 receptor antagonist, and high-dose corticosteroids are frequently used to treat HBV reactivation[60]. These treatments may be utilized in severe COVID-19 patients to manage the cytokine storm and to lessen the immune-mediated multiorgan damage. The impaired balance between the host's immune system and viral replication is the main cause of the progression to HBV reactivation after infection with SARS-CoV-2. The dosage of glucocorticoids or immunosuppressive medications is a major risk factor for the reactivation of HBV during the treatment of COVID-19, together with the host baseline virological markers[61].

The COVID-19 pandemic may delay the global commitment to eradicate HBV infection for several years because of the decrease in chronic hepatitis B prevention, diagnosis, and treatment. According to estimates, during the COVID-19 pandemic, half of low- and middle-income families were unable to access healthcare facilities for the diagnosis, clinical evaluation, and treatment of HBV. This was primarily due to travel restrictions, job and income losses, and patients' fear of contracting SARS-CoV-2 [62]. Only 18% of the patients with hepatocellular carcinoma (HCC) and 32% of those with decompensated cirrhosis had continuity of care, while 23% of the health centers postponed HBV infection therapy initiation[63].

Regarding hepatitis C virus (HCV), some studies have revealed that corticosteroid-treated individuals might experience significant viral reactivation, which is mostly caused by immunosuppression[64-66]. Steroid therapy can lead to HCV reactivation through two different mechanisms: first, they increase the capacity of the virus to replicate by upregulating two essential HCV entry factors:

occludin and scavenger receptor class B type I; and second, they do so by inhibiting the immune response against the virus, including T lymphocytes and plasmacytoid dendritic cells[67-69]. In individuals with persistent HCV infection, corticosteroid therapy can aggravate the course. Evidence suggests that HCV viremia rises in response to corticosteroids and falls back to normal levels in response to their cessation[70]. Therefore, it is best to avoid using corticosteroids in individuals with HCV infection[71].

However, the synergism between SARS-CoV-2 and chronic viral HBV and HCV has not been clearly elucidated. Hence, more widespread studies are required to evaluate the use of this therapy.

COVID-19 IN NON-INFECTIOUS CHRONIC LIVER DISEASE PATIENTS

Currently, decompensated liver disease and HCC cause 2 million deaths annually as a result of liver cirrhosis, which affects 112 million people globally[72]. High COVID-19 death rates in cirrhotic patients have been found in numerous recent reports[73,74]. The baseline Child-Pugh score also showed to be substantially correlated with death. The most frequent cause of mortality in COVID-19 patients is lung damage. Liver dysfunction is a possible cause of persistent lung damage. Indeed, liver failure plays a significant role in individuals with bacterial chest sepsis[75]. Cirrhosis and SARS-CoV-2 may have a fatal synergy because of alterations in the immune system caused by viral infection and coagulation. Dysregulation of pulmonary dynamics has been attributed to a number of factors, including ascites or deteriorating encephalopathy, immunological dysfunction in viral infection, a rise in the burden of venous thromboembolic illness, and concurrent lung disease. According to Marjot *et al*[52], mortality rates in patients with cirrhosis was 32% compared to 8% in those without it, while the mortality rate rose in connection with the Child-Pugh class [A (19%), B (35%), and C (51%)] in patients with cirrhosis. The principal cause of decease was respiratory failure (71%).

Alcohol-associated liver disease and COVID-19

There is very little effect of COVID-19 on patients with alcoholic hepatitis or alcoholic liver disease[74,76]. However, research on cirrhotic patients has revealed that, like other cirrhotic patients, those with alcohol-related cirrhosis have higher mortality rates[74,76]. Chronic kidney disease, obesity, and diabetes are common comorbidities among patients with alcohol-related cirrhosis, leading to higher risks of COVID-19 complications[77]. In a study involving 867 patients with COVID-19 and chronic liver disease, Kim *et al*[78] found that alcohol-associated liver disease (ALD) was independently associated with a higher risk of poor survival and a higher COVID-19 mortality rate. ALD is connected to the inflammatory state prompted by danger-associated molecular patterns which trigger the secretion of pro-inflammatory cytokines by particular immune cells[74,79]. It was hypothesized that the superimposed cytokine storm produced by SARS-CoV-2 in patients with ALD could exacerbate the heightened inflammatory process, leading to worse outcomes[80].

Non-alcoholic fatty liver disease

Diabetes, hypertension, cardiovascular disease, and obesity are well-known risk factors for severe COVID-19[81]. These metabolic comorbidities are closely related to non-alcoholic fatty liver disease (NAFLD).

Wide-ranging effects of the course of COVID-19 make distinguishing the independent effect on NAFLD a challenge. The difficulty is raised by a number of confounding cofactors, reverse causality from steatosis caused by SARS-CoV-2, as well as population heterogeneity and the diagnostic conditions studied. Consequently, results from clinical studies have been ambiguous. In a retrospective study of 202 SARS-CoV-2 infected patients, 35% were individuals with NAFLD. When compared to SARS-CoV-2 infected patients without NAFLD, patients with NAFLD displayed a higher risk of severe COVID-19, as evidenced by a higher probability of liver abnormalities in the hospitalized patient and long-term viral shedding[33].

Seventy-one consecutive COVID-19 patients from a different case-control study were divided into groups based on whether or not they had NAFLD. After reviewing all medical records, including demographic, clinical, and laboratory data, this study concluded that NAFLD poses a significant risk for developing severe COVID-19[82]. Patients with NAFLD were more probable to be admitted to the intensive care unit, according to a retrospective multicenter study with a cohort of hospitalized adults with COVID-19[83]. These results were supported by two thorough systematic reviews, as well as a meta-analysis[84,85].

On the other hand, NAFLD was not linked to severe COVID-19, as shown by a study conducted by Mushtaq *et al*[86] in a Middle Eastern cohort. The authors revealed that gene variants associated with NAFLD (transmembrane 6 superfamily 2 [TM6SF2], patatin-like phospholipase domain-containing protein 3 [PNPLA3], glucokinase regulator, and membrane-bound O-acyltransferase domain-containing protein 7 [MBOAT7]) and the severity of COVID-19 disease (TM6SF2, PNPLA3, and MBOAT7) is associated with genetic variation as a mechanism to establish a genetic risk score[87]. Additionally, other studies have concluded that some PNPLA3 allelic variants may act protectively by lowering the

risk of COVID-19 mortality[88]. Finally, a study that used a Mendelian randomization approach to investigate the correlations between COVID-19 severity and NAFLD found scant evidence supporting such a relationship[89].

COVID-19 AND HCC

At the beginning of the SARS-CoV-2 pandemic, serious measures were taken to preserve cancer patients from morbidity and mortality by restraining hospital presence and submitting anti-cancer therapy. The European Association for the Study of the liver recommended postponing treatments and evaluating the possibility of gradually removing anti-cancer immunological therapy[90].

In the context of rapidly escalating viral transmission, a series of precautionary principles were dictated. These measures rested on the hypothesis that cancer and active anti-cancer therapy have an unfavorable effect on the consequences of SARS-CoV-2 infection. It is widely known that cancer patients are commonly immunosuppressed as a consequence of chemotherapy. Therefore, different studies indicated that patients with subjacent cancer were at major risk of acquiring SARS-CoV-2 infection and severe outcomes could eventually evolve[91,92].

Recently, a multicenter retrospective revision including 250 non-vaccinated patients with liver cancer and COVID-19 infection showed that the mortality rate was 12.96% in patients with a diagnosis of HCC simultaneously with SARS-CoV-2 infection, and 20.25% in individuals with HCC history[74].

However, the prevalence of SARS-CoV-2 infection did not display a real rise in patients with chronic liver disease or in HCC patients. Nevertheless, HCC cirrhotic patients with COVID-19 may have a worse prognosis than the general population regarding severe disease, complications of SARS-CoV-2 infection, and mortality. Hereafter, the significance of applying actions to decrease the possibility of infection in these patients[93].

COVID-19 AMONG LIVER TRANSPLANT RECIPIENTS

Managing liver transplantation in the curse of SARS-CoV-2 pandemic was difficult as numerous hospitals had to essentially stop or drastically scale back their transplantation operations owing to a sudden drop in donor numbers and were forced to convert several care facilities into COVID-19 units. Due to the limited information available and the necessity of continuing immunosuppressive medication in these patients, the medical staff faced difficult challenges to manage post-liver transplant receivers in the course of COVID-19 pandemic while patients were at risk for a more severe COVID-19 infection and possible continued viral shedding. Qin *et al*[94] described the first instance of SARS-CoV-2 infection in a patient with hepatocellular cancer who had undergone liver transplantation, and discovered a higher viral load with an increasing immunosuppressive dosage. Immunosuppressive medications had no effect on the frequency of COVID-19 severity, according to Bhoori *et al*[95]. Early studies from Italy claimed that transplant patients experienced low death rates of less than 5%[96]. However, later assessments revealed that liver recipients and other solid organ transplants experienced mortality rates of over 25%[97,98]. Recently, findings from a prospective European trial comprising 57 liver transplant patients with proven SARS-CoV-2 infection and 19 transplant facilities were released. These results are consistent with is consistent with the projected mortality rate because patients with severe COVID-19 infection had overall and in-hospital case fatality rates of 12% and 17%, respectively. Five of the seven patients who passed away had a cancer history at the time of their deaths[99]. The evidence currently available does not support the idea that transplantation or certain immunosuppressive therapies have a significant impact on the likelihood of disease severity. Nevertheless, patients with underlying malignancies may need special care[97]. A number of COVID-19 vaccines have lately approved and have demonstrated effectiveness in healthy individuals. However, careful assessment and immunization of immunocompetent individuals are still required due to the potential immunological imbalance brought on by their illness or immunosuppressive medication. According to Boyarsky *et al*[100], solid organ transplant recipients who were fully vaccinated with the mRNA vaccine showed an appropriate humoral response, while the subpar response was linked to the use of antimetabolite immunosuppression.

CONCLUSION

It is widely acknowledged that the effects of SARS-CoV-2 infection on the liver have played a significant role during the COVID-19 pandemic. In patients with pre-existing infectious and non-infectious liver disease, who are at an extra high risk of developing severe COVID-19 and death, this feature is most medically relevant. This review aims to provide an overview of the current research on the molecular mediators responsible for inflammatory liver injury during SARS-CoV-2 infection. To fully comprehend

the pathogenic pathways that cause clinical deterioration of patients with COVID-19 due to an imbalanced interaction between the liver, systemic inflammation, disrupted coagulation, and the lung, further research should be conducted.

FOOTNOTES

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MINIREVIEWS

Current status of novel biologics and small molecule drugs in the individualized treatment of inflammatory bowel disease

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Abstract

Treatment strategies for inflammatory bowel disease (IBD) are rapidly evolving with the development of biologics and small molecule drugs (SMDs). However, these drugs are not guaranteed to be effective in all patients, and a "ceiling effect" of biologic monotherapy may occur. This issue highlights an unmet need for optimizing the use of biologics and predicting therapeutic responses. Thus, the development of new drugs with novel mechanisms of action is urgently needed for patients with primary nonresponse and secondary loss of response to conventional biologics and SMDs. In addition, combining different biologics or SMDs has been proposed as a novel strategy to enhance treatment efficacy in IBD, which theoretically has multidimensional anti-inflammatory potential. Based on the current evidence available for IBD, dual targeted therapy may be a promising strategy for refractory IBD patients who have failed in multiple biologic treatments or who have extraintestinal manifestation. Additionally, identifying the subgroup of IBD patients who are responding to biological combination therapies is also equally important in stable disease remission. In this review, we summarize the newly developed biologics and SMDs and the current status of biologics/SMDs to highlight the development of individualized treatment in IBD.

Key Words: Inflammatory bowel diseases; Biologic; Dual targeted therapy; Therapeutic drug monitoring; Bispecific antibodies

Core Tip: The emergence of biologics and small molecules has significantly changed the therapies used for inflammatory bowel disease (IBD). However, the efficacy of these drugs is not satisfactory for every patient, which indicates an unmet need for optimizing the use of biologics/small molecules and for predicting therapeutic responses. Here, we describe the current status of novel biologics and small molecules and new treatment strategies to combat IBD by using more than one biologic.

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INTRODUCTION

The inflammatory bowel diseases (IBD) ulcerative colitis (UC) and Crohn's disease (CD) are progressive inflammatory diseases with the gastrointestinal tract being the major site of inflammation. Patients require lifelong medical therapy in the context of the complicated aetiology of IBD[1]. Encouragingly, the advent of biologics and small molecule drugs (SMDs) has fundamentally changed patient prognoses and improved their quality of life. Strong evidence has indicated that early treatment with these drugs might lead to more favorable outcomes, such as deeper inflammation control and longer steroid-free remission[2]. Despite the optimization of biological therapies, the proportion of patients who exhibit primary nonresponse and secondary loss of response to biologics remains high, and approximately only 40% of patients who respond to biologic therapies maintain clinical remission in one year[3]. This highlights a potential "ceiling effect" of biological monotherapy and an unmet need for optimizing the use of biologics and for predicting therapeutic responses. Thus, patients need not only new drugs but also optimized treatment strategies. In the last decade, increasing numbers of new biologics and SMDs have been developed for IBD treatment[4], and a novel therapy combining different biologics and/or SMDs targeting multiple inflammatory signalling pathways, which is called dual targeted therapy (DTT), has begun to emerge in recent years[5]. However, whether DTT is superior to monotherapy in achieving the new target of long-term deep healing is uncertain. Additionally, DTT might only work in a selected subgroup of IBD patients, and indiscriminate use of DTT is expensive, ineffective, and unsafe [6]. Thus, in the era of biologics, it is important to identify eligible patients and treat them with individualized therapy. In this review, we describe newly emerging drugs and advanced strategies to provide insight for optimizing the current treatments for IBD in the context of individualized medicine.

EMERGING BIOLOGICS AND SMDS IN IBD

Currently, the goal of IBD treatment is not only to maintain clinical remission but also to achieve transmural healing to prevent further structural damage. Therefore, biologics and/or SMDs are recommended for patients with moderate to severe IBD. To date, the approved biological and small molecule therapies for IBD consist of the following anti-tumor necrosis factor (TNF) agents [infliximab (IFX), adalimumab (ADA), certolizumab (CZP), golimumab (GOL)], anti-adhesion agents [vedolizumab (VDZ), natalizumab (NAT)], anti-interleukin (IL)-12/23 agents [ustekinumab (UST)], and Janus kinase (JAK) inhibitors (tofacitinib). However, the current biological monotherapies are efficacious only in a certain proportion of patients. For example, only 30%-50% of active patients can achieve clinical or mucosal remission after biological inducing therapy. Besides, the rates of long-term corticosteroid-free remission are even lower and are less than 30%[7]. Thus, new drug development is rapidly advancing to meet the needs of patients with primary nonresponse, loss of response or intolerance to conventional biologics and SMDs (Table 1).

Anti-TNF agents

Anti-TNF agents were the first class of biologics to be approved for IBD treatment, and since then, they have tremendously changed IBD management. However, even in patients who respond to anti-TNF agents, the scope of anti-TNF use is limited due to systemic effects, such as infection and immunosuppression[8]. In addition, immunogenicity is another complex problem in anti-TNF-based treatment. Although some randomized controlled trials (RCTs) have shown that adding immunomodulators (IMs), such as the thiopurines azathioprine and 6-mercaptopurine, may reduce the immunogenicity of anti-TNF agents, and then improve the efficacy of anti-TNF therapy, only a minority of patients will benefit from this strategy[9,10]. Gut-selective anti-TNF agents might overcome these defects. An oral anti-TNF agent is currently in development. Since the antibodies comprising this therapy are derived from cow

Table 1 Summary of emerging biologics and small molecule drugs in inflammatory bowel disease treatments

Drug class	Agent	Target	Route	IBD type	Ref.
Anti-TNF	AVX470	Anti-TNF	Oral	UC	[11]
Anti-IL-23	Risankizumab	IL-23/p19 subunit	IV/SC	CD/UC	[17]
	Brazikumab	IL-23/p19 subunit	IV/SC	CD/UC	[15]
	Mirikizumab	IL-23/p19 subunit	IV/SC	CD/UC	[18]
	Guselkumab	IL-23/p19 subunit	IV/SC	CD/UC	[16]
Anti-lymphocyte trafficking	Etrolizumab	α4β7 and αEβ7 integrins	SC	CD/UC	[22]
	AJM300	α4 integrin	Oral	UC	[23]
	Ontamalimab	MAdCAM	SC	CD/UC	[38]
S1P receptor modulators	Ozanimod	S1PR1 and S1PR5	Oral	CD/UC	[24]
	Etrasimod	S1PR1, S1PR4 and S1PR5	Oral	CD/UC	[39]
JAK inhibitor	Filgotinib	JAK1	Oral	CD/UC	[27]
	Upadacitinib	JAK1	Oral	CD/UC	[28]
PDE4 inhibitor	Apremilast	PDE4	Oral	CD/UC	[30]

Anti-TNF: Anti-tumour necrosis factor; CD: Crohn's disease; IBD: Inflammatory bowel disease; IL-23: Interleukin-23; IV: Intravenous; MAdCAM: Mucosal addressin cell adhesion molecule-1; PDE4: Phosphodiesterase 4; SC: Subcutaneous; SMDs: Small molecule drugs; SIP: Sphingosine-1-phosphate; UC: Ulcerative colitis.

colostrum, this agent can act on the small intestine and colon in a delayed-release manner. A preclinical study that assessed the efficacy of AVX-470 showed a higher clinical response rate in the treatment group at week 4 than in the control group (25.9% *vs* 11.1%)[11]. Additionally, serious systemic side effects and formation of anti-drug antibodies were not observed. The current new oral formulation of anti-TNF agents might bring gut specificity to anti-TNF treatments. However, many more clinical studies are needed to confirm the efficacy of this novel formulation.

Anti-IL-12/23 agents

IL-12/23 signalling pathways are the key in regulating the differentiation and maturation of Th17 cells, which results in intestinal inflammation in IBD[12]. The conventional anti-IL-12/23 agent, UST, prevents activation of the IL-12/23 signalling pathway by targeting the shared subunit of cytokine p40 of IL-23 and IL-12[13,14]. At present, several monoclonal antibodies are in development that targets other subunits of IL-12/23.

Briakinumab is a human monoclonal antibody that acts specifically against the p19 subunit of IL-23 and exerts no effect on IL-12. In a clinical phase II study, 119 CD patients who failed anti-TNF therapy received brazikumab or placebo randomly at the beginning of the trial and 4 wk later. A higher clinical response rate was observed in brazikumab-treated patients than for those in the placebo group (49% *vs* 27%, $P = 0.01$)[15]. Guselkumab is another anti-p19 human mAb that was assessed in a phase II study in 250 patients with moderate-to-severe CD. Patients in all guselkumab groups treated with different doses exhibited a significant reduction in inflammatory activity at week 12. In addition, more patients in guselkumab treatments achieved clinical response [200 mg: 54%, 600 mg: 65%, 1200 mg: 50% *vs* 15.7% placebo ($P < 0.001$, respectively)] and safety events were similar between the groups[16]. Risankizumab, another anti-p19 monoclonal antibody, resulted in a 31% remission rate in treated CD patients in a phase III study, which was much higher than that in the control group (15%)[17]. Similarly, another anti-p19 antibody, mirikizumab, seemed to be effective in inducing remission in patients with moderate to severe UC[18]. Inhibition of the IL-12/23 signalling pathway is a promising therapeutic option for IBD, especially if the safety of the new anti-IL-12/23 agents targeting the p19 subunit can be confirmed.

Anti-lymphocyte trafficking agents

Inhibition of immune cell migration to inflamed tissue has emerged as a novel therapeutic mechanism for IBD[19]. VDZ is the most commonly used antiadhesion agent with a selective blocking effect of the α 4β7 integrin in the intestine[20]. In addition to this agent, etrolizumab is a newly developed monoclonal antibody that targets the β7 subunit of the α4β7 and αEβ7 integrins. In a study that included 1081 patients with moderate to severe UC, the rate of remission induction in the etrolizumab group was 18.5% compared with only 6.3% in the placebo group[21]. Etrolizumab was also reported to be effective in CD patients[22]. In addition, inhibition of the integrin-α4 subunit might also be useful for inflam-

mation control in IBD. AJM300 is a small molecule inhibitor of the α4 subunit of integrin that led to disease remission rate of 63% compared with a 26% remission rate in the placebo group among 102 UC patients in a randomized controlled study[23]. Another mechanism that limits immune cell migration is the inhibition of the sphingosine-1-phosphate receptor (S1PR). The S1P signalling network is mediated by 5 S1P G-protein coupled receptors (S1PR1-5). Ozanimod is a new class of S1PR modulators that shows activity against S1PR1 and S1PR5. In the TOUCHSTONE study, ozanimod therapy showed excellent efficacy in remission induction and maintenance in moderate to severe UC, and mucosal healing was better (34% with ozanimod *vs* 12% with placebo)[24]. Anti-lymphocyte migration might be an attractive therapeutic strategy in some situations, and these drugs may be promising and powerful in IBD management.

JAK inhibitors

The JAK family comprises important intracellular signalling molecules consisting of 3 subtypes (*e.g.*, JAK1, JAK2, and JAK3). Tofacitinib is the only SMD targeting JAK1 and JAK3 for moderate to severe UC that is approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA)[25]. However, some studies have revealed an association between tofacitinib and systemic side effects, such as malignancies, cardiovascular events, and venous thromboembolism, in patients with rheumatoid arthritis[26]. Thus, more selective JAK inhibitors are needed for IBD. Currently, filgotinib is a selective JAK1 inhibitor that has shown promising effects in the induction of disease remission in CD patients in the phase II FITZROY study[27]. More patients treated with filgotinib achieved endoscopic response, remission and healing compared with those who received placebo (47% *vs* 23%, $P = 0.0077$). However, data from that study showed that patients in the filgotinib group experienced more serious adverse events (9% *vs* 4%) and more serious infections (3% *vs* 0%) than those in the placebo group. Upadacitinib is another oral selective JAK1 inhibitor. The CELEST trial recently assessed upadacitinib in patients with moderate to-severe CD. At week 16, clinical remission was notable in the 6 mg group (upadacitinib 27% *vs* 11% placebo, $P < 0.1$). However, at week 52, patients in the upadacitinib groups had a higher incidence of serious infections. In addition, patients treated with 12 or 24 mg twice daily had increased serum lipids[28]. Generally, these new selective JAK inhibitors provide a promising prospects in IBD treatments, but their safety profiles should not be ignored.

Phosphodiesterase 4 inhibitors

Phosphodiesterase 4 (PDE4) is involved in intracellular cAMP transformation and activation of the nuclear transcription factor kappaB and promotes inflammation in the intestine[29]. Thus, PDE4 inhibition may reduce cytokine release syndrome. A phase II RCT assessed the efficacy of the PDE4 inhibitor apremilast in 170 adult UC patients. The results showed a higher clinical remission rate in patients treated with apremilast *vs* placebo[30]. In addition, significant decreases in inflammatory markers, such as C-reactive protein (CRP) and faecal calprotectin, were observed in this study.

Taken together, although the understanding of the pathogenesis of IBD is rapidly evolving and increasing numbers of new biologics and SMDs that have been developed, none of these drugs is effective in all patients. Thus, there is increasing interest in the therapeutic potential of the combination of biologics and/or SMDs with different mechanisms of action in patients with refractory IBD.

THE CURRENT STATUS OF BIOLOGICAL COMBINATIONS IN IBD

The immune response in IBD is multifaceted and accompanied by multiple activated inflammatory pathways in the intestinal mucosa. Single-targeted therapy consisting of biological monotherapy blocks only one inflammatory pathway, which is inadequate to control inflammation completely. Combinations of biologics with different mechanisms may have synergistic effects and contribute to the control of refractory IBD[3,31]. Currently, an emerging strategy, DTT, which is a combination of two biologics or a biologic and tofacitinib has been applied in patients with refractory disease. However, most studies on DTT are case reports and case series, and therefore, we could not summarize data to provide a comprehensive understanding of experiences with this strategy. From the limited evidence available, we briefly discuss the current paradigms of DTT in treating patients with refractory IBD who have failed multiple biologics.

As a prominent anti-TNF biologic, IFX was the first biologic agent used in IBD and has achieved great success[20]. In the last decade, several RCTs have demonstrated the efficacy of IFX combined with immunosuppressive agents[9,32,33]. However, approximately one-third of IBD patients exhibit no response to anti-TNF biologics, and another third need to switch to different agents within one year due to the secondary loss of response[20,34]. With the permission of the FDA and EMA, VDZ has become the first choice among second-line biologics for moderate to severe CD and UC patients who have experienced failure with conventional medications or anti-TNF agents[31]. Thus, in clinical practice, anti-TNF + VDZ is the most common combination paradigm used in DTT. A meta-analysis consisting of 30 studies of dual biologics or SMDs in IBD management revealed that the proportion of anti-TNF + VDZ algorithms ranked first among the various DTT paradigms and accounted for 48% of all

algorithms used. The combination of UST + VDZ was the second most popular paradigm and accounted for 19%. The clinical response rates and endoscopic response rates were comparable for different DTT groups in this meta-analysis[35]. Yang *et al*[36] reported that the rates of endoscopic improvement [reduction of simple endoscopic score for CD (SES-CD) > 50%] for anti-TNF + VDZ and anti-TNF + UST were both 33%. Additionally, VDZ + UST had the highest rates of endoscopic improvement (63%) compared with other combinations, but all DTT paradigms had similar efficacy in terms of endoscopic remission (SES-CD < 3). To date, the broadest experience with IBD patients treated with various DTTs is reported by a retrospective study[37]. Fifty patients with IBD [31 CD, 18 UC and 1 IBD-unclassified (IBD-U)] were included in this study. VDZ + UST was the most used combination paradigm (25/50), followed by VDZ + ADA (3/50), VDZ + GOL (2/50), and VDZ + CZP (2/50). Notably, 20 patients received tofacitinib combined with biologic treatment, but no specific data on this subgroup were provided in this report. The results from this study showed that CRP levels were significantly reduced from baseline (2.35 mg/dL *vs* 5.00 mg/dL, $P = 0.002$), 56% (18/32) of patients treated with dual biologic therapy maintained clinical remission after 3 mo, and that 11 of 32 patients were still in endoscopic remission after 8 mo[37]. Currently, growing numbers of newer biologics and SMDs are included in the candidate pools for DTTs, including anti-IL-23 agents such as mirikizumab, risankizumab, brazikumab and guselkumab, anti-integrin agents such as etrolizumab and ontamalimab [38], new SMDs such as PDE-4 inhibitors, as well as IL-6 inhibitors and S1PR agonists[4,39,40].

Although the use of DTTs that address different targets is increasingly applied to treat patients with refractory CD or UC, no strong evidence has shown that DTT might be effective in all patients. An early RCT included 79 patients with active CD who failed to respond to IFX treatment and did not report a statistically significant difference in efficacy between the IFX + NAT group and the IFX + placebo group [41]. Another observational study conducted on 16 paediatric patients with refractory IBD (7 CD, 9 UC, 1 IBD-U) showed that 75% achieved steroid-free clinical remission 6 mo after DTT but that 19% of patients discontinued DTT treatment because of inflammation control failure[42]. Additionally, some low-quality evidence suggested that DTT is more effective in CD patients with a penetrating phenotype [36,43]. Among the patients enrolled in a study conducted by Kwapisz *et al*[43], the median disease duration was 12.5 years, 86.7% (13/15) had penetrating disease, and 3.8 types of biologics were ineffective for these patients. Despite the disease severity, more than half of patients exhibited improved clinical symptoms and had less steroid use after DTT[43]. However, it is still unclear which combinations of biologics work best in specific IBD subgroups. Understanding of the pathophysiology of IBD and identifying prognostic biomarkers may significantly optimize DTT therapy.

WHAT CAN WE DO TO IMPROVE THE RESPONSE TO DTT

Heterogeneity among patients is one of the main features of IBD and is reflected by different disease behaviors and responses to therapeutics[44]. Although remarkable progress has been achieved in the development of new agents with novel mechanisms of action assisted by advanced management strategies, the current treatment pattern for IBD still relies on clinical symptoms and endoscopy examinations[45]. In addition, as mentioned above, many drugs are effective only in selected patients with IBD, and even DTT strategies cannot guarantee a response in all patients. Thus, the identification of patients who can benefit from DTT is urgent so that individualized treatment with biological agents can be provided.

Therapeutic drug monitoring enhanced the response to DTT

DTT is mainly used as an add-on therapy for patients who exhibit a partial response to monotherapy or who relapse during maintenance therapy. A major problem associated with failure of biological therapy is a loss of response, and therapeutic drug monitoring (TDM) may be a useful auxiliary tool in the management of patients treated with DTT[6,46].

TDM was originally suggested as a way to monitor the response to monotherapy and is divided into two categories: Proactive TDM (performed regularly to target an appropriate drug trough concentration) and reactive TDM (performed upon loss of response)[46]. Strong evidence indicates that TDM implementation is associated with higher rates of clinical remission, better endoscopic mucosal healing, and lower rates of secondary loss of response to biologics[47,48]. To date, TDM has achieved great success in optimizing the management of a combined biologics approach in patients treated with anti-TNF agents. The most convincing evidence comes from the management of secondary failures for IFX and ADA. The personalized anti-TNF therapy in CD study (PANTS), which included 1610 anti-TNF-naive patients with exposure to IFX or ADA, demonstrated that monitoring drug concentrations helps greatly in predicting therapeutic responses. The results showed that the only risk factor associated with primary nonresponse was low drug concentrations at week 14 [IFX: Odds ratio (OR) = 0.35, 95% confidence interval (CI): 0.20-0.62, $P = 0.00038$; ADA: OR = 0.13, 95%CI: 0.06-0.28, $P < 0.0001$] and that ideal drug concentrations at week 14 (7 mg/L for IFX and 12 mg/L for ADA) were a strong predictor for clinical remission at week 54. With the guidance of proactive TDM, dose intensification of initial biologics or combinations with IM (thiopurine or methotrexate) therapy improved outcomes of patients

with suboptimal drug concentrations at week 14[47]. Additionally, an expert consensus statement by Cheifetz *et al*[49] recommended a proactive TDM strategy during remission induction with anti-TNF agents and at least once during maintenance.

Reactive TDM could help distinguish patients who need to switch or combine with another class of biologic due to anti-drug antibodies (immunogenicity) from those who might benefit from dose escalation of monotherapy[50]. Actually, identifying patients with pharmacokinetic failure in biologics therapy is extremely useful in the guidance of biologics regimens, especially in DTT. Inadequate trough concentrations of drugs can not only lead to an insufficient efficacy of biologics, but patients may also become insensitive to the mechanism of action. For example, it was recently found that anti-TNF resistance in CD patients may be related to increased numbers of CD4+ T cells that overexpress the IL-23 receptor. Thus, it is possible that combinations with IL-23 inhibitors may help restore the sensitivity to the mechanism of action of anti-TNFs in such patients[51].

At present, there is little research focusing on the role of TDM for biologics other than anti-TNF agents, such as VDZ (anti-α4β7 integrin) or UST (anti-IL-12/23)[52]. Although the relationship between drug concentrations and clinical outcomes has been demonstrated, the value and cost-effectiveness of TDM in optimizing these biologic therapies are uncertain, and all the information given regarding TDM is derived from studies performed in patients treated with monotherapy. Therefore, relevant guidelines about TDM implementation in DTT have not been recommended by any academic association. Overall, TDM has great value in optimizing biologics therapy and providing individualized treatment for IBD but still has very significant problems and challenges in clinical practice.

Biomarkers help predict responses to biological therapies

IBD treatments are a long-term process, and disease monitoring is essential once treatment has started. A growing number of studies have put great effort into identifying prognostic and predictive biomarkers[53,54]. To date, various biomarkers have been proposed as clinical predictors of the response to biologics, including serological and faecal proteins, cytokines, proteomic-related and microbiome-related factors as well as metabolomic and genetic factors[55].

Serum and faecal markers have been widely applied in evaluating the efficacy of biologics. Serum CRP and fecal calprotectin, as inflammatory markers, have been shown helpful in response detection of anti-TNF agents. Although fecal markers are more sensitive than serum markers, such as CRP, in monitoring intestinal inflammation, there is no solid evidence demonstrating the association between fecal biomarkers and the response to anti-TNF agents[56,57]. Some proteins in the intestinal mucosa can also play a predictive role in response to biologics, such as Piwi-like protein 1, MYCBP associated and testis expressed 1, regulators of G-protein signaling 13 and Dachsous 2. Elevated expressions of these cytokines or proteins are beneficial for achieving a stable response to anti-TNF therapy[58,59].

Exploration of the genetic factors that predict the responses to anti-TNF therapy has also made great progress. The genetic polymorphisms in TNFRSF1A (rs4149570), IL-6 (rs10499563), IL-1β (rs4848306), toll-like receptors 2 (TLR2) (rs3804099), and TLR4 (rs5030728) are associated with the response to anti-TNF agents[60]. In addition, an observational study including 1240 European patients with CD found that the human leukocyte antigen-DQA1*05 mutation increased the risk of developing anti-TNF antibodies[61]. Single-cell sequencing revealed that some activated cells [*e.g.*, macrophages, immunoglobulin G (IgG) cells, T cells, and dendritic cells] in patients with failure to receive anti-TNF therapy are dysfunctional with genetic variation[62].

Many studies have suggested that the composition of the gut microbiota is related to the response to therapies. In the STORI study, Rajca *et al*[63] found that lower levels of *Faecalibacterium prausnitzii* (*F. prausnitzii*) were associated with early recurrence of CD after IFX withdrawal. Additionally, higher abundances of *F. prausnitzii* were associated with better responses to anti-TNF treatments[64,65]. Nevertheless, this relationship could not be confirmed in other studies[66], and when we examine the effects of other bacteria, studies with conflicting results are common[65,67]. To date, the microbiome has not been shown to be a predictive indicator of the response to biologics due to the very high heterogeneity in different individuals.

Metabolomics is a novel method that can quantify small metabolite sugars, such as lipids and amino acids, and thus offers a promising opportunity to identify candidate markers. Through metabolomic analysis, Nikolaus *et al*[68] found that the serum tryptophan levels increased in IBD patients who responded to IFX therapy but were unchanged in patients who did not respond to IFX or VDZ therapy. In addition, another study including 76 CD patients found that responders and non-responders have distinctive patterns of bile acids derived from feces, serum, and urine. By combining these representative markers, the responses to anti-TNFs may be predicted[69]. The biomarkers mentioned above could assist only with the accuracy of disease monitoring and response to biologics in IBD, and more studies are needed to identify a gold standard in the DTT strategy of IBD.

BISPECIFIC ANTIBODIES: THE NEXT GENERATION OF DTT

It has been proven that targeting multiple inflammatory signaling pathways by combining different

biologics has better outcomes for IBD patients than monotherapies[41,70]. However, the doses used in the DTT strategy are based on those for individual therapies, which might have unfavorable benefit-risk ratios, such as placing patients at greater risk of serious infections or malignancies[6,35]. The advent of bispecific antibodies (BsAbs) may provide new insights to help avoid some of these problems in DTT.

BsAbs are antibody formats that can bind to two different antigens or two different epitopes of the same antigen. Broadly, they can be classified as a special type of DTT. To date, BsAbs are divided into two major structural classes: IgG-like BsAbs carrying an Fc domain and non-IgG-like formats, which rely entirely on their antigen-binding capacity to exert therapeutic effects[71]. BsAbs can function by: (1) Impacting specific cell types by targeting multiple receptors; (2) Activating novel signaling *via* receptor colocalization or hyper crosslinking; and (3) Destroying pathogenic T cells through redirection[72]. Currently, different BsAbs are in different stages of clinical trials, and three BsAbs have been approved for clinical practice globally, namely, catumaxomab (for malignant ascites), blinatumomab (for leukemia) and emicizumab (for hemophilia)[71].

Although there are currently no BsAbs approved for IBD patients in clinical practice, several promising BsAbs are under investigation. For instance, BsAb drugs targeting both TNF and IL-23 are in the preclinical stage for autoimmune diseases, including IBD[72]. These BsAbs showed synergistic efficacy in alleviating colitis in a CD40-induced colitis model compared with anti-TNF and anti-IL-23 agents alone. Another ongoing phase I trial is investigating the effects of APVO210 in treating UC. These BsAbs are composed of an anti-CD86-IL-10 fusion protein and selectively deliver IL10 to CD68+ antigen-presenting cells, in which they have been demonstrated to induce a tolerogenic phenotype to relieve inflammation[73].

The unique mechanism of BsAbs provides an opportunity to target multiple molecular pathways with a single therapeutic agent. With careful dose adjustments, IBD patients can achieve maximal benefits from BsAbs and also good benefit-risk ratio[72]. However, this therapeutic approach is not without defects. On the one hand, the formulation of the two antibody binding domains of BsAbs is fixed, so it is impossible to change the single administration dose of different monoclonal antibodies according to patient needs, as we usually do in traditional dual biologic combination paradigms[74,75]. On the other hand, immunogenicity is an ever-present concern during the development of biological medication in IBD, especially in BsAbs. The large antibody complexes on the surface of BsAbs could act as "danger signals" that induce immunogenicity and eventually lead to loss of response[76].

CONCLUSION

Medical treatment patterns for IBD are rapidly evolving with the increased understanding of disease pathogenesis and development of new drugs that target various pathways. This review describes novel biological agents and SMDs that are in development and highlights the current status of DTT strategies in IBD management. Although drugs and therapeutic strategies that can cure all patients have not yet emerged, the efficacy of DTT in inducing and maintaining disease remission has been dramatically improved by taking advantage of new biological combination paradigms, modern TDM strategies, and novel predictive biomarkers. In future work, the identification of biomarkers that can predict subsets of patients and a more profound comprehension of the immunological landscape with IBD would help to enable more specific individualized medicine.

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MINIREVIEWS

Confusion and prospects for carcinogenesis of gastric adenoma and dysplasia: What is the correct answer currently?

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Abstract

There are differences in the diagnoses of superficial gastric lesions between Japan and other countries. In Japan, superficial gastric lesions are classified as adenoma or cancer. Conversely, outside Japan, the same lesion is classified as low-grade dysplasia (LGD), high-grade dysplasia, or invasive neoplasia. Gastric carcinogenesis occurs mostly *de novo*, and the adenoma-carcinoma sequence does not appear to be the main pathway of carcinogenesis. Superficial gastric tumors can be roughly divided into the *APC* mutation type and the *TP53* mutation type, which are mutually exclusive. *APC*-type tumors have low malignancy and develop into LGD, whereas *TP53*-type tumors have high malignancy and are considered cancerous even if small. For lesions diagnosed as category 3 or 4 in the Vienna classification, it is desirable to perform complete *en bloc* resection by endoscopic submucosal dissection followed by staging. If there is lymphovascular or submucosal invasion after mucosal resection, additional surgical treatment of gastrectomy with lymph node dissection is required. In such cases, function-preserving curative gastrectomy guided by sentinel lymph node biopsy may be a good alternative.

Key Words: Gastric adenoma; Low-grade dysplasia; High-grade dysplasia; Intramucosal carcinoma; Submucosal carcinoma; Endoscopic submucosal dissection

Core Tip: Gastric carcinogenesis occurs mostly *de novo*. Superficial gastric tumors can be roughly divided into the *APC* mutation type and the *TP53* mutation type, which are mutually exclusive. *APC*-type tumors have low malignancy and develop into low-grade dysplasia, whereas TP53-type tumors have high malignancy and are considered cancerous even if they are small. For lesions diagnosed as category 3 or 4 in the Vienna classification system, endoscopic submucosal dissection and staging should be performed. If the tumor is diagnosed with lymphovascular or submucosal invasion, additional surgical treatment of gastrectomy with lymph node dissection is required.

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INTRODUCTION

Gastric cancer is one of the most common cancers worldwide. However, the incidence of gastric cancer declined in many Western countries during the 20th century. Japan was one of the countries with a high incidence of gastric cancer, but the incidence is also decreasing. This fact proves that *Helicobacter pylori* (*H. pylori*) infection is deeply involved in the development of gastric cancer[1]. In Japan, the water supply and sewerage systems were completed in the 1960s, and the *H. pylori* infection rate has decreased among the generations born subsequently[2,3]. Most patients with gastric cancer in Japan are elderly, and the incidence of gastric cancer among age groups with low *H. pylori* infection rates is low. Besides *H. pylori*, many factors are known to be involved in gastric carcinogenesis. These include salt intake, smoking, exposure to N-nitroso compounds, and Epstein-Barr virus infection[4-7]. However, the molecular mechanisms leading to gastric carcinogenesis are not well understood.

In contrast, the molecular mechanism leading to colorectal cancer has been clarified to some extent. Colorectal cancer is one of the most common cancers worldwide. Many colorectal cancers are thought to develop from adenomas and serrated polyps through the adenoma-carcinoma sequence. The molecular mechanism of colorectal carcinogenesis has long been a subject of interest and has been well-studied, with genetic and epigenetic changes in oncogenes and tumor suppressor genes identified in considerable detail.

There are various reasons for this difference in the understanding of the molecular mechanisms between gastric carcinogenesis and colorectal carcinogenesis. The most important is that gastric carcinogenesis is often of the *de novo* type and does not necessarily follow the adenoma-carcinoma sequence, making it difficult to examine the genetic changes from benign lesions to carcinoma in a sequential manner. Another reason is that the diagnostic criteria for gastric adenomas are vague and differ between countries in the East and West.

In this article, we describe the issues surrounding gastric adenomas, the molecular mechanisms of carcinogenesis that have been identified to date, and future perspectives.

DIAGNOSTIC CRITERIA FOR GASTRIC ADENOMA

It has long been known that some benign superficial gastric lesions are difficult to distinguish from adenocarcinoma. They are conventionally called atypical epithelial lesions or IIa-subtype[8,9]. These cases were organized and given the diagnostic name "gastric adenoma[10]" approximately during the time the World Health Organization (WHO) histological classification of gastric cancer was established in the 1970s. In Japan, superficial gastric lesions are classified into adenoma and cancer and a treatment policy is adopted: The cancer is resected, small adenomas are followed up, and large adenomas are regarded as early gastric cancer (EGC) and treated by mucosal resection. In Japan, gastric adenomas are classified mainly according to glandular structure, with occasional reference to immunohistochemical mucin staining. Recently, foveolar-type gastric adenomas with a raspberry-like appearance in *H. pylori*-negative cases have become a contentious issue[11,12]. Conversely, outside Japan, dysplasia is used to describe lesions that are difficult to distinguish from benign to malignant. Dysplasia is defined as a histologically probable neoplastic lesion without evidence of invasive growth within the specimen. Intraepithelial neoplasia is a synonymous condition. Dysplasia is classified into low-grade dysplasia (LGD) and high-grade dysplasia (HGD) according to the degree of cellular atypia[13]. Gastric adenoma exists outside Japan but mainly refers to a protruding tumor.

Therefore, there are differences in the diagnoses of superficial gastric lesions between Japan and other countries. Table 1 also shows the classification of gastric lesions according to the WHO classification, Vienna classification proposed at the worldwide pathologists' consensus meeting[14], and revised

Table 1 The classifications of gastric adenoma of the Japanese Gastric Cancer Association and gastric superficial lesions of the WHO classification and the Vienna classification

Classification	Code	Diagnosis	Subtype	Subtype 2
JGCA[16]		Gastric adenoma	Intestinal type	
			Gastric type	Pyloric gland type
				Foveolar type
WHO 2019[13]	8148/0	Glandular intraepithelial neoplasia, low grade		
	8148/2	Glandular intraepithelial neoplasia, high grade		
	8213/0	Serrated dysplasia, low grade		
	8213/2	Serrated dysplasia, high grade	Intestinal-type dysplasia	
			Foveolar-type (gastric type) dysplasia	
			Gastric pit/crypt dysplasia	
	8144/0	Intestinal-type adenoma, low grade		
	8114/2	Intestinal-type adenoma, low grade	Sporadic intestinal-type gastric adenoma	
			Syndromic intestinal-type gastric adenoma	
	8210/0	Adenomatous polyp, low-grade dysplasia		
	8210/2	Adenomatous polyp, high-grade dysplasia		
Vienna[14]	3	Non-invasive low-grade neoplasia	Low-grade adenoma/dysplasia	
	4	Non-invasive high-grade neoplasia		
	4.1		High-grade adenoma/dysplasia	
	4.2		Non-invasive carcinoma (carcinoma *in situ*)	
	4.3		Suspicion of invasive carcinoma	
	5	Invasive neoplasia		
	5.1		Intramucosal carcinoma	
	5.2		Submucosal carcinoma or beyond	
Revised Vienna[15]	3	Mucosal low-grade neoplasia	Low-grade adenoma/dysplasia	
	4	Mucosal high-grade neoplasia		
	4.1		High-grade adenoma/dysplasia	
	4.2		Non-invasive carcinoma (carcinoma *in situ*)	
	4.3		Suspicious for invasive carcinoma	
	4.4		Intramucosal carcinoma	
	5	Submucosal invasion by carcinoma		

JGCA: Japanese Gastric Cancer Association.

Vienna classification[15]. Figure 1 shows the relationship between the diagnosis of gastric lesions and the Japanese classification of gastric carcinoma[16], WHO classification, and Vienna classification. The diagnosis of adenomas in Japan is probably the most limited. It is difficult to determine the correct classification system because they all have advantages and disadvantages, and there are also differences in the frequency of encountering lesions and treatment strategies. In Japan, where the prevalence of gastric cancer is high, clinicians perform numerous endoscopic screenings. They often find EGCs, and targeted biopsy is frequently used for definitive diagnosis. Pathologists and clinicians must determine benign or malignant lesions from biopsies and determine cancer based on cellular and structural atypia. As the presence or absence of submucosal invasion is not required under the Japanese classification,

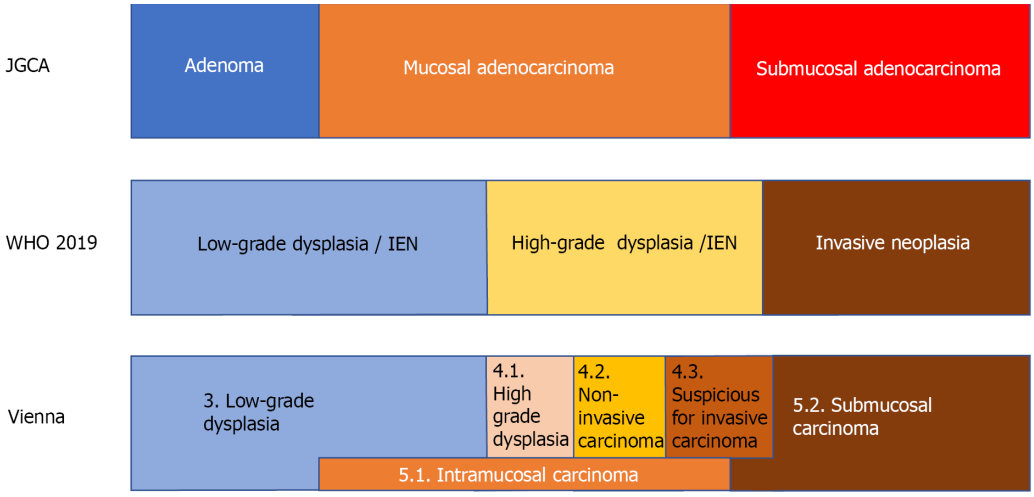

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Figure 1 The relationship of the diagnosis of superficial gastric lesions between the Japanese classification of gastric carcinoma by the Japanese Gastric Cancer Association, the WHO classification, and the Vienna classification. In Japan, gastric cancer is diagnosed based on cellular and structural atypia. On the other hand, outside Japan, dysplasia is used to describe lesions that are histologically probable neoplastic lesions without evidence of invasive growth. Intraepithelial neoplasia is a synonymous condition. Therefore, all mucosal and some submucosal cancers diagnosed by the Japanese Gastric Cancer Association criteria are diagnosed as dysplasia outside Japan. The original Vienna classification is the answer to this discrepancy by setting non-invasive carcinoma and intramucosal carcinoma. IEN: Intraepithelial neoplasia; JGCA: Japanese Gastric Cancer Association.

cancer can be diagnosed without performing complete resection and determining the presence or absence of submucosal invasion. However, intramucosal carcinoma is also considered a cancer, although this is not accepted by some pathologists in Western countries. Conversely, according to criteria other than those of the Japanese Gastric Cancer Association (JGCA), it is difficult to obtain tissue from the submucosal layer with endoscopic biopsy; therefore, pathological judgment using biopsy material can only be performed in dysplasia, making it difficult to diagnose cancer using biopsy. Pathologists cannot determine gastric cancer without complete resection of the lesion, and intramucosal cancer is not defined as cancer. Although intramucosal carcinoma has a good prognosis and rarely metastasizes, lymph node metastasis still occurs in 2% of cases[17]; if left untreated, it can progress to submucosal and advanced cancer[18]. Therefore, intramucosal cancer should still be considered life-threatening. In Japan, adenomas are regarded as "benign lesions"; therefore, even among Japanese pathologists, it is difficult to distinguish high-grade intestinal-type adenomas and foveolar-type adenomas from cancer, and discrepancies in diagnosis sometimes occur.

DOES GASTRIC ADENOMA BECOME CANCER?

Many colorectal cancers are thought to develop from adenomas and serrated polyps. Do gastric adenomas become cancers, similar to colorectal cancer?

Some gastric lesions might be cancerous after mucosal resection, even if the preoperative diagnosis is adenoma using targeted biopsy. The frequency of such cases varies in the literature; however, there are reports of a reasonably high rate; therefore, caution should be exercised[19]. It is sometimes difficult to distinguish between high-grade intestinal-type adenomas and very well-differentiated tubular adenocarcinomas, and sampling errors may occur in targeted biopsies[19]. In contrast, adenocarcinoma in adenoma, unlike colorectal cancer, is rarely observed in low-grade intestinal-type adenoma, and it is rare for low-grade adenoma of Vienna classification category 3 to become malignant[20]. In addition, gastric minute carcinomas without adenomatous components are common. Gastric carcinogenesis is mostly *de novo*, and the adenoma-carcinoma sequence does not appear to be the main pathway of carcinogenesis.

The Cancer Genome Atlas system classifies gastric cancer into four categories based on molecular biological characteristics[21]. A summary of this classification system is presented in Table 2. The molecular biological features revealed here help in the consideration of treatment strategies for advanced gastric cancer; however, this system does not provide insight into genetic alterations in the early stages of carcinogenesis.

The pathway for the accumulation of gene mutations leading to gastric carcinogenesis is not as clear as that in colorectal cancer. This may be mainly due to the lack of a clear adenoma-carcinoma sequence in the stomach and the discrepancy in the diagnostic criteria for gastric adenoma and intramucosal carcinoma between countries in the East and West.

Table 2 Brief summary of The Cancer Genome Atlas classification

Type	Chromosomal instability	EBV	Microsatellite instability	Genomically stable
Percentage	50%	9%	21%	20%
Profile of patients		Male prevalence	Elderly age	Younger age
Location	GEJ, cardia	Corpus or fundus	Antrum	Distal location
Lauren type	Intestinal		Intestinal	Poorly cohesive
Other pathological feature	DNA aneuploidy	Carcinoma with lymphoid stroma		
Prognosis			Favorable	Worst
Genetic features	*TP53* mutation	Extensive DNA promoter methylation	*MLH1* promoter hypermethylation	Low copy number alterations and mutational burden
	Amplification of *TKR*	*CDKN2A* promoter hypermethylation	High mutational burden	*ARID1*, *RHOA*, *CDH1* mutations
		PIK3CA, *ARID1A*, *BCOR* mutations		*CLDN18-ARHGAP26* fusion in 15%

TCGA: The Cancer Genome Atlas; EBV: Eptein-Barr virus; GEJ: Esophagogastric junction.

GENETIC MUTATION AND CANCERIZATION OF GASTRIC ADENOMA AND DYSPLASIA

Recent advances in genetic analysis have provided insights into gene mutations in adenomas and dysplasia, and the pathway to carcinogenesis in adenomas and dysplasia is becoming more clear.

Fassan *et al*[22] investigated the mutational status of HGD and EGC using high-throughput mutation profiling. Mutations in *APC*, *ATM*, *FGFR3*, *PIK3CA*, *RB1*, *STK11*, and *TP53* were confirmed in both HGD and EGC. Lim *et al*[23] examined the mutation profiles of LGD using whole-exome sequencing and confirmed that *APC* mutations occur in LGD. Lee *et al*[24] examined *APC* mutations in adenomas, dysplasias, and adenocarcinomas. They found that *APC* mutations play an essential role in the pathogenesis of adenoma and dysplasia but have a limited role in the progression to adenocarcinoma. Rokutan *et al*[25] investigated the mutational status of LGD, HGD, and intramucosal carcinoma using targeted deep DNA sequencing. They found that *APC* mutations and *TP53* mutations were highly prevalent in these lesions and were the initial mutations in the tumors. *TP53* mutations were also found in microscopic intramucosal carcinomas of 1 mm and 3 mm. *APC* mutations were found in all the LGDs examined. In contrast, no *TP53* mutations were detected in the LGD group. *APC* mutations and *TP53* mutations are frequently observed in patients with HGD, but they are mutually exclusive.

Based on these results, superficial gastric tumors can be roughly divided into the *APC* mutation type and the *TP53* mutation type. *APC*-type tumors have low malignancy and develop into LGD, whereas *TP53*-type tumors have high malignancy and are judged as cancerous even if they are small[25]. It is still unclear whether *APC*-type LGD progresses into HGD or whether *APC*-type HGD progresses into cancer. In contrast, it is reasonable to treat *TP53*-type HGD as cancer. This finding is illustrated in Figure 2; it also shows the translation of this to the JGCA criteria. Many Japanese gastric cancer specialists believe that all mucosal cancers progress from submucosal to advanced. However, some mucosal cancers may not progress to submucosal cancer, although they may progress laterally.

HOW SHOULD GASTRIC TUMORS BE TREATED?

Superficial gastric tumors are often observed in *H. pylori*-positive stomachs under numerous gastroscopies. There is still no consensus regarding the treatment of these tumors.

In Japan, EGC is often detected, and endoscopic submucosal dissection (ESD) is frequently performed; therefore, most superficial gastric tumors, including gastric adenomas, are resected by ESD [19]. The treatment policy is the same in China and South Korea, where there are many *H. pylori*-positive individuals. In contrast, in Western countries, the treatment of dysplasia is not always standardized due to the small number of *H. pylori*-positive patients, low number of gastroscopies performed, and lack of widespread use of ESD. In the Vienna classification[14,15], a target biopsy diagnosis is set from category 1 to 5, with category 1 being negative for neoplasia and should undergo no treatment, category 2 is indefinite for neoplasia and should undergo repeat biopsy, and category 5 is indicated for surgical resection. The problem is the treatment of categories 3 and 4. The revised Vienna classification[15] recommends endoscopic resection or follow-up for category 3 and endoscopic or surgical local resection

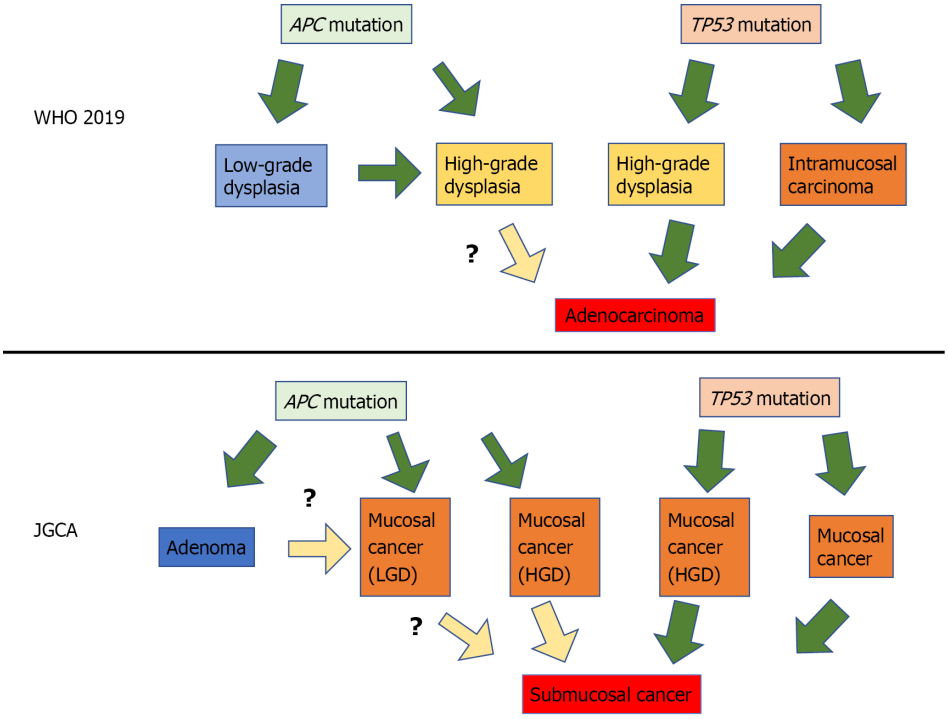

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Figure 2 The diagram assuming the relationship between gene mutations and gastric carcinogenesis. Superficial gastric tumors can be roughly divided into two types by specific gene mutations: The *APC* mutation type and the *TP53* mutation type. *APC*-type tumors have low malignancy and develop into low-grade dysplasia, whereas *TP53*-type tumors have high malignancy and are considered cancerous even if small. JGCA: Japanese Gastric Cancer Association; HGD: High-grade dysplasia; LGD: Low-grade dysplasia.

for category 4. The 2012 MAPS guideline[26] states that "patients with endoscopically visible HGD or carcinoma should undergo staging and adequate management". According to this, category 3 should be followed up, and category 4 should undergo excision. In contrast, the 2019 MAPS II guideline[27] states that "patients with an endoscopically visible lesion harboring LGD, HGD, or carcinoma should undergo staging and treatment". Due to the uncertainty of biopsy diagnosis[28,29], it is assumed that LGD would be upgraded to HGD or adenocarcinoma after resection. Therefore, treatment is also required for LGD [27], and category 3 is targeted for diagnostic treatment. However, staging and treatment methods have not been described. Considering the invasiveness of surgical resection, it is desirable to perform complete *en bloc* resection by ESD first[30] and then perform staging. Subsequent treatment should follow the Japanese Gastric Cancer Treatment Guidelines[31]. If the tumor is a well to moderately differentiated mucosal cancer with no lymphovascular invasion, treatment is completed, and if lymphovascular invasion or submucosal invasion is found, additional surgical treatment of gastrectomy with lymph node dissection is required.

Conversely, do we need ESD for all category 3 cases? Endoscopic resection of colorectal adenomas reduces the incidence of colorectal cancer, which provides evidence that the adenoma-carcinoma sequence is an essential pathway for colorectal carcinogenesis. In contrast, low-grade intestinal adenomas, which are rarely associated with adenocarcinomas, are unlikely to become cancerous even if left untreated. Upgrading to HGD or adenocarcinoma has been reported to be less than 10% after follow-up for adenoma and LGD[20,32], and the possibility of regression with *H. pylori* eradication therapy has also been reported[32]. For these reasons, category 3 adenomas can be safely treated with observation; if the adenoma meets the intestinal type in the JGCA criteria and is less than 2 cm in size, resection may not be necessary. However, a case of gastric-type adenoma that was adenocarcinoma in adenoma with submucosal invasion has been reported[33], and follow-up of gastric-type adenoma may not always be safe. In addition, the safety of observing LGDs that fall into mucosal cancer in the JGCA criteria is not guaranteed. In the future, further understanding of the relationship between genetic mutations in LGD and the natural history of lesions will provide profiles for safe follow-up of category 3 Lesions. Category 3 patients with *APC* mutations may be observed. However, at this time, category 3 adenomas, other than intestinal-type adenomas, seem to have no choice but to undergo complete diagnostic resection with ESD.

The results are summarized in Figure 3. Since category 3 and 4 Lesions are highly likely to be mucosal adenocarcinomas according to the JGCA criteria, complete *en bloc* resection of the mucosal layer is desirable even for diagnostic purposes, and ESD is appropriate. However, ESD is a complicated procedure. Surgical mucosal resection and laparoscopic intragastric surgery may also be acceptable in

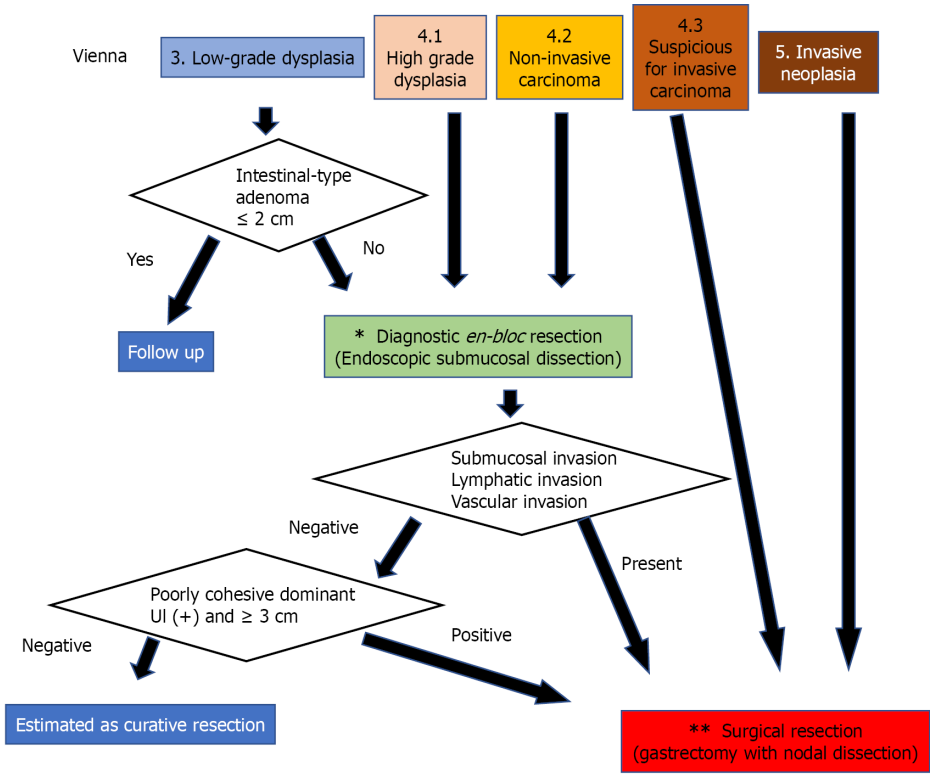

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Figure 3 The strategy for diagnosis, staging, and treatment of gastric dysplasia and cancer according to the Vienna classification. Since category 3 and 4 Lesions are highly likely to be mucosal adenocarcinomas according to the Japanese Gastric Cancer Association (JGCA) criteria, complete *en bloc* resection of the mucosal layer is desirable for diagnosis and initial treatment. However, a small part of category 3, such as a small intestinal-type adenoma judged by the JCGA criteria, can be followed up. In contrast, category 5 corresponds to submucosal adenocarcinoma according to the JGCA criteria; therefore, curative surgery is necessary. Category 4.3 was also treated surgically. The asterisk (*): For *en bloc* mucosal resection, endoscopic submucosal dissection is appropriate; however, laparoscopic intragastric surgery may also be acceptable in cases where there is no skilled endoscopist. The two asterisks (**): Gastrectomy with lymph node dissection up to $D1^+$ is recommended for surgical treatment. However, since the possibility of lymph node metastasis is only 15%-20% even for such lesions, function-preserving curative gastrectomy guided by sentinel lymph node biopsy can be performed by a specialist.

cases where there is no skilled endoscopist[34]. In contrast, category 5 corresponds to submucosal adenocarcinoma in the JGCA criteria; therefore, gastrectomy with lymph node dissection is necessary [17,30]. Category 4.3. also has a high possibility of developing similar lesions; thus, surgery should be performed from the beginning. In addition, since the possibility of lymph node metastasis is only 15%-20% even for such lesions, not only gastrectomy with nodal dissection up to $D1^+$ but also function-preserving curative gastrectomy guided by sentinel lymph node biopsy may be a good indication[17].

CONCLUSION

Gastric carcinogenesis occurs mostly *de novo*, and the adenoma-carcinoma sequence does not appear to be the main pathway of carcinogenesis. Superficial gastric tumors can be roughly divided into the *APC* mutation type and the *TP53* mutation type, which are mutually exclusive. For lesions diagnosed as category 3 or 4 in the Vienna classification, it is desirable to perform ESD for accurate diagnosis and staging. If there is lymphovascular or submucosal invasion, additional surgical treatment of gastrectomy with lymph node dissection is required.

FOOTNOTES

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MINIREVIEWS

Nuclear factor erythroid 2-related factor 2-mediated signaling and metabolic associated fatty liver disease

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Abstract

Oxidative stress is a key driver in the development and progression of several diseases, including metabolic associated fatty liver disease (MAFLD). This condition includes a wide spectrum of pathological injuries, extending from simple steatosis to inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma. Excessive buildup of lipids in the liver is strictly related to oxidative stress in MAFLD, progressing to liver fibrosis and cirrhosis. The nuclear factor erythroid 2-related factor 2 (NRF2) is a master regulator of redox homeostasis. NRF2 plays an important role for cellular protection by inducing the expression of genes related to antioxidant, anti-inflammatory, and cytoprotective response. Consistent evidence demonstrates that NRF2 is involved in every step of MAFLD development, from simple steatosis to inflammation, advanced fibrosis, and initiation/progression of hepatocellular carcinoma. NRF2 activators regulate lipid metabolism and oxidative stress alleviating the fatty liver disease by inducing the expression of cytoprotective genes. Thus, modulating NRF2 activation is crucial not only in understanding specific mechanisms underlying MAFLD progression but also to characterize effective therapeutic strategies. This review outlined the current knowledge on the effects of NRF2 pathway, modulators, and mechanisms involved in the therapeutic implications of liver steatosis, inflammation, and fibrosis in MAFLD.

Key Words: Nonalcoholic fatty liver disease; Metabolic-associated fatty liver disease; Nuclear factor erythroid 2-related factor 2; Oxidative stress; Antioxidants; Liver injury

Core Tip: This updated literature review contributes to the role of nuclear factor erythroid 2-related factor 2 in combating inflammation, oxidative stress, steatosis, and fibrosis in metabolic associated fatty liver disease. There are several reviews that elucidated the advantages of nuclear factor erythroid 2-related factor 2 in human diseases, but this is the first review reporting the broad range of nuclear factor erythroid 2-related factor 2 modulators and their therapeutic implications in metabolic associated fatty liver disease.

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INTRODUCTION

Nonalcoholic fatty liver disease is the most frequent chronic liver disease, affecting about 25% of the global population. Due to the reappraisal in its nomenclature, a group of experts changed the terminology from nonalcoholic fatty liver disease to metabolic-associated fatty liver disease (MAFLD), strengthening the link of this disease to metabolic alterations[1]. MAFLD is defined as a condition where hepatic fat accumulation exceeds 5% of the liver weight without alcohol consumption (< 30 g per day). It covers a wide spectrum of pathological conditions, extending from simple steatosis (deposit of fat in hepatocytes) to nonalcoholic steatohepatitis (characterized by the presence of 5% hepatic steatosis and inflammation with hepatocellular damage, with or without fibrosis), cirrhosis, and ultimately leading to hepatocellular carcinoma[2]. MAFLD is emerging with the prevalence of type 2 diabetes mellitus, obesity, and metabolic syndrome[3]. Of note, patients with MAFLD, and particularly with nonalcoholic steatohepatitis, exhibit an increased liver-related mortality rate and higher incidence of cardiovascular-related morbidity and mortality[2].

MAFLD is the hepatic expression of metabolic syndrome, but its pathogenesis is still not clearly known. Insulin resistance (IR) seems to play a key role in the initiation and progression of the disease from simple fatty liver to advanced forms[4]. MAFLD pathogenesis is complex and multifactorial. The first theory was based on a two-hit hypothesis, where the first hit is liver steatosis, which is due to increased hepatic lipogenesis and reduced free fatty acid degradation caused by IR. This alteration is followed by the second hit of oxidative stress, which induces hepatocyte inflammation and cell death[5, 6]. However, this simplistic theory has been recently replaced by the multiple hit hypothesis, where many factors including systemic and hepatic IR, intestinal microbiota, genetic predisposition, and oxidative stress act simultaneously resulting in a cascade of detrimental effects such as hepatic inflammation, free radical production from gut and adipose tissue, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and hepatocyte apoptosis[7]. Among all the contributing factors of MAFLD, oxidative stress plays a major role. Oxidative stress promotes inflammation by activating Kupffer cells and stimulating the release of proinflammatory cytokines, directly leading to lipid, protein, and DNA/RNA damage. Nuclear factor erythroid 2-related factor 2 (NRF2) is the most important transcription factor in preserving redox homeostasis in the cell and counteracting oxidative or electrophilic stress by producing antioxidant and cytoprotective enzymes such as heme oxygenase 1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and those involved in glutathione (GSH) metabolism[8].

Thus, due to its antioxidative and detoxicant properties, it is currently accepted that NRF2 plays a pivotal role and has been recognized as a potential target to prevent the pathological spectrum of MAFLD. Even though the beneficial role of NRF2 in human diseases has been the topic of several recent reviews, the broad range of NRF2 modulators and their therapeutic implications in MAFLD were not completely summarized in recent literature. In this review, we described the current knowledge on the effects of NRF2-dependent mechanisms involved in the therapeutic implications of liver steatosis, inflammation, and fibrosis in MAFLD.

NRF2 PATHWAY

NRF2 belongs to the basic leucine zipper transcription factors in the Cap "n" Collar subfamily including seven functional domains, Nrf2-ECH homology (Neh) 1 to Neh7[9]. Neh2 is important for interaction between NRF2 and Kelch-like ECH-associated protein 1 (Keap1), a negative modulator of NRF2[10]. Keap1 is a substrate for Cullin based E3 ubiquitin ligase. During homeostatic conditions, Keap1 targets NRF2 that is localized in cytoplasm, causing its polyubiquitination and degradation. The binding and regulation of NRF2 by Keap1 has been defined as the "hinge and latch model"[11]. During oxidative stress, hyperactive cysteine residues of Keap1 undergo thiol modification and NRF2 is dissociated from Keap1, preventing ubiquitination and proteasomal degradation (Figure 1). The newly generated NRF2

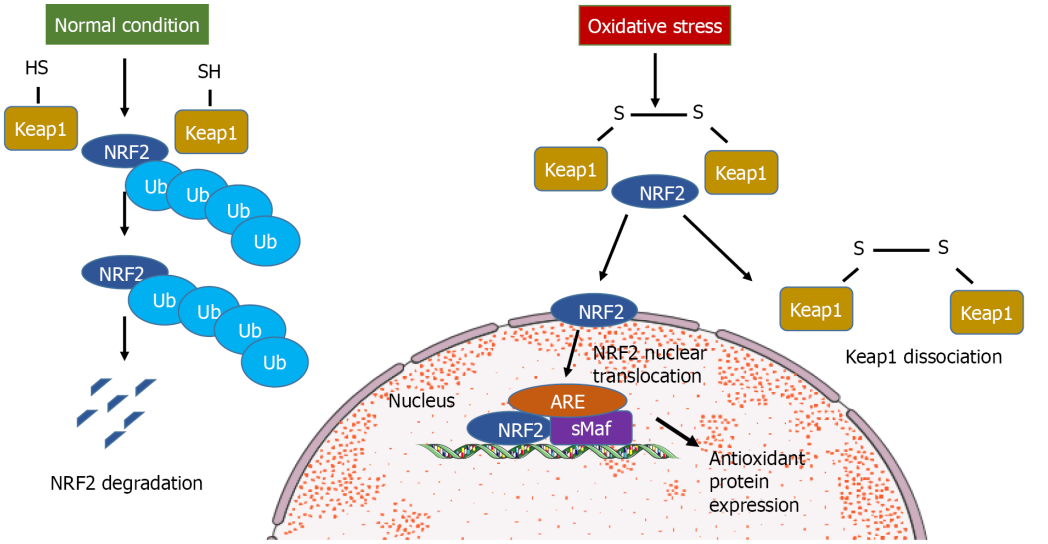

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Figure 1 Kelch-like ECH-associated protein 1-dependent nuclear factor-erythroid 2-related factor 2 signaling. During oxidative stress, nuclear factor-erythroid 2-related factor 2 (NRF2) detaches from kelch-like ECH-associated protein 1 (Keap1) and translocates to the nucleus to bind the target genes. In normal conditions, NRF2 is ubiquitinylated and undergoes degradation. ARE: Antioxidant responsive element; SMaf: Small musculoaponeurotic fibrosarcoma oncogene homologue; Ub: Ubiquitin.

escaped from Keap1 control translocates to the nucleus and heterodimerizes with the Maf proteins, promoting the expression of antioxidant response element genes like *HO-1*, superoxide dismutase (*SOD*), catalase, glutathione-S-transferase, glutathione reductase, glutathione peroxidase (*GSH-Px*), *NQO1*, *etc*[12].

Of note, emerging evidence revealed Keap1-independent novel mechanisms of NRF2 regulation. The phosphatidylinositol 3'-kinase/protein kinase B pathway is protective against oxidative stress and is able to activate NRF2 signaling[13]. Phosphatidylinositol 3'-kinase-protein kinase B-NRF2 signaling pathway involves the glycogen synthase kinase-3β as a key mediator. Glycogen synthase kinase 3β can phosphorylate the NRF2 domain Neh6, containing serine residues that can be recognized by the β-transducin repeats-containing protein. β-transducin repeats-containing protein is a substrate receptor for ubiquitin ligase complex, which targets NRF2 for ubiquitination and proteasomal degradation[14,15]. During autophagy, NRF2 is stabilized by the binding of p62 (autophagy substrate) to Keap1 at the NRF2 binding site, resulting in the transcriptional activation of NRF2-target genes[16,17]. In addition, oxidative stress-induced protein kinase C phosphorylates Neh2 at serine and threonine residue on Ser40, dissociating the Keap1 homodimer and transferring NRF2 to the nucleus, thus binding to the antioxidant response element-mediated cytoprotective genes[18] (Figure 2).

NRF2 IN THE PATHOGENESIS OF MAFLD

MAFLD is the most widespread chronic liver condition worldwide, potentially leading to end stage disease, which requires liver transplantation[19,20]. MAFLD is a lipotoxic disease characterized by both structural and functional mitochondria abnormalities and oxidative stress. Impairment in mitochondrial electron transport chain causes excessive production of reactive oxygen and nitrogen species (ROS and RNS)[21]. ROS and RNS play a crucial role in cellular signaling, proliferation and differentiation, metabolism, and immune defense mechanisms. Besides mitochondria, ROS and RNS are continuously produced by the ER and peroxisomes as byproducts during their normal physiological processes. Oxidative stress is described as the imbalance between production of ROS/RNS and antioxidant systems[22]. Oxidative stress is intrinsically linked to the pathogenesis of MAFLD, and NRF2 has been found to be a key regulator to protect against the hepatocellular injury. Since MAFLD development and progression are characterized by alterations of redox balance, NRF2 is involved in every stage of disease, from simple steatosis to inflammation, advanced fibrosis, and initiation/progression of hepatocellular carcinoma[8].

NRF2 and liver steatosis

Accumulation of lipids in hepatocytes is the first step characterizing MAFLD development. This process is the result of increased fatty acid uptake/synthesis and decreased fatty acid oxidation/removal[23]. Fatty acid oxidation in peroxisomes produces H_2O_2, which in turn decreases the expression of enzymes

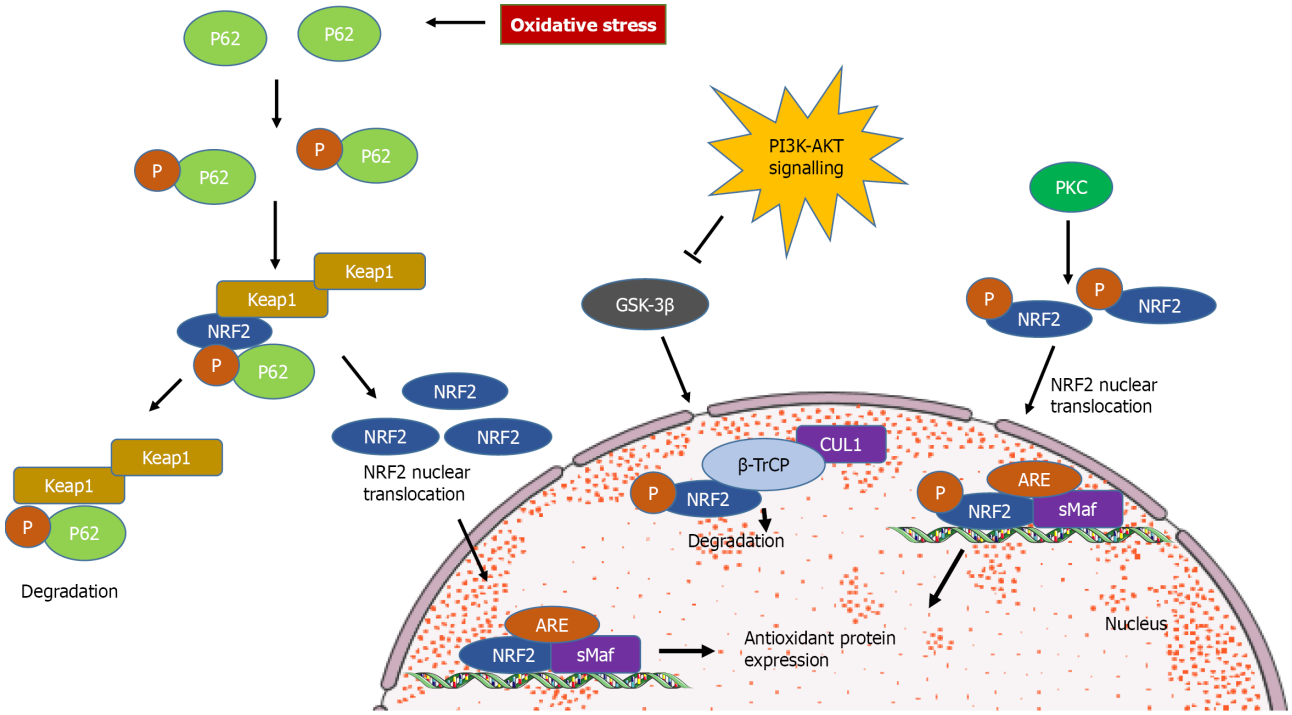

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Figure 2 Kelch-like ECH-associated protein 1-independent nuclear factor-erythroid 2 signaling. During oxidative stress, selective autophagy substrate p62 competes with nuclear factor-erythroid 2 (NRF2) to bind with kelch-like ECH-associated protein 1 (Keap1). As a consequence, NRF2 dissociates from Keap1 and translocates to the nucleus to induce target genes. Glycogen synthase kinase 3β (GSK-3β) phosphorylates the NRF2 subunit Nrf-ECH homology (Neh) 6, leading to degradation by β-transducin repeats containing protein (β-TrCP). Phosphatidylinositol 3'-kinase-protein kinase B (AKT) signaling could inhibit GSK-3β. Protein kinase C phosphorylates Ser40 in Neh2, inducing NRF2 translocation to the nucleus. ARE: Antioxidant responsive element; SMaf: Small musculoaponeurotic fibrosarcoma oncogene homologue.

involved in fatty acid oxidation as carnitine palmitoyltransferase 1A and acyl-CoA oxidase through their regulatory factor peroxisome proliferator activated receptor alpha. Besides, H_2O_2 promotes lipid accumulation by upregulating the expression of sterol regulatory element-binding protein-1c (SREBP-1c), which further activates fatty acid synthase, and stearoyl coenzyme-A desaturase 1, contributing to MAFLD pathogenesis[24]. In addition, ER-stress activates SREBP-1c and increases the expression of hepatic very-low density lipoprotein receptor, leading to deposition of triglycerides (TG)[12,24].

NRF2 is a key player in maintaining cellular homeostasis, suppressing MAFLD promotion and progression. A microarray analysis of mouse hepatic gene expression revealed that pharmacologic and genetic activation of *NRF2* suppresses key enzymes involved in lipid synthesis and reduces hepatic lipid storage. NRF2$^{-/-}$ mice fed a high-fat diet (HFD) are more prone to develop steatosis and oxidative stress than wild-type mice[25]. Consistent to this, NRF2-knockout mice fed a methionine- and choline-deficient (MCD) diet developed a severe form of micro- and macrovesicular steatosis and neutrophil recruitment compared to wild-type mice[26-28]. Studies on hepatic protein expression in NRF2-null and wild-type mice found two major groups of NRF2-modulated proteins. One group of proteins in NRF2 wild-type animals was implicated in phase II drug metabolism and antioxidant defense, while the other group of proteins in NRF2-null animals was involved in lipid and fatty acid synthesis and metabolism [29]. Another study in NRF2-null 8-wk old mice revealed a higher expression of SREBP-1c and fatty acid synthase than wild-type mice[30]. Nonetheless, NRF2 has little effect on hepatic fatty acid metabolism in 12-25 wk old mice[31,32].

In addition, flavonoid glycoside scutellarin ameliorates MAFLD pathogenesis by reducing blood lipid levels and enhances antioxidant capacity by activating peroxisome proliferator-activated receptor gamma (PPAR-γ) and its cofactor-1α as well as NRF2-dependent enzymes HO-1 and glutathione-S-transferase. Moreover, scutellarin suppresses nuclear factor κ B (NF-κB) and Keap1 mitigating MAFLD [33]. Another study revealed that scutellarin contains breviscapine as its active component, possibly exerting its antioxidant effects through phosphatidylinositol 3'-kinase/protein kinase B activation and subsequent enhancement of NRF2 nuclear translocation, increasing the expression of HO-1 and NQO1. Thus, breviscapine could be used in MAFLD and hyperlipidemia due to its potential therapeutic effects [34].

In addition, the food-derived compound apigenin is a modulator of PPAR-γ, which attenuates the NRF2-associated antioxidative response and hepatocyte lipid metabolism in MAFLD[35]. The specific deletion of NRF2 in mice diminished the signs of MAFLD induced by HFD, decreasing the accumulation of TGs. Hepatic NRF2 deficiency dampens the expression of PPAR-γ, suggesting that the NRF2-

dependent expression of PPAR-γ is critical in initiation and progression of MAFLD[36].

Liver X receptors are a family of nuclear receptors implicated in the modulation of lipid homeostasis. Directly or *via* SREBP-1c, liver X receptor α triggers the expression of lipogenic genes involved in the uptake and synthesis of fatty acids, TGs, cholesterol, and phospholipids. Treatment with the NRF2 activator sulforaphane suppresses T0901317-induced lipogenesis, promoting deacetylation of farnesoid X receptor (FXR) by competitive binding of p300, a protein necessary for the acetylation of FXR. The FXRE chromatin immunoprecipitation assay confirmed that NRF2 may complex with p300 and, as a result, dissociate from the FXR complex[37-39]. Moreover, NRF2 activator inhibits SREBP-1c and lipogenic genes by promoting deacetylation of FXR and inducing small heterodimer partner, which accounts for the repression of liver X receptor α-dependent gene transcription, protecting the liver from excessive fat accumulation[40].

NRF2 and liver inflammation

NRF2 is further involved in the regulation of pro- and anti-inflammatory mediators. NRF2 is known for its anti-inflammatory effects as it inhibits the expression of proinflammatory cytokines like interleukin-6 (IL-6), tumor necrosis factor, and inducible nitric oxide synthase. Moreover, NRF2-dependent antioxidant genes, such as *HO-1*, *NQO1*, and glutamate cysteine ligase catalytic and modifier subunits, inhibit the transcription of proinflammatory mediators by blocking NF-κB activation[41-43]. Of note, NRF2 also triggers the NLR family pyrin domain containing 3 inflammasome, which cleaves caspase-1 and initiates the processing of pro-IL-1β to mature IL-1β[44]. NLR family pyrin domain containing 3-dependent production of proinflammatory response can be inhibited by activation of NRF2 through dimethyl fumarate in alcoholic liver disease[45], and 4-acetylantroquinonol B in mice fed with a methionine- and choline-deficient diet[46] inducing the expression of NQO1, which inhibits the ROS/RNS-dependent priming.

NRF2-KO mice fed the methionine- and choline-deficient diet lose the antioxidant and detoxification enzymes and show an increase in steatosis, inflammation, oxidative stress, lipid peroxidation, and fibrinogenesis[26,28]. In line with these results, feeding the NRF2-KO mice with the HFD yielded significantly greater amounts of lipids and inflammation compared to wild-type mice. NRF2-KO mice fed a diet containing 4% soyabean oil and 16% lard for 12 wk exhibited massive lipid accumulation, inflammation, oxidative stress, and iron accumulation when compared to their wild-type counterparts [47]. NRF2-KO mice fed a diet containing 45 kcal% fat (0.02% cholesterol) for 24 wk displayed a higher MAFLD activity score compared to wild-type animals. In HFD-fed NRF2-KO mice, livers scored higher for steatosis, ballooning, inflammation, and fibrosis when compared to $Nrf2^{+/+}$ mice. The biochemical characterization studies of such mice revealed higher expression of sterol regulatory element binding transcription factor 1 and 2 and carbohydrate response element binding protein also known as MLX-interacting protein-like in HFD-fed NRF2-KO mice, suggesting exaggerated lipogenic transcription[48]. In another study, NRF2-KO mice fed a high-fat plus 30% fructose in drinking water exhibited a higher MAFLD score than wildtype. Moreover, these NRF2-KO mice overexpress lipogenic transcription factor sterol regulatory element binding transcription factor 1, fatty acid synthase, stearoyl coenzyme-A desaturase 1, and CD36 and exhibited higher proinflammatory factors as NF-κB p65 and p50 subunits [49].

In another investigation, NRF2-KO mice fed a chow diet were subjected to scanty inflammation with minimal increases in *IL-1β*, *Cox2*, and *Nos2* mRNA[26,28]. This is due to the compromised expression of zonula occludens-1 and claudin-1, which are responsible for the translocation of lipopolysaccharides from the gut microbiota to the liver through the portal vein. In addition, the phagocytic ability of Kupffer cells is diminished in NRF2-KO due to lower expression of the macrophage receptor with collagenous structure that restricts TLR4 signaling and boosts the inflammatory response on exposure to lipopolysaccharide[50].

NRF2 and liver fibrosis

Liver fibrosis is a reversible wound healing response and degenerative condition caused by extensive deposition of extracellular matrix proteins like collagen fibrils[51]. Mechanisms underlying liver fibrosis include the activation of both hepatic stellate cells and Kupffer cells, resulting in functional and biological alterations[52]. Oxidative stress is a serious process involved in liver damage, and the activation of the Keap1/NRF2 pathway plays a protective role in liver fibrosis[12]. NRF2 activation triggers the reverse IR and attenuates liver fibrosis by inhibiting hepatic steatosis. These noticeable effects during NRF2 activation are due to the disruption of JAK2/STAT3 signaling and higher expression of suppressor of cytokine signaling 3[53]. Moreover, administration of fibroblast growth factor 1 variants carrying substitutions of heparin-binding sites in 9-mo-old mice inhibited activity and expression of lipogenic genes, improving both steatohepatitis and fibrosis[54].

CCl_4-induced hepatic fibrosis is accompanied by elevated serum transaminases, alkaline phosphatase, and bilirubin, decreased albumin, and increased proinflammatory cytokines. In addition, CCl_4-intoxicated rats display an increase in NF-κB, p65, malondialdehyde and a decrease in antioxidants. Bone marrow-derived mesenchymal stem cells show favorable effects in ameliorating the hepatic effects of CCl_4 through NRF2/HO-1 signaling, suppressing liver fibrosis, inflammation, and oxidative stress [55].

A major bioactive extract from the plant *Schisandra chinesis*, known as Schisandrin B, exerts anti-inflammatory, anti-tumor, antioxidative, and hepatoprotective properties. Schisandrin B effectively improves liver function and decreases collagen deposition in the CCl_4-induced liver fibrosis in rats through the modulation of NRF2-antioxidant response element and transforming growth factor-β /Smad signaling pathways[56]. Tanshinol, a water-soluble compound isolated from *Salvia miltiorrhiza Bunge*, is known to exert a variety of biological effects, including anti-fibrotic effects. Rats with CCl4-induced liver fibrosis treated intraperitoneally with tanshinol show lower serum levels of aspartate aminotransferase, alanine aminotransferase, and total bilirubin, as well as circulating hyaluronic acid, laminin, type IV collagen, and procollagen III peptide as compared to controls. Tanshinol is also able to suppress the expression of inflammatory cytokines such as transforming growth factor-β, tumor necrosis factor, Cox2, IL-1β, and IL-6 through regulation of the NF-κB pathway. In addition, tanshinol treatment is able to regulate the NRF2/HO-1 signaling pathway increasing SOD and GSH-Px and decreasing malondialdehyde levels. In this regard, tanshinol exerts protective effects on CCl4-induced liver fibrosis by activating the NRF2 pathway[57].

Asiatic acid (AA), a bioactive compound extracted from *Centella asiatica*, is known to have anti-inflammatory, antioxidative, and hepatoprotective properties[19-22]. Fan *et al*[34] showed that treatment with AA in the CCl_4-induced liver fibrosis dramatically ameliorates oxidative stress, inflammation, and fibrosis in rats. The nuclear NRF2 levels were increased after AA treatment, and the NRF2-dependent proteins like HO-1, NQO-1, and Glutamate cysteine ligase catalytic subunit were significantly increased to counteract oxidative stress. Furthermore, AA inhibited the NF-κB/IkBα and JAK1/STAT3 signaling pathway to suppress the activation of hepatic stellate cells and the production of inflammatory markers, suggesting that AA could be used for the treatment of liver fibrosis[58]. Another water soluble compound, salvianolic acid A, extracted from a traditional Chinese herb *Radix Salvia miltiorrhiza*, was found to have anti-fibrotic effects. salvianolic acid A is able to modulate the NRF2/HO-1, NF-κB/IkBα, p38 MAPK, and JAK1/STAT3 signaling pathways, and to ameliorate the CCl4-induced liver fibrosis, improve morphology and attenuate collagen deposition in the fibrotic liver. Besides, salvianolic acid A is able to increase the levels of SOD and GSH-Px and decrease the malondialdehyde levels, indicating the effectiveness in preventing liver fibrosis by inhibiting inflammation and oxidative stress[59].

Pharmacological stimulation of NRF2 by acetylenic tricyclic bis (cyano enone) TBE-31 reverses IR in wild-type mice, decreases liver steatosis by increasing hepatic fatty acid oxidation and reducing ER stress, and lessens markers of oxidative stress, apoptosis, and fibrosis. Of note, histology studies showed that TBE-31 decreases the fibrosis score and MAFLD activity score[59]. In another study, NRF2 activator NK-252 (1-(5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)-3-(pyridin-2-ylmethyl)urea) significantly reduced markers of fibrosis like COL1A1, TIMP-1, and transforming growth factor-β in rats, suggesting that this compound could be used as a therapeutic agent to reverse liver fibrosis. In addition, NK-252 attenuated the serum aspartate aminotransferase and alanine aminotransferase levels in male Fischer rats and upregulates *NQO1* gene expression[60].

THERAPEUTIC IMPLICATIONS OF NRF2 IN MAFLD

Currently, there is no medicine that can treat MAFLD, but some therapeutic agents are useful in managing the problems associated with the disease (Table 1). Thus, it is necessary to develop and test drugs for the prevention and treatment of MAFLD, and it is conceivable that NRF2-activating compounds can attenuate MAFLD progression. Plant-derived compounds including resveratrol, curcumin, quercetin, and synthetic molecules like oltipraz and pirfenidone could be used to prevent oxidative stress by modulating the NRF2 pathway[12,21].

Flavonoids represent a class of bioactive antioxidants extracted from vegetables, plants, and fruits known to exhibit therapeutic properties in MAFLD. The flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside activates NRF2 and improves the ratio of GSH/glutathione disulfide and increases the expression of HO-1 and GSH-Px3[61,62]. The flavonoid scutellarin (4′,5,6-trihydroxy flavonoid-7-glucuronide) increases NRF2 protein in C57BL/6J mice, increases the expression of HO-1, glutathione-S-transferase, and NQO1, and inhibits both NF-κB and Keap1[33]. Furthermore, 7,8-dihydroxyflavone upregulates NRF2 activity to counteract alcohol-induced and HFD-induced liver toxicity[63]. Apigenin (4′,5,7-trihydroxyflavone), a flavonoid derived from fruits, inhibits lipid peroxidation and exerts protective effects against hepatic steatosis. Moreover, apigenin increases the activities of SOD, CAT, and GSH-Px [35,64].

Gastrodin is a water-soluble extract of *Gastrodia elata* BI that exerts antioxidative activity and improves lipid metabolism in MAFLD mice by promoting NRF2 nuclear translocation[65]. Clusterin, a glycoprotein extracted from ram rete testis fluid, improves steatosis and hepatitis induced by methionine and choline-deficient diet by triggering NRF2 and HO-1 expression[66]. Osteocalcin treatment improves hepatic TG accumulation, promotes NRF2 nuclear translocation, and inhibits phosphorylation of c-Jun N-terminal kinase pathway[67].

In addition, compounds like scutellarin containing breviscapine, hesperitin, apigenin, scoparone, Schisandrin B, tanshinol, and AA and other tabulated compounds are known to exert antioxidative and

Table 1 Modulators of nuclear factor erythroid 2-related factor 2 pathway in metabolic associated fatty liver disease

Compound name	Species	Diet/duration	Treatment	Key findings	Reference
MonoHER	Female C57BL/6J mice ($Ldlr^{-/-}$)	High fat and high cholesterol/13 wk	Administered daily subcutaneously at a dosage of 500 mg/kg of body weight (25 μL/g of body weight)	NRF2 activation, ↑GSH/GSSG ratio, ↑HO-1, GSH-Px	[62]
Scutellarin	Male C57BL/6 mice, hepaG2 cells	High fat/10 wk	Administration of 12.5, 25.0, and 50.0 mg/kg per day	↑PPARγ, PGC-1α, NRF2, HO-1, NQO1, Keap1, NF-κB	[33]
	Sprague-Dawley rats	High fat/12 wk	Administered orally 50, 100, and 300 mg/kg/d	NRF2, HO-1, NQO1; PI3K/AKT activation	[34]
Apigenin	Male C57BL/6J mice	High fat/16 wk	Injected intraperitonially 30 mg/kg daily for 3 wk	NRF2 activation; PPARγ inhibition; SOD, CAT, GSH-Px	[35]
7,8-dihydroxyflavone	Male wistar rats	High fat, ethanol/12 wk	Administered intraperitonially at 5 mg/kg/d for 4 wk	Amelioration of liver architecture, vescicular changes, infiltration; restored serum biomarkers like AST, ALT, and TC; ↑NRF2; ↓NF-κB	[63]
Resveratrol	Male C57BL/6 mice	High fat/16 wk	Supplemented with 0.4% resveratrol in HFD for 16 wk	Attenuated liver steatosis; ↑ NRF2 activation; attenuated HFD induced methylation of NRF2 promoter; ↓oxidative stress	[68]
Quecertin	HepG2 cells	-	Treated with quecertin at 5-50 μM concentrations for 0, 10, 30, 60, 120, 240, and 1080 min	↑GSH, GSH-Px, GCS; p38-MAPK is involved in NRF2 modulation; ↓oxidative stress	[69]
Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione)	Male C57BL/6 mice	High fat and high fructose/8 wk	Administered orally 50 and 100 mg/kg/d for 4 wk	↑CYP3A, CYP7A; regulation of NRF2/FXR/LXRα pathway; ↓ SREBP-1C, FAS	[70]
	Male Sprague-Dawley rats	High fat/6 wk	Administered orally 50 mg/kg daily for 6 wk	↓Steatosis and inflammation; ↓ Serum aminotransferases, lipids, and insulin resistance; ↓ TNF, IL-6, MDA; ↑NRF2, GSH, HO-1, SOD	[71]
Oltipraz	Male Fischer 344 rats	Choline-deficient L-amino acid–defined/10 wk	Administered orally at 60 mg/kg/d for 9 wk	↑NRF2 activation; antifibrotic and anti-inflammatory; ↓AST and ALT; ↑*NQO1* gene expression	[61]
GSTD	HL-7702 cells, male C57BL/6J, male Sprague-Dawley rats	Oleic acid (OA)/24 h, high fat/10 wk; high fat and high cholesterol/10 wk	Cells were treated with GSTD for 24 h, administered orally at 10, 20, 50 mg/kg per day for 10 wk, administered orally at 20, 50 mg/kg per day for 10 wk	↑NRF2, HO-1, SOD; activate AMPK/NRF2; ↓proinflammatory response, and hepatic steatosis; ↓MDA, ROS	[65]
NK-252 1-(5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)-3-(pyridin-2-ylmethyl)urea)	Male Fischer 344 rats	Choline-deficient L-amino acid–defined/10 wk	Administered orally at 20, 60 mg/kg/d for 9 wk	Attenuated histological abnormalities; ↑antifibrotic effects; ↓TGF-β1, collagen α1; NRF2 activation; ↑NQO1 expression	[61]
Clusterin	Male hCLU-tg mice	MCD/3 wk	Generated hepatocyte-specific clusterin overexpression transgenic mice and fed with MCD diet	↓Hepatic TGs; less infiltration of macrophages; ↓TNF; ↑NRF2 activation and mRNA of *HO-1*	[66]
Osteocalcin	Male C57/BL6J mice	High fat/12 wk	Injected intraperitonially at concentration 3 ng/μL/d for 12 wk	↓Hepatic TG accumulation; ↑ NRF2 activation; ↑CAT, SOD, GSH-Px; ↓JNK activation	[67]
Orlistat	Male Sprague-Dawley rats	High fat/12 wk	Administered at 10 mg/kg/d for 12 wk	↑NRF2 activation; protection against insulin resistance, hyperlipidemia, oxidative stress, and liver injury	[72]
Garcinia Cambogia	Male C57BL/6N mice	High fat/8 wk	Administered 200, 400 mg/kg/d for 8 wk	↑NRF2 activation; ↓ROS production; suppressed lipogenic factors C/EBPα and	[73]

				PPARγ; suppressed apoptosis by normalizing Bcl-2/BAX ratio and PARP cleavage	
HTT	Male Sprague-Dawley rats, 3T3-L1 murine embryo fibroblast cells	High fat/4 wk, 3T3-L1 cells treated with FBS/DMEM for 8 d	Administered orally HTT at 350, 700, and 1400 mg/kg/d, 3T3-L1 cells treated with HTT at 500 μg/mL for 24 h or 48 h	↑NRF2-HO-1 activation, antioxidant activities; HTT inhibited liver weight gain; reduced lipid profile; improved liver function; HTT promoted lipolysis and increased antioxidant activities in 3T3-L1 cells	[74]
Hesperitin	HepG2 cells, male wistar rats	OA/24 h, high fat/16 wk	Treated cells at 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 μM; administered 100 mg/kg in 0.5% CMC-Na	Alleviated hepatotoxicity and oxidative stress by increasing SOD, GSH-Px, GCLC, and HO-1; ↑NRF2 activation; suppressed OA induced inflammation; reduced TC, TGs, and LDLC in a dose-dependent manner	[75]
Glucoraphanin	Male C57BL/6JSlc mice	High fat/14 wk	Administered 0.3% glucoraphanin orally for 14 wk	Decrease in weight gain; improved insulin resistance; reduced hepatic steatosis and oxidative stress; decrease in circulating LPS; ↑NRF2 activation; ↑energy expenditure and; UCP1 protein expression	[76]
Scutellaria baicalensis extract	Male KK-A^{y} mice	1% Orotic acid and 33% sugar/7 d	Supplemented with diet for 7 d	Diminished increase in liver weight; attenuated hepatic steatosis; ↑NRF2 expression; suppress *SREBP-1c* gene and protein expression	[77]
Ginkgolide B	Male C57/BL6 ApoE$^{-/-}$mice, HepG2 cells	High fat/5 wk, 100 μM palmitic acid (PA) and 200 μM OA/24 h	Administered orally at 20, 30, and 1.3 mg/kg/d; treated cells at dosages 0, 1, 2, 4, 8, 16, and 32 μg/mL	NRF2 activation; inhibition of oxidative stress and lipid peroxidation through NRF2 pathway; increase in HO-1 and GSH-Px4	[78]
Scoparone	Male C57BL/6 J mice, AML2 and RAW264.7 cells	MCD/4 wk; AML12/300 μM PA and RAW264.7/10 μM/ Chloroquine	Administered daily intraperitonially for 4 wk at 20, 40, and 80 mg/kg; AML12 and RAW264.7 cells were pretreated with scoparone for 2 h	Ameliorated hepatic inflammation; improved hepatic autophagy; suppressed inflammation by inhibiting ROS/P38/NRF2 axis and PI3K/AKT/mTOR pathway	[79]
DA	Male C57BL/6J mice, HL7702 cells	High fat/12 wk, 0.6 mM OA/24 h	Administered by gavage at 10 and 20 mg/kg/d for 9 wk; treated with 2.5, 5.0, and 10.0 μM DA	Ameliorated liver ferroptosis in mice and cells; improved oxidative stress and lipid peroxidation *in vivo*; ↑NRF2-HO-1 expression; ↑GSH, GSH-Px4	[80]
Silibinin	Male C57BL/6 mice, NCTC-1469 cells	MCD/6 wk, OA plus PA/24 h	Administered by gavage at 10 and 20 mg/kg/d for 6 wk, 0.25 mM/L PA and 0.5 mM/L OA/24 h	Prevented CFLAR-JNK pathway; ↑β-oxidation and efflux of fatty acids; ↑ expression of CAT, GSH, GSH-Px, and HO-1; ↓expression of CYP2E1 and CYP4A; ↑NRF2 activation	[81]
Chicoric acid	Male C57BL/6 mice	High fat/9 wk	Administered by gavage at 15 and 30 mg/kg/d for 9 wk	Attenuated hyperglycemia, dyslipidemia, and systemic inflammation; alleviated hepatic lipid accumulation and oxidative stress; suppressed hepatic inflammation and NF-κB pathway; ↑NRF2/Keap1 activation; improved gut microbiota	[82]
Carbon monoxide releasing molecule-A1	Male C57BL/6J mice	High fat/16 wk	Administered intraperitonially 2 mg/kg/d for 7 wk	↑NRF2/ARE activation; improved lipid homeostasis; ↑ ATP production; improved mitochondrial biogenesis; ameliorated oxidative stress	[83]

NRF2: Nuclear factor erythroid 2-related factor 2; GSH: Reduced glutathione; GSSG: Oxidized glutathione; HO-1: Heme oxygenase-1; GSH-Px: Glutathione peroxidase; PPAR-γ: Peroxisome proliferator-activated receptor-γ; PGC-1α: Proliferator-activated receptor gamma coactivator-1α; NQO1: NAD(P)H quinone oxidoreductase 1; DA: Dehydroabietic acid; PA: Palmitic acid; NF-κB: Nuclear factor κ B; PI3K: Phosphatidylinositol 3′-kinase; SOD: Superoxide dismutase; CAT: Catalase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TC: Total cholesterol; HFD: High-fat diet; GCS: Glutamylcysteine-synthetase; MAPK: Mitogen-activated protein kinase; CYP3A: Cytochrome P450, family 3, subfamily A, CYP7A Cytochrome P450, family 7, subfamily A; FXR: Farnesoid-X-receptor; LXRα: Liver X receptor α; SREBP-1C: Sterol regulatory element-binding protein-1c; FAS: Fatty acid synthase; TNF: Tumor necrosis factor; IL-6: Interleukin-6; MDA: Malondialdehyde; AMPK: AMP kinase; ROS: Reactive oxygen species; TGF-β1: Transforming growth factor-β1; TG: Triglycerides; JNK: c-Jun N-terminal kinase; C/EBPα: CCAAT/enhancer binding protein α; Bcl-2: B-Cell Leukemia/Lymphoma 2; BAX: BCL2 associated X protein; PARP: Poly-ADP ribose polymerase; HTT: Hedansanqi Tiaozhi Tang; GCLC: Glutamate cysteine ligase catalytic; LDLC: Low density lipoprotein cholesterol; LPS: Lipopolysaccharide; UCP1: Uncoupling protein 1; GSH-Px4: Glutathione peroxidase 4; mTOR: Mammalian target of rapamycin; CFLAR: CASP8 And FADD like apoptosis regulator; CYP2E1: Cytochrome P450 family 2 subfamily E member 1; CYP4A: Cytochrome P450 family 4 subfamily A; ARE: Antioxidant response element; AKT: Protein kinase B; MCD: Methionine- and choline-deficient; GSTD: Gastrodin; Keap1: Kelch-like ECH-associated protein 1.

hepatoprotective activity by modulating the NRF2 pathway.

CONCLUSION

Oxidative stress can be a potent inducer of inflammation and fibrosis in the spectrum of chronic liver diseases. Among them, MAFLD is the most widespread chronic liver condition worldwide. The transcription factor NRF2 has gained importance in recent years as a possible therapeutic target for the treatment of liver diseases. The expression of antioxidant protective genes through the NRF2 pathway counteracts oxidative stress and prevents progression of liver damage in MAFLD. The different antioxidative molecules modulating the NRF2 pathway have exerted beneficial effects in ameliorating liver damage. Currently, there is no efficient treatment to counteract the complex pathophysiology of liver diseases. Thus, compounds having antioxidative properties could be useful candidates for the treatment of liver diseases by modulating the NRF2 signaling pathway. NRF2 activators could improve and prevent the advanced stages of MAFLD such as liver fibrosis and liver cirrhosis. Natural plant-derived and synthetic NRF2 activators require further experimental validation to be promoted as efficient therapeutic agents. Some drugs have entered clinical trials, and further attempts are ongoing to find NRF2 inducers with high bioavailability, safety, and specificity.

FOOTNOTES

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MINIREVIEWS

Current and future perspectives on acute-on-chronic liver failure: Challenges of transplantation, machine perfusion, and beyond

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Abstract

Acute-on-chronic liver failure (ACLF) is a syndrome that occurs in patients with chronic liver disease and is characterized by acute decompensation, organ failure and high short-term mortality. Partially due to the lack of universal diagnostic criteria, the actual ACLF prevalence remains unclear; nevertheless, it is expected to be a highly prevalent condition worldwide. Earlier transplantation is an effective protective measure for selected ACLF patients. Besides liver transplantation, diagnosing and treating precipitant events and providing supportive treatment for organ failures are currently the cornerstone of ACLF therapy. Although new clinical specific therapies have been researched, more studies are necessary to assess safety and efficacy. Therefore, future ACLF management strategies must consider measures to improve access to liver transplantation because the time window for this life-saving therapy is frequently narrow. Thus, an urgent and global discussion about allocation and prioritization for transplantation in critically ill ACLF patients is needed because there is evidence suggesting that the current model may not portray their waitlist mortality. In addition, while donor organ quality is meant to be a prognostic factor in the ACLF setting, recent evidence suggests that machine perfusion of the liver may be a safe tool to improve the donor organ pool and expedite liver transplantation in this scenario.

Key Words: Acute-on-chronic liver failure; Liver cirrhosis; Liver transplantation; Machine perfusion; Hypothermic oxygenated machine perfusion

Core Tip: Acute-on-chronic liver failure (ACLF) is characterized by high short-term mortality. Although new clinical specific therapies have been researched, more studies are necessary to assess safety and efficacy. Conversely, earlier transplantation is effective for selected patients. Therefore, future ACLF management strategies must consider measures to improve access to liver transplantation. Discussions about donor organ allocation and recipient prioritization are necessary because there is evidence suggesting the current model may not portray the waitlist mortality of these patients. In this scenario, machine perfusion of the liver may prove to be a safe tool to improve the donor organ pool.

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INTRODUCTION

Despite the heterogeneity in the diagnosis criteria, consensually, acute-on-chronic liver failure (ACLF) is a condition that occurs in patients with chronic liver disease developing multi-organ failure in the presence of one or more hepatic or extrahepatic precipitant events. In addition, it is associated with high 28-d mortality[1-4]. Several international consortiums proposed different definitions of ACLF, reflecting their own types of underlying liver disease and precipitant events. The Asian Pacific Association for the Study of the Liver (APASL), the European Association for the Study of the Liver (EASL), formed by the Chronic Liver Failure (CLIF) Consortium and the North American Consortium for the Study of End-Stage Liver Disease (NACSELD) are the most widely accepted[1,5,6].

Because of the lack of universal diagnostic criteria and study design limitations, the actual ACLF prevalence remains unclear. However, it is expected to be a highly prevalent condition worldwide. For example, in the CANONIC study (EASL-CLIF Acute-on-Chronic Liver Failure in Cirrhosis study), a prospective evaluation of 1343 patients admitted for acutely decompensated cirrhosis, 22.6% met the EASL-ACLF criteria for ACLF, and 8.3% developed the condition during hospitalization. In another study using APASL criteria, 12% of complicated cirrhotic patients were diagnosed with ACLF[1,7]. Recently, a large meta-analysis of global epidemiological data found about 35% of ACLF in patients admitted due to acutely decompensated cirrhosis using EASL-ACLF criteria, with a higher prevalence in South Asia, reaching 65%[8]. Notably, the overall mortality in ACLF is about 32% and increases in parallel with the number of organ failure (OF). The 28-d mortality rate for ACLF - grades 1 to 3 - ranges from 20% to 80%, 49% to 77%, and 13% to 86% for EASL-CLIF Consortium, NACSELD, and APASL-ACLF, respectively[4,9].

In recent years, several studies have investigated the pathophysiology, prognosis, and treatment options for ACLF. Although the benefit of timing liver transplantation (LT) in ACLF is undeniable, issues still exist with assessing the mortality risk of patients on the waiting list to avoid futile LT. In addition, another urgent and global discussion is about allocation and prioritization for LT in critically ill ACLF patients, as the time window for transplantation is frequently narrow[2,10,11].

This review provides an overview of ACLF management, focusing on the current challenges of LT in this scenario and future perspectives, including machine perfusion of the liver.

DIAGNOSTIC CRITERIA FOR ACLF

The natural history of cirrhosis is characterized by a long asymptomatic phase called compensated cirrhosis. The increase of portal pressure above the hepatic venous pressure gradient of 10 mmHg plays a central role in the transition to decompensated cirrhosis. Acute decompensation (AD) is defined by the new development of ascites, hepatic encephalopathy, hepatorenal syndrome, variceal bleeding, or infection[1,9].

In the PREDICT study (PREDICTing ACLF), a prospective and observational analysis of 1071 patients hospitalized with AD, 218 (20%) developed ACLF in 90 d (3-mo mortality rate of 53.7%), called pre-ACLF. Two hundred and thirty-three patients (22%) required frequent hospitalization unrelated to ACLF, called unstable decompensated cirrhosis (3-mo mortality rate of 21.0%), and 620 (58%) reached a state called stable decompensated cirrhosis (1-year mortality rate of 9.5%). These three clinical courses revealed by the PREDICT Study group originated from data of the CANONIC study, which created the EASL-CLIF definition of ACLF - probably the most widely accepted definition used to date[1,12]. Therefore, cirrhotic patients with pre-ACLF should be promptly identified due to the high risk of death in the short term.

THE EASL-CLIF CONSORTIUM DEFINITION OF ACLF

The EASL-CLIF definition considers ACLF in cirrhotic patients with or without prior episodes of decompensation and one or more OF. The CANONIC study enrolled 1343 patients from 29 centers in 12 European countries. Chronic hepatitis C virus and alcohol were the most frequent underlying causes of chronic liver diseases. The OF definitions were adapted from the Sequential Organ Failure Assessment (SOFA) score and called the CLIF-SOFA score. The CLIF-SOFA score includes subscores ranging from 0 to 4 for each of the six components (liver, kidney, brain, coagulation, circulation, and lungs). Aggregated scores range from 0 to 24 and provide information on overall severity[13].

OF was defined by liver failure (level of serum bilirubin) or extrahepatic failure. The latter includes kidney failure (defined by serum creatinine), cerebral (by grade of encephalopathy according to West-Haven classification), coagulation (by the international normalized ratio [INR]), circulatory (by blood pressure or need of vasopressors), and respiratory failure (by partial pressure of oxygen [PaO_2]/fraction of inspired oxygen [FiO_2] or oxygen saturation [SpO_2]/FiO_2) (Table 1). The most prevalent OF was kidney (43.6%), followed by coagulation (27.7%) and cerebral (24.1%). At presentation, the prevalence of ACLF grades 1, 2, and 3 were 49%, 35%, and 16%, respectively. The mortality in 28 d without LT was 32.8% in the seminal study.

THE APASL ACLF RESEARCH CONSORTIUM DEFINITION

This definition was based on an expert opinion on ACLF from the APASL, published in 2009 with updates in 2014 and 2019, using data from 1402 and 3300 patients, respectively[5,14,15]. This definition considers patients with compensated cirrhosis (diagnosed or not) and non-cirrhotic liver disease patients with a first episode of acute liver deterioration due to an insult directed to the liver. This reflects most of the patient population seen in Asia. In Asia, there is mainly hepatitis B virus reactivation or superinfection with hepatitis viruses A, D, or E. Patients with extrahepatic precipitants and those with kidney, circulatory or respiratory failures are excluded, meaning the liver dysfunction is the basis of the APASL definition. Extrahepatic OF may subsequently develop but are not needed for diagnostic criteria. The acute hepatic insult can be manifested by jaundice (serum bilirubin ≥ 5 mg/dL), coagulopathy (INR ≥ 1.5) and complications within 4 wk such as ascites, encephalopathy, or both. With these criteria, there is an estimated 25% to 37% 30 d-mortality[16].

THE NACSELD DEFINITION OF ACLF

ACLF was defined as the development of two or more OF (maximum of four) in patients with AD cirrhosis with or without prior episodes of decompensation. They used a prospective multicenter Canadian and American cohort of 507 patients with non-elective hospitalization in 18 centers. The OF criteria were: Kidney if dialysis is required; brain if encephalopathy grades 3 or 4 as West Haven classification occurs; respiratory if mechanical ventilation is needed; circulatory if vasopressor support is required or a reduction in systolic blood pressure by 40 mmHg from baseline despite adequate fluid resuscitation. The most prevalent OF was cerebral (36%), followed by circulatory (16%), kidney (13%) and respiratory (9%)[6]. Lately, these criteria were validated in a cohort of 2675 patients with or without infections, and mortality was higher in patients with infections, whatever the number of OF[17].

PRECIPITANTS FACTORS FOR ACLF AND PROGNOSIS

This section will discuss the most frequent precipitants for ACLF, its pathophysiology, and prognosis; nevertheless, a more thorough review of these subjects is outside the scope of this manuscript. Regardless of the ACLF definition employed, the precipitant factors can be recognized in only about 50% of patients with ACLF[9]. In 2021, the EASL-CLIF Consortium published a second paper derived from the PREDICT study reporting the precipitant factors which could influence the clinical course and the prognosis of ACLF patients. The most prevalent, more than 96% of cases, were bacterial infections (documented) and severe alcoholic hepatitis, whereas gastrointestinal bleeding with shock and toxic encephalopathy were rare. Although not so common, other precipitant factors like drug-induced liver injury, surgery, viral hepatitis, and ischemia can also be considered[18]. Mezzano *et al*[8] in a recent review and meta-analysis using ACLF-EASL-CLIF criteria in patients from Europe, East/South Asia and North/South America, showed that bacterial infections (35%), followed by gastrointestinal bleeding (22%) and acute alcohol consumption (19%) were the most frequent triggers to ACLF, with kidney dysfunction being the most common organ failure (49%). Although it is crucial to identify precipitant factors for ACLF, a recent study reported that they could not be detected in up to one-third of patients [12,19].

Table 1 European Association for the Study of the Liver Chronic Liver Failure Consortium acute-on-chronic liver failure grades

ACLF grades	Number of organ failures
1	Single organ kidney failure
	Single liver, coagulation, circulatory or lung failure with creatine levels ranging from 1.5 mg/dL to 1.9 mg/dL or encephalopathy grade 1 or 2, or both
	Single brain failure with creatinine ranging from 1.5 mg/dL to 1.9 mg/dL
2	2 organ failures
3	3 or more organ failures

ACLF: Acute-on-chronic liver failure.

Data from the CANONIC cohort showed that ACLF is a dynamic syndrome and may evolve to resolution, improvement or worsening in a short period[1]. ACLF grade 1 could be reversible in most patients (54.0%), while 21.0% remain stable in grade 1, and 24.5% progress to a higher grade. The clinical course after 3-7 d from diagnosis of ACLF may be a better predictor of outcome than its initial severity. Indeed, patients with grade 3 ACLF 3-7 d after diagnosis showed the worst prognosis[20,21]. This was called the opportunity window, wherein LT could reach the best results.

In severely ill patients, the prognosis differed according to the number of OF. For example, three OF had lower 28-d transplant-free mortality than those with four OF, 53.0% *vs* > 90.0%, respectively[20,21]. Indeed, the number of OF - according to the EASL-CLIF definition - along with age and white cell count compose the EASL-CLIF ACLF score. This predictive tool developed by the CANONIC study group proved superior to older models)Acute Physiology and Chronic Health Evaluation [APACHE] II, Child-Turcotte-Pugh, Model for End-stage Liver Disease [MELD]) to predict ACLF patients' mortality [22]. Accordingly, a recent study demonstrated that patients with an EASL-CLIF ACLF score of greater than 70 had 90% mortality at 90 d, regardless of care setting[23]. Another study reported a 28-d mortality rate of 100% for patients with a score greater than 70 at 48 h post-intensive care unit (ICU) admission[24].

Nevertheless, importantly, a 90-d and a 1-year post-LT survival rate of 90% and 81%, respectively, were reported for ACLF patients with 5 to 6 OF[25]. Conversely, without LT, survival rates dropped dramatically[25]. Therefore, although application and reassessment of the EASL-CLIF ACLF score may prevent prolongation of futile therapy, especially after a short trial of ICU stay, withdrawal of care must only be considered if the patient is not a LT candidate.

Recent evidence suggests systemic inflammation is the key to AD and ACLF disease progression[3,10,26]. Briefly, in patients with sepsis as the precipitant event, the inflammatory response is triggered by the recognition of pathogen-associated molecular patterns by pattern recognition receptors. The inflammatory response is then exacerbated, resulting in organ damage, cell death and release of damage-associated molecular patterns, which could aggravate and accelerate OF development in the ACLF setting. In addition, cirrhotic patients have portal hypertension, secondary intestinal congestion, and splanchnic endothelial dysfunction. These features can enhance gut permeability and facilitate bacterial translocation, driving local and systemic inflammation. Traditionally, inflammatory response causes organ dysfunction and stimulation of nitric oxide production, worsening pre-existing circulatory collapse and activation of immune cells. Another mechanism involved is mitochondrial metabolic impairment, resulting in metabolic disorder and cellular dysfunction with a preferential allocation of circulant nutrients to innate immune cells due to high metabolic demands. Secondarily, this process decreased mitochondrial energy production and enhanced organ dysfunction. So, inflammation and immunoparesis are thus key features of ACLF[3,26].

CURRENT MANAGEMENT OF ACLF

Besides LT, diagnosing and treating precipitant events and providing supportive treatment for OF are currently the cornerstone of ACLF therapy.

TREATMENT OF PRECIPITANT EVENTS

Infections: antimicrobial therapy should commence as quickly as possible based on the suspected site involved, existing culture results and local antimicrobial sensitivity patterns. Empirical broad-spectrum antimicrobials should be promptly initiated and deescalated once the results are available. Empirical

antifungals should be considered in patients without clinical improvements within 48-72 h. In addition, antifungal therapy may also be used in ACLF patients with multiple risk factors, such as corticosteroid use, prolonged antimicrobial therapy, long-term central venous access devices, parenteral nutrition, renal replacement therapy, sarcopenia and malnutrition[4,17,21]. Recent studies also recommended avoiding proton pump inhibitors unless there is a clear indication (like stress-ulcer prophylaxis) because they increase the risk of infection, mainly due to *Clostridioides*. Nonselective beta-blockers, when tolerated, and rifaximin may be beneficial by reducing bacterial translocation and intestinal dysbiosis.

Alcoholic hepatitis: although corticosteroids are indicated in patients with alcoholic hepatitis, especially when Maddrey's discriminant function score is higher than 32, the response negatively correlates with the number of OF in ACLF. The risk of new infections is one of the most critical factors in the decision-making process for steroid therapy[4,21]. The response to steroids should be assessed with the Lille score on day 7.

Acute viral hepatitis or reactivation: potent nucleotide or nucleoside analogues should be started in the event of hepatitis B infection or reactivation.

Surgical procedures: surgery of any type in patients with cirrhosis is associated with a significant risk of OF and ACLF. Consequently, it must be carefully considered. For example, open abdominal non-liver surgery, high preoperative cardiovascular risk, or hepatic venous pressure gradient greater than 16 mmHg were frequently associated with ACLF. Recently, a new score, the VOCAL PENN score, demonstrates an excellent ability to predict 30-d mortality when surgery is needed in patients with ACLF[18,27].

SUPPORTIVE THERAPY

ACLF patients frequently require admission to an ICU for advanced OF support and assistance from a multidisciplinary team.

Hemodynamic: Early goal-directed therapy using intravenous fluid resuscitation, preferably with crystalloids, must target mean arterial pressure > 65 mmHg. If vasopressors are required, norepinephrine is the first option, and a low dose of vasopressin can be necessary. Next, terlipressin or epinephrine can be added, though they are no longer the second option. Finally, intravenous hydrocortisone can be indicated in refractory septic shock, whereas no long-term survival benefit exists [28].

Acute kidney injury (AKI): It is essential to eliminate or avoid nephrotoxic drugs. The assessment of AKI severity using the modified Kidney Disease Improving Global Outcomes (KDIGO) criteria established the use of albumin in patients with stages 2-3 and albumin plus terlipressin or norepinephrine in type-1 hepatorenal syndrome. In non-responders, it should be necessary to start renal replacement therapy, mainly in patients with LT perspectives[29,30].

Lungs and respiratory failure: The airway should be protected in West-Haven grade 3 or 4 hepatic encephalopathy patients with elective intubation. Patients should be sedated with short-acting agents such as propofol. Benzodiazepines should be avoided. Hypoxemia (PaO_2 < 80 mmHg) should be prevented, and paracentesis is clinically indicated in case of tense ascites[28].

Gastrointestinal: Consider the use of stress-ulcer prophylactic drugs. As soon as possible, initiate oral or enteral feedings[18].

Coagulation: Consider prophylaxis for deep-vein thrombosis in the absence of severe coagulopathy. However, avoid correction of INR alterations with fresh frozen plasma. Instead, assessing the risk of bleeding and thrombosis in ACLF patients should be done with viscoelastic testing (rotational thromboelastography or rotational thromboelastometry), which must also guide correction when needed or before invasive procedures[31,32].

NEW PERSPECTIVES FOR ACLF MANAGEMENT

The use of albumin has been well recommended to prevent AKI and renal failure in spontaneous bacterial peritonitis, besides preventing post-paracentesis circulatory dysfunction[18,26,33,34]. Recent data have highlighted the non-oncotic properties of albumin as homeostatic effects, antioxidants, immunomodulation, endothelial stabilization, and toxic binding metabolites, including bile acids. Three studies recently evaluated the routine outpatient administration of intravenous albumin. One of them, the ANSWER trial, which included outpatients in an early stage of liver disease, showed improvement in mortality and reduction in cirrhosis-related complications, mainly when the albumin level was maintained above 4 g/dL[35]. However, further studies are necessary to indicate albumin infusion use routinely[18,28].

Various artificial and bioartificial extracorporeal liver support systems have been attempted to treat ACLF. However, artificial liver support such as molecular adsorbent recirculating system and fractionated plasma separation and adsorption system (Prometheus) have failed to show any survival benefit in this setting. Some bioartificial liver supports with a source of cells, traditionally human or

porcine hepatocytes, are under investigation, but the clinical benefit is still unclear[18,21].

While the use of plasma exchange has been shown to improve survival in acute liver failure, the actual effect in the ACLF scenario is unknown. Some studies in Asia using selected patients with ACLF showed improvement in 30 and 90-d survival in non-transplanted patients. Still, randomized trials are needed on the duration and amount of plasma exchange required[36].

One potential therapy for ACLF is the administration of granulocyte colony-stimulating factor (G-CSF). Several trials, mainly in patients with hepatitis B from Asia and India, have studied the efficacy of G-CSF and showed an increased leukocyte and neutrophil count, reduced severity of the disease and a protective effect on the development of sepsis, hepatorenal syndrome and hepatic encephalopathy[3]. However, trials in Western cohorts did not demonstrate survival benefits, CLIF OF scores modification or recurrence of infections. Therefore, to date, G-CSF cannot be recommended as part of routine treatment for ACLF[3,18,33,37].

Another promising therapy is mesenchymal stem cell transplantation, which can be a bridge to stabilization in patients with ACLF until LT. However, if the concept is interesting in theory, the studies were made with a few patients, and consequently, many questions remain open[38].

In addition to treatment for hypercholesterolemia, statins may have a role in ACLF therapy because of their potential hepatoprotective and anti-inflammatory properties. Currently, two clinical trials are underway to address the benefits and safety of statin (simvastatin and atorvastatin) in cirrhotic patients [26,39,40].

The gut microbiota plays an important role in complications associated with cirrhosis, with specific intestinal microbiomes being associated with adverse outcomes. The changes in the gut microbiome parallel the disease stages reaching their peak in ACLF. ACLF patients were shown to present an increase of *Enterococcus* and *Peptostreptococcus* sp and a reduction of some autochthonous bacteria[41]. Individual microbiome signatures could possibly identify ACLF patients and their prognosis, leading to more personalized treatment, a topic under investigation in the MICROB-PREDICT study (https://microb-predict.eu/). Manipulation of the gut microbiome using fecal microbiota transplantation may positively impact the course of cirrhosis, as shown in patients with severe alcoholic hepatitis. In addition, one novel-engineered carbon bead (Carbalive™) designed to absorb toxins from the gut and prevent translocation is under investigation[26]. Whereas promising, more studies are necessary to assess the clinical significance of gut microbiome manipulation in managing ACLF.

PRIORITISATION CRITERIA FOR LIVER TRANSPLANTATION

Although the MELD has enhanced equity in organ allocation in LT, there is evidence suggesting it may not portray the waitlist mortality of ACLF patients. This is in accordance with distinct pathological mechanisms in decompensated cirrhosis and ACLF[42].

Sundaram *et al*[43], in a large retrospective study, reported that irrespective of the MELD-Na score, ACLF-3 patients had a worse prognosis (ACLF grades refer to the EASL-CLIF classification hereafter unless contrarily stated). They found that 43.8% of patients with ACLF-3 and MELD-Na < 25 died or were removed from the waitlist at 28 d, having the worst prognosis among ACLF groups. This is probably associated with the more frequent occurrence of extrahepatic failures, which, although not fully captured by the MELD score, result in a high mortality rate. In addition, the authors report that LT within 30 d of listing was the only significant independent protective factor for 1-year patient survival after transplantation. More importantly, in this study, yet ACLF-3 patients had a greater mortality risk, they presented a similar probability of being transplanted than non-ACLF-3 patients with similar MELD scores[44].

In the CANONIC study, Jalan *et al*[22] developed an organ function scoring system (CLIF Consortium Organ Failure score, CLIF-C OFs) to diagnose ACLF and the prognostic EASL-CLIF ACLF score. The latter score discussed previously revealed significantly higher mortality 28-d predictive accuracy than the MELD and MELD-Na[22].

Allocation systems must privilege ACLF patients once so far earlier transplantation is the cornerstone of their successful management. The question remains whether ACLF-3 patients must be prioritized or whether a new scoring system that depicts better OF must be implemented for these cases[44-46].

DONOR CHARACTERISTICS AND OUTCOMES AFTER TRANSPLANTATION

Donor organ selection in the ACLF scenario can be a real conundrum for transplant teams to solve. This is because there is evidence that donor characteristics may be associated with a poor outcome after transplantation[25,47,48], which adds complexity to the decision of whether to accept or not a donor organ offer. This aspect is troubling, considering that strict donor organ selection may even postpone further transplantation to this threatened population, especially in regions with frequent high MELD recipients.

A recent retrospective analysis from the United Network for Organ Sharing (UNOS) involving 50552 transplanted ACLF patients reported the donor risk index (DRI) above 1.7 as an independent risk factor for mortality within 1 year after LT (hazard ratio [HR] 1.22; 95% confidence interval [CI] 1.09-1.35)[47]. On the other hand, independently of the MELD score, they described LT within 30 d of the ACLF diagnosis as a predictor of improved 1-year survival after transplantation[47]. Other authors also reported similar results[48,49]. These findings together pressurize even more transplant teams, which face the dilemma of whether to proceed with a high-risk donor or wait for an ideal donor which might not come on time.

It is ideally argued that the benefit of transplantation to this population surpasses the potential negative impact of a suboptimal graft when comparing this risk with a 1-year survival probability without transplantation. This is especially true for ACLF-3 patients, whose reported 1-year survival probability with and without transplantation is 83.9% and 7.9%, respectively[50,51]. Nevertheless, the scarce literature available also reports extra caution during donor organ selection for ACLF-3 patients in real life. For example, in the UNOS database retrospective study, ACLF-3 patients received organs from younger donors (mean age 38.7 years) with the cause of death predominantly related to head trauma (38.0%) and a small percentage of organs from high-risk donors with DRI ≥ 1.7 (22.9%)[47].

Kitajima *et al*[52] using the Organ Procurement and Transplantation Network and the UNOS registry, recently analyzed 17300 transplanted ACLF patients between 2002 and 2019. They grouped the patients by eras, Era 1 (2002-2007, n = 4,032), Era 2 (2008-2013, n = 6,130), and Era 3 (2014-2019, n = 7,138). Donor characteristics were classified according to the DRI (DRI < 1.2, 1.2-1.6, 1.6-2.0, and > 2.0). They have shown a significant improvement in overall patient survival and transplant outcomes throughout eras. However, although donors with DRI > 2.0 were associated with a lower risk of patient death in Eras 2 and 3 than Era 1 in ACLF-1 and 2, this was not confirmed for ACLF-3[52]. Therefore, the authors advise the need for particular caution for high-risk donors (DRI > 2.0) for ACLF-3 patients.

Comparatively, ACLF grades 1 and 2 are associated with lower mortality than ACLF grade 3. The reported 28-d and 90-d mortality rates for ACLF grades 1 and 2 are 25.8% and 28.6%, and 41.1% and 65.4%, respectively[1]. Therefore, more judicious donor organ selection could be applied, mainly when disease progression is evaluated concomitantly. Whereas data regarding the impact of donor organ selection on postoperative outcomes are scarcer for this population, the results of the study mentioned above support a wider acceptance of suboptimal grafts in ACLF-1 and 2 patients[52]. Nevertheless, the quality of evidence (retrospective study) must be considered before drawing definitive conclusions.

In the setting of donor organ shortage, another question to consider is the allocation of an ideal organ to a very sick recipient who may not survive the procedure. Again, the coexistence of additional risk factors and the real-time change in OF may guide the decision to accept a non-ideal donor organ offer rather than wait (Figure 1). Accordingly, there is evidence suggesting that in ACLF-3 patients, a better survival rate can be achieved if the transplant occurs after organ failure recovery[48].

Indeed, the timing for LT in ACLF patients is critical. In a recent study involving specifically ACLF-3 patients, authors investigated the optimal timing for transplantation and the impact of extended criteria donor (ECD) organs (defined exclusively by the DRI ≥ 1.7)[49]. They analyzed three variables to define the groups of patients (age ≤ 60 or > 60 years, 3 OF or more, and hepatic or extrahepatic ACLF-3). Through two-way sensitivity analyses, they found that overall survival is optimized by earlier transplantation, especially among candidates > 60-years-old or with 4-6 OF[49]. These findings are in accordance with the proposal mentioned above and reinforce the need to consider early transplantation, even with suitable suboptimal grafts, to this population of ACLF patients.

To date, just the DRI was evaluated as a donor parameter within ACLF studies. The DRI is a quantitative score developed to predict the risk of graft failure[53]. It identified seven donor characteristics associated with graft failure, donor age, donation after circulatory death (DCD), split/partial grafts, race, height, and cause of brain death[53]. Yet, the DRI has known limitations which must be considered in this analysis. First, the DRI does not account for steatosis, a known risk factor for postoperative graft dysfunction. Second, cold ischemia time cannot be anticipated in all cases, especially in challenging logistical scenarios, such as in countries with long territorial extensions and complex surgical cases. Consequently, a more thorough evaluation of the impact of donor features within ACLF studies is needed.

Living-donor liver transplantation (LDLT) could expand the donor organ pool and expedite transplantation in ACLF patients. Whilst studies in countries where deceased donors are scarce for cultural reasons demonstrated good LDLT postoperative outcomes[54,55], concerns regarding the prognosis of the sickest patients leading to stringent patient selection criteria hinder the applicability of this option thus far. In a retrospective analysis of 60 patients with EASL-CLIF grade 1 and 2 ACLF, LDLT transplanted patients exhibited a 1-year survival rate of 92% *vs* 11% in those who did not undergo transplantation[55]. In another retrospective study involving 218 ACLF patients, employing strict selection criteria -no high vasopressors or respiratory failure- for LDLT transplantation, the 1-year postoperative patient survival was 92.9% for EASL-CLIF grade 1, 85.4% for grade 2, and 75.6% for grade 3[54]. Despite suggesting the benefit of LDLD in this setting, the justifiable caution patient selection may have biased the conclusions. In addition, right lobe LDLT is most often required, leading to a right hepatectomy in the donor, which increases their morbidity and mortality.

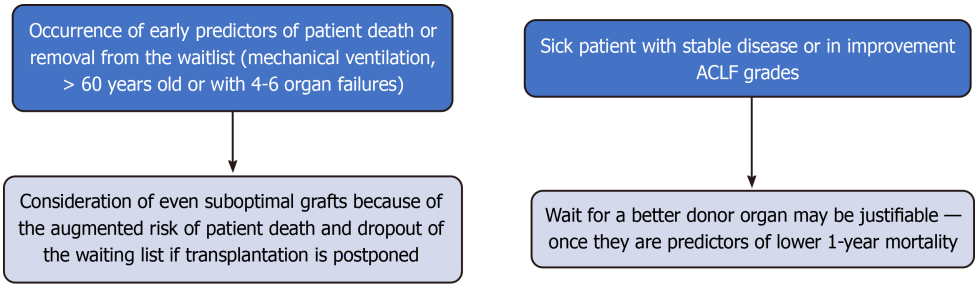

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Figure 1 Suggested flowchart on when to accept a non-ideal donor organ offer for transplantation in the acute-on-chronic liver failure setting. In this scenario, the coexistence of additional risk factors and the real-time change in organ failures are key components in deciding to proceed with transplantation. ACLF: Acute-on-chronic liver failure.

While nowadays, consensually amongst experts, ECD organs must be considered in ACLF patients due to the transplant benefit, further studies detailing the real impact of donor characteristics on posttransplant patient survival are awaited. This is particularly valid for ACLF-3 patients, in which the limited literature available urges caution before proceeding with a high-risk donor. Thus far, this general concept is more theoretical than practical and originates from the need to provide timely transplantation to these patients. Therefore, this is a warranted subject of deeper investigation for future studies.

WHAT ROLE DOES MACHINE PERFUSION OF THE LIVER MAY PLAY IN THE ACLF SETTING

Machine perfusion of the liver (MPL) is currently a hot topic in LT. It has gained growing attention from the transplant community with the expansion of the ECD population and, therefore, the need to prevent the frequent ischemia-reperfusion injury (IRI)-ECD postoperative-related complications. IRI is an intrinsic consequence of solid organ transplantation and the basis of major postoperative complications. Although ECD organs are highly vulnerable to IRI, surpassing the protective capacity of traditional static cold storage (SCS) preservation solutions and, consequently, associated with higher morbidity and mortality rates after transplantation, their increased utilization is needed to attend to the rising number of patients on the waiting list. Thus far, the two most studied modalities of MPL in LT are *ex situ* hypothermic and normothermic machine perfusion.

The hypothermic oxygenated machine perfusion (HOPE) of the liver was shown to enhance mitochondrial respiratory function[56]. The optimized mitochondrial respiratory chain and oxidative phosphorylation system increase cellular energy production -replenishing the exhausted stores of adenosine triphosphate-, avoid the reverse flow of electrons with the production of reactive oxygen species, and prevent the activation of the inflammatory cascade with subsequent tissue damage[56].

The normothermic machine perfusion of the liver (NMP) allows the recovery of the full metabolism of the organ at 37 °C. Consequently, it requires an oxygen carrier to attend to the cellular metabolic demand. NMP permits the assessment of parameters that traditionally indicate appropriate liver function such as bile production, vascular flow, lactate metabolism, glucose metabolism, and hepatocellular injury such as transaminases released into the perfusate[57]. In addition, NMP enables prolonged organ preservation and, potentially, *ex situ* organ treatments[57,58].

So far, most clinical trials on MPL in LT were intended to demonstrate the safety and feasibility of the technique and were centered in European countries. Arguably, the sickest patients with very high morbidity and mortality risk were not included in these studies and were not even on the waiting list in many of these countries. Therefore, regional divergence and particularities amongst geographical areas, such as mean MELD on the waiting list and territorial extensions, must also be considered.

Hurdles to timely access of ACLF patients to LT and their disadvantage in receiving a donor organ offer in the MELD allocation system were presented herein. In addition, concerns about accepting ECD organs to these sick patients were discussed beforehand. Consequently, none of the MP clinical trials has encompassed ACLF patients thus far. Nevertheless, hypothetically, MPL can be even more advantageous for this population.

Studies suggest that MP may recondition ECD organs before transplantation, preventing further deterioration or even improving their quality. Although DCD LT has not been reported for ACLF patients, clinical trials described similar results for patients transplanted with DCD organs treated with HOPE and those transplanted with low-risk donors after brain death (DBD)[59]. Furthermore, in a randomized clinical trial, hypothermic machine perfusion reduced the occurrence of postreperfusion syndrome and early allograft dysfunction (EAD) after DCD LT. These factors contribute to the early

recovery of the sickest patients after the procedure[60]. Accordingly, concerning the applicability of the technique in ECD DBD LT, a multicenter randomized clinical trial recently reported that, compared to SCS, HOPE led to a significant reduction in 90-d complications with a shorter hospital stay and a trend toward a reduced rate of EAD[61].

Prolonged organ preservation and assessment of organ viability are two critical features related to NMP which may benefit ACLF transplantation. Whereas it could not find a difference in graft survival or patient survival compared to SCS, the first randomized clinical trial on NMP demonstrated that it could safely extend the organ preservation time[62]. The median total preservation time was close to 12 h for NMP-preserved livers. Driven mainly by the difference in peak aspartate transaminase, NMP also reduced the occurrence of EAD[62]. The VITTAL clinical trial (NCT02740608), from Birmingham, United Kingdom, transplanted twenty-two donor livers discarded by all United Kingdom centers of 31 meeting specific high-risk criteria based on the lactate clearance to levels ≤ 2.5 mmol/L within 4 h on NMP with 100% 90-d patient and graft survival[63]. Nevertheless, applying the Birmingham criteria, NMP could not prevent non-anastomotic biliary strictures in DCD livers, and 4 (18%) patients needed re-transplantation[63].

Our group recently reported for the first time the successful transplantation of an ACLF patient using an ECD DBD liver graft treated with HOPE[64]. The autoimmune hepatitis-related cirrhosis ACLF-2 patient (liver and coagulation failure) with a MELD-Na score of 42 was offered an ECD DBD organ with a DRI of 2.79 after 8 d from hospital admission (well above the previously identified threshold of 1.7). HOPE started after 06 h and 19 min of cold ischemia time and lasted 5 h and 19 min. The flavine mononucleotide was measured in the perfusate to assess the viability of the organ and revealed a low value after 30 min of perfusion (3097 A.U.) - permissive for transplantation in any recipient. During transplantation, the reperfusion was uneventful. Postoperatively, the graft recovered well, without EAD, according to the Olthoff criteria, and the patient developed AKI KDIGO stage 3 with complete recovery after 1 wk[64].

While the case suggests the feasibility and safety of employing MP within this setting, more conclusive evidence to prove the benefit of the technique is still needed. So far, because of the scarce existing literature, the evidence of the impact of ECD transplantation in ACLF grades 1 and 2 is still anecdotal. In addition, there are no reports on the application of MPL in ACLF-3 patients, those with greater risk and the subject of more concerns in the literature. Earlier transplantation was shown to improve overall survival in ACLF-3 patients > 60-years-old or with 4-6 OF[49]. This population and those with the coexistence of additional risk factors and worsening organ failures may be the target population for future MPL studies.

Yet the evidence is very limited currently, MPL may play a game-changing role in ACLF transplantation. First, it can expedite LT because it allows more liberal acceptance of ECD organs based on the properties of the technique. The proven capacity of MPL to recondition ECD livers reassures surgeons in their decision to accept an ECD organ. This effect is amplified with the application of biomarkers for organ viability assessment during perfusion. Second, rescuing discarded high-risk organs or prolonging the preservation of organs compromised by logistics *via* MPL may increase the donor organ pool, which may also help tackle the shortage of donor organs for transplantation. This is especially important in countries with frequent high MELD score patients and long cold ischemia time, which may need to adapt their organ preservations systems.

CONCLUSION

Although new clinical specific therapies have been researched for ACLF management, earlier transplantation -within the frequently narrow opportunity window- is a proven effective therapy for selected ACLF patients. Thus far, other options encompass diagnosing and treating precipitant events and supportive treatment for organ failures. Therefore, current and future perspectives on ACLF management must envisage improved access to LT. Accordingly, discussions about allocation and prioritization for transplantation in critically ill ACLF patients are awaited because there is evidence suggesting the current model may not portray their waitlist mortality. Furthermore, whereas donor organ quality is meant to be a prognostic factor in the ACLF setting, recent evidence suggests that MPL may be a safe tool to improve the donor organ pool and expedite access to this life-saving procedure.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Bladder-colon chronic cross-sensitization involves neuro-glial pathways in male mice

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Abstract

BACKGROUND

Irritable bowel syndrome and bladder pain syndrome often overlap and are both characterized by visceral hypersensitivity. Since pelvic organs share common sensory pathways, it is likely that those syndromes involve a cross-sensitization of

the bladder and the colon. The precise pathophysiology remains poorly understood.

AIM

To develop a model of chronic bladder-colon cross-sensitization and to investigate the mechanisms involved.

METHODS

Chronic cross-organ visceral sensitization was obtained in C57BL/6 mice using ultrasound-guided intravesical injections of acetic acid under brief isoflurane anesthesia. Colorectal sensitivity was assessed in conscious mice by measuring intracolonic pressure during isobaric colorectal distensions. Myeloperoxidase, used as a marker of colorectal inflammation, was measured in the colon, and colorectal permeability was measured using chambers. c-Fos protein expression, used as a marker of neuronal activation, was assessed in the spinal cord (L6-S1 level) using immunohistochemistry. Green fluorescent protein on the fractalkine receptor-positive mice were used to identify and count microglia cells in the L6-S1 dorsal horn of the spinal cord. The expression of NK1 receptors and MAPK-p38 were quantified in the spinal cord using western blot.

RESULTS

Visceral hypersensitivity to colorectal distension was observed after the intravesical injection of acetic acid *vs* saline ($P < 0.0001$). This effect started 1 h post-injection and lasted up to 7 d post-injection. No increased permeability or inflammation was shown in the bladder or colon 7 d post-injection. Visceral hypersensitivity was associated with the increased expression of c-Fos protein in the spinal cord ($P < 0.0001$). In green fluorescent protein on the fractalkine receptor-positive mice, intravesical acetic acid injection resulted in an increased number of microglia cells in the L6-S1 dorsal horn of the spinal cord ($P < 0.0001$). NK1 receptor and MAPK-p38 levels were increased in the spinal cord up to 7 d after injection ($P = 0.007$ and 0.023 respectively). Colorectal sensitization was prevented by intrathecal or intracerebroventricular injections of minocycline, a microglia inhibitor, by intracerebroventricular injection of CP-99994 dihydrochloride, a NK1 antagonist, and by intracerebroventricular injection of SB203580, a MAPK-p38 inhibitor.

CONCLUSION

We describe a new model of cross-organ visceral sensitization between the bladder and the colon in mice. Intravesical injections of acetic acid induced a long-lasting colorectal hypersensitivity to distension, mediated by neuroglial interactions, MAPK-p38 phosphorylation and the NK1 receptor.

Key Words: Cross-organ sensitization; MAPK-p38; Microglia; NK1 receptor; Pain; Visceral hypersensitivity

Core Tip: A model of chronic cross-organ visceral sensitization in mice was developed using ultrasound-guided intravesical injections of acetic acid. Visceral hypersensitivity to colorectal distension was observed as early as 1 h post-injection and lasted up to 7 d. Visceral hypersensitivity was associated with an increased expression of c-Fos protein in the spinal cord. The NK1 receptor and MAPK-p38 levels were upregulated in the spinal cord 7 d post-injection. Colorectal sensitization was prevented by intrathecal or intracerebroventricular injections of minocycline, a microglia inhibitor, by intracerebroventricular injection of CP-99994, a NK1 antagonist, and by intracerebroventricular injection of SB203580, a MAPK-p38 inhibitor.

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INTRODUCTION

Irritable bowel syndrome (IBS) and bladder pain syndrome (BPS) are two functional disorders that affect the gastrointestinal tract and the urinary tract, respectively[1,2]. Their prevalence in the general

population is 4.6%[3] and 4.2%[4], respectively, and recent studies have shown a strong overlap between both syndromes[1,2]. Indeed, BPS is found in 40%-60% of IBS patients[5], and IBS is observed in 25.4%-38.6% of BPS patients[1,2]. Both syndromes are characterized by visceral mechanical hypersensitivity at the urinary tract level for BPS and at the intestinal level for IBS[6,7]. The involvement of several mechanisms has been suggested in the onset and/or the maintenance of visceral hyperalgesia, including urothelial and/or intestinal epithelial permeability, mucosal immune activation and altered brain-gut interaction[8].

Despite increasing knowledge of the pathophysiology of IBS and BPS, limited mechanistic data is available in the context of BPS-IBS overlap. Based on the fact that pelvic organs share common sensory pathways, a few studies have offered evidence that cross-sensitization between the bladder and the colon may explain sensitization of both organs[9]. This may involve primary extrinsic afferents or central sensitization, both at the spinal and supraspinal levels[10]. All these studies involved acute sensitization models, often in anesthetized animals[11]; therefore, there is not yet any data regarding the chronicization of pelvic organ cross-sensitization.

The role of spinal glia has recently been highlighted in the sensitization of the bladder or colon[12] and even in cross-organ sensitization between both organs[13]. The role of spinal glia, however, has never been demonstrated in the maintenance of such sensitization using models of bladder-colon cross-sensitization induced chronic visceral hyperalgesia. The aims of our study were therefore to develop a model of chronic cross-organ sensitization between the bladder and the colon and to investigate the mechanisms involved in the development and persistence of this cross-sensitization.

MATERIALS AND METHODS

Ethics

The experiments were carried out in accordance with the ethical guidelines of the International Association for the Study of Pain[14] and in accordance with the guidelines of the French Ministry of Agriculture and Fisheries (Decree No. 874848). Our protocol was approved by the local Ethics Committee for Animal Experiments (CENOMEXA No: N/02-01-13/02/01-16).

Animals

Adult male wild type C57Bl/6 mice (Janvier Laboratories, Le Genest-Saint-Isle, France) and transgenic mice expressing the green fluorescent protein on the fractalkine receptor of microglial cells (Inserm Laboratory U1239, Dr. David Vaudry Team, Mont Saint Aignan, France[15]) were 8 wk old on the day of the experiment (weight range: 22-26 g). The animals were randomized by their weight in several cages, with five mice per cage, housed in a 12 h light/dark cycle in an animal housing facility free of specific pathogenic organisms and maintained at room temperature (22 ± 2 °C). The animals received a standard diet (RM1 diet; SDS, Witham, Essex, United Kingdom). Drinking water and food were available *ad libitum*. Each manipulation or experiment took place after at least 1 wk of acclimatization to the housing conditions. All animals were euthanized by cervical dislocation after anesthesia with ketamine (100 mg/kg, Imalgene 1000; Merial, Lyon, France) and xylazine (10 mg/kg, Rompun® 2%; Bayer, Berlin, Germany) administered intraperitoneally before tissue collection.

Study design

The animal protocol was designed to minimize pain or discomfort to the animals. Eight series of mice were used in this work. Each set was comprised of different groups of mice, and each group was formed of 5-8 mice. The first series was used to develop the cross-organ sensitization model. Once the model was validated, a second series was used to analyze inflammatory parameters in the colon and the bladder and to assess colonic and bladder permeabilities. We then focused on the central nervous system, especially at lamina I and II of the dorsal horn at the L6-S1 segment, where pelvic extrinsic primary afferent neurons form synapses with spinal neurons. Our third series assessed the neuronal activity in this model using c-Fos expression, and inflammatory parameters were measured in the spinal cord of a fourth series. The model was then transposed on transgenic mice to assess changes in microglia cells. Finally, three different inhibitors/antagonists were used in the sixth, seventh and eighth series to gain a better understanding of which pathway could be involved in the cross-sensitization process.

Acetic acid sensitization

An injection of acetic acid (0.75%, 200 µL) or saline (NaCl 0.9%, 200 µL; Baxter, Deerfield, IL, United States) was made into the urinary bladder using ultrasound monitoring in mice under brief anesthesia (isoflurane: 3% in 1.5 L/min of air, Iso-Vet®; Piramal Critical Care, Voorschoten, The Netherlands).

Colorectal distension and visceral sensitivity measurement

We measured visceral pain to colorectal distension (CRD) using a non-invasive method, as previously

reported in mice[16]. Changes in intracolonic pressure, reflecting viscero-motor responses induced by CRD (nociceptive stimulus), were used as a surrogate marker of colorectal sensitivity[16].

An infinitely compliant distension balloon (diameter 0.7 cm) was made using a polyethylene bag attached to a PE-50 catheter (Intramedics, France) drilled in its end and taped 2 cm below the pressure sensor of a miniaturized pressure transducer catheter (SPR-524 Mikro-Tip catheter; Millar Instruments, Houston, TX, United States). Polypropylene 4-0 Ligatures (Prolène®; Ethicon Inc., Somerville, NJ, United States) were covered with parafilm to prevent any air leak.

On the experimental day, mice were briefly anesthetized with isoflurane (3% in air), and the lubricated "balloon-pressure sensor" was introduced into the colorectum, so that the balloon was inserted 2 cm upstream of the anal margin into the colon. Each mouse was placed in an adjustable mouse restrainer (30 mm diameter × 90 mm length, LE5016; *In Vivo* Research Instruments, Perkin Elmer, Waltham, MA, United States) and left to rest for 30 min before the CRD procedure. The balloon was then secured to the tail with tape and connected to an electronic barostat (Distender Series II; G & J Electronics Inc., Toronto, ON, Canada) to perform isobaric CRD. Our distension protocol consisted of a set of graded phasic distensions of 15, 30, 45 and 60 mmHg (two times each, 20 s duration, 4 min inter-stimulus interval) (Protocol Plus Deluxe; G & J Electronics, Toronto, ON, Canada). Voltage output was converted digitally using CED digital-analogic converter (Micro 1401; Cambridge Electronic Design, Cambridge, United Kingdom) and Spike 2 software (CED, Ltd., Cambridge, United Kingdom). The pressure sensor allowed the assessment of visceral pain *via* a custom-made script that allowed signals to be specifically extracted from abdominal muscle contractions (excluding those from colonic contractions).

Colonic sensitivity was measured in awake mice at 60 min, 3 d and 7 d following acetic acid urinary bladder injections.

Measurement of bladder and colonic paracellular permeabilities

After euthanasia, bladder and distal colon samples were removed on day 7. Samples were cut along the mesenteric border. Bladder and colonic permeabilities were assessed by measuring fluorescein isothiocyanate (FITC)-dextran (4 kDa) fluxes in Ussing chambers with an exchange surface of 0.07 cm^2 (Harvard Apparatus, Holliston, MA, United States) as previously described[17]. FITC-dextran (5 mg/mL) was loaded on the mucosal side. After 3 h at 37 °C, the medium from the contralateral side (serosa) was removed and stored at -80 °C. The fluorescence level of FITC-dextran (excitation at 485 nm, emission at 535 nm) was measured in a 96-well black plate using spectrometer Chameleon V (Hidex Co, Turku, Finland). The results were converted to concentrations of FITC-dextran (mg/mL) for analysis.

Myeloperoxidase measurement

After euthanasia, bladder and distal colon samples were removed on day 7. Colonic and vesical tissues (around 50 mg) were washed in phosphate-buffered saline and then homogenized (50 mg/mL) in 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, Steinheim, Germany) with 50 mmol/L of phosphate-buffered saline (pH 6.0). They were frozen at -80 °C and thawed at 37 °C three times, then sonicated (Vibra Cell ultrasonic processor 75115; Bioblock Scientific, Illkirch, France) and finally centrifuged (14000 rpm at 4 °C for 15 min). Myeloperoxidase (MPO) was assayed in the supernatant by adding 1 mg/mL of dianisidine dihydrochloride (Sigma-Aldrich) and 5 × 10^{-5}% of hydrogen peroxide (Sigma-Aldrich). The change in optical density was measured at 450 nm. One unit of MPO activity was defined as the amount that degraded 1.0 μmol of hydrogen peroxide per minute at 25 °C, and human neutrophil MPO (Sigma-Aldrich) was used as standard, as previously described[18,19].

c-Fos immunofluorescence

Seven days after the intrabladder injection of acetic acid 0.75%, c-Fos immunohistochemistry was performed after 120 min of CRD at 45 mmHg (20 s of distension every 4 min for 120 min). Under ketamine (100 mg/kg i.p.)/xylazine (10 mg/kg) anesthesia and upon thoracotomy, mice were perfused through a cardiac-aorta cannula with saline followed by 150 mL/mouse of ice-cold 4% paraformaldehyde and 14% saturated picric acid in a 0.1 M phosphate buffer solution (pH 7.2). After decapitation, the lumbo-sacral spinal cord (L6-S1) was post-fixed in the same fixative solution overnight at 4 °C, cryoprotected by immersion in 10% sucrose overnight and transferred to 30% sucrose overnight. The spinal cords were then embedded in Tissue-Tek® optimal cutting temperature compound (Sakura Finetek United States, Inc., Torrance, CA, United States), snap-frozen and cut with a cryostat. Fluorescent microscopy was used to identify activated neurons. The expression of c-Fos was assessed by immunofluorescence. We applied anti-c-Fos antibody (1:2000; Calbiochem, Darmstadt, Germany) overnight at 4 °C and then incubated Cy3-conjugated goat anti-rabbit IgG (1:400; Fisher, Invitrogen, Carlsbad, CA, United States) for 2 h at room temperature. Pictures were taken using a fluorescence microscope (DM5500 B; Leica Microsystem Ltd., Wetzlar, Germany) at magnification × 10, and the number of c-Fos immunoreactive cells in lamina I and II of the dorsal horn at the L6-S1 segment of the spinal cord was counted for each mouse. The average number of stained nuclei in three 20 μm thick slices for each mouse was used for analysis.

Semiquantitative PCR for the detection of TNF-α, IL-1β and IL-10 mRNA in the spinal cord

Total RNA from L6-S1 spinal cord segments was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). RNA was purified according to the manufacturer's instructions. Total RNA was treated with DNase I (Invitrogen) to remove any contaminating DNA. DNase I was stopped with DNase inactivation reagent (Invitrogen) according to the manufacturer's instructions. The quality and quantity of total RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, United States). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA. A ratio of ≥ 2.0 was accepted for analysis.

After reverse transcription of 1.5 µg total RNA into cDNA by using 200 units of SuperScript II Reverse Transcriptase (Invitrogen), quantitative PCR was performed using SYBR Green technology on a Bio-Rad CFX96 real time PCR system (Bio-Rad Laboratories, Marnes-la-Coquette, France). The *GAPDH* gene was chosen as the reference gene. All samples were performed in duplicate in a single 96-well reaction plate. Serially diluted cDNA samples were used as external standards. The absolute quantification of mRNA was performed by converting the sample Ct values to concentration (copies per µL) based on standard curves. The identity and purity of the amplified products were assessed using melting curve analysis at the end of amplification. The technique was used to assay *TNF-α*, *IL-1β* and *IL-10* mRNA in the spinal cord. The primer sequences for the targeted mouse genes are presented in Supplementary Table 1.

Analysis of microglia cells

Green fluorescent protein on the fractalkine receptor-positive transgenic mice were perfused as described for c-Fos immunofluorescence, and the dorsal horn of L6-S1 spinal cord was embedded in Tissue-Tek® optimal cutting temperature compound, snap-frozen and cut with a cryostat. Pictures were then taken with the Leica photonic microscope used for c-Fos experiments at magnification × 10, and the number of microglia cells expressing microglia green fluorescent protein in lamina I and II of L6-S1 dorsal spinal cord was counted using NIH ImageJ software (version 2.0.0-rc-43/1.51u)[20]. The average number of stained nuclei per field in three 20 µm thick slices for each mouse was used for statistics.

Pharmacologic studies

Minocycline (2.5 mg/mL; 1.25 mg/kg; Sigma-Aldrich), a microglia blocker, CP 99994 (15 mg/mL; 7.5 mg/kg; Sigma-Aldrich), a NK1R antagonist, SB203580 (5 mg/mL; 2.5 mg/kg; Calbiochem, Merck, EMD Millipore Corp., Billerica, MA, United States), a MAPK-p38 blocker, or saline were injected in the intracerebroventricular (ICV) region using a Hamilton syringe (Neuros™, Gastight, 1705, 33 gauge; Dutscher, Bernolsheim, France) 1 h before injecting acetic acid (0.75%) into the bladder. Intrathecal injections of minocycline at the L6-S1 Level of the spinal cord were also performed to demonstrate that microglia activation occurred at that level.

Western blot analysis

After euthanasia, the L6-S1 segment of the mice spinal cord was removed on ice and quickly frozen in liquid nitrogen. After thawing on ice, the spinal cord samples were homogenized at 4 °C in a lysis buffer (100 µL of buffer A × 2, 2 µL of 100 mmol/L dithiothreitol, 50 µL of 1% NP40, 1 µL of 1 × P8340 protease inhibitors, 2 µL of 1 × P2850 phosphatase inhibitors and 200 mL of H_2O). Samples were displayed on ice for 15 min, then centrifuged at 12000 × g for 15 min at 4 °C. The protein-containing supernatant was collected and stored at -80 °C until analysis. The proteins (25 µg) were loaded on a 4%-20% gradient polyacrylamide gel (Bio-Rad) and transferred onto a nitro-cellulose membrane (GE Healthcare, Orsay, France). Membranes were then blocked for 1 h at room temperature with 5% (w/v) nonfat dry milk in Tris buffered saline containing 0.05% Tween® 20 (Sigma-Aldrich). An overnight incubation at 4 °C was then performed with primary antibodies: anti-P-p38 (1:500; Cell Signaling Technology®, Leiden, The Netherlands; P/N 4511) or anti-NK1R (1:200; Atlas Antibodies, Bromma, Sweden; P/N HPA074573) from rabbit or anti-GAPDH (1:5000; Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France) from goat. All antibodies were diluted in a blocking solution. After three washes, a 1-h incubation was performed with a peroxidase-conjugated IgG secondary antibody from goat anti-rabbit or from rabbit anti-goat (1:5000; Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France). After three additional washes, immunocomplexes were revealed using the ECL detection system (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). Proteins were quantified by densitometry using ImageScanner III and ImageQuant TL software (GE Healthcare Life Sciences), and standardized against the intensity of GAPDH.

Statistical analyses

The data were expressed as mean ± standard error of the mean. Quantitative data was compared between groups using an unpaired *t*-test, with Welch's correction in case of unequal variances. Comparisons of multiple groups were performed using one-way analysis of variance (Kruskal-Wallis test) with post-hoc analysis (Dunn's multiple comparison post-test to compare all pairs of columns to each other or Dunnett's post-test to compare all pairs of columns to controls) to assess the difference among the groups, and two-way analysis of variance with post-hoc analysis (Bonferroni's correction)

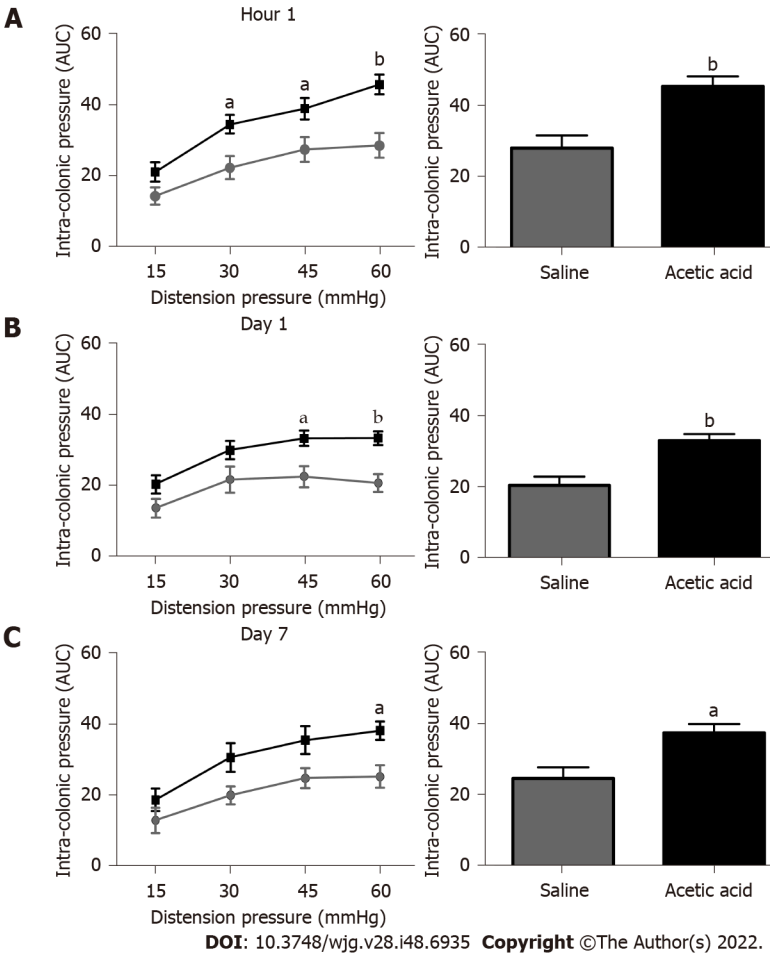

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Figure 1 Assessment of visceromotor response to colorectal distensions. A-C: Changes in intracolonic pressure in response to isobaric colorectal distensions (15, 30, 45 and 60 mmHg) after intravesical injections of acetic acid (0.75%) *vs* saline (NaCl 0.9%) in mice at 1 h (A), 1 d (B) and 7 d (C) after injections, with details of the comparison at 60 mmHg of distension on the right. n = 8 mice per group. $^{a}P < 0.05$ and $^{b}P < 0.001$. Variability in the results is represented by the standard error of the mean (area under the curve ± standard error of the mean). AUC: Area under the curve.

was used to assess groups with repeated measures. Individual data for visceral sensitivity in mice, especially the kinetics of visceromotor responses to increasing CRD, was visually assessed for each experimental group separately; tracings of animals with aberrant or outlier responses to distensions were excluded from the analysis. A P value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software Inc., San Diego, CA, United States, www.graphpad.com). The statistical methods used in this study were reviewed by Fabien Wuestenberghs from CHU UCL Namur.

RESULTS

Development and validation of a mouse model of chronic bladder-colon cross-sensitization

Based on our previous rat study[9], we tested the intravesical administration of a 0.75% acetic acid solution in mice. A single intravesical injection of 0.75% acetic acid under ultrasound monitoring induced an increase of the colonic nociceptive response during CRD at 30 mmHg ($P < 0.05$), 45 mmHg ($P < 0.05$) and 60 mmHg ($P < 0.001$) 1 h after injection (Figure 1). An increased colonic nociceptive response during CRD was still observed at 60 mmHg ($P < 0.05$) on 7thd in the acetic acid group compared to the control group (Figure 1), confirming that colonic hypersensitivity persists up to 7 d after the intravesical injection in our model. Further experiments were therefore designed to understand the mechanisms of the chronicization of cross-sensitization in this model. No serious adverse events occurred during the experiments.

Permeability and inflammatory parameters of bladder and colon

No differences were found at day 7 in either bladder or colon permeabilities or MPO activities between animals treated with acetic acid or saline intravesically (Figure 2).

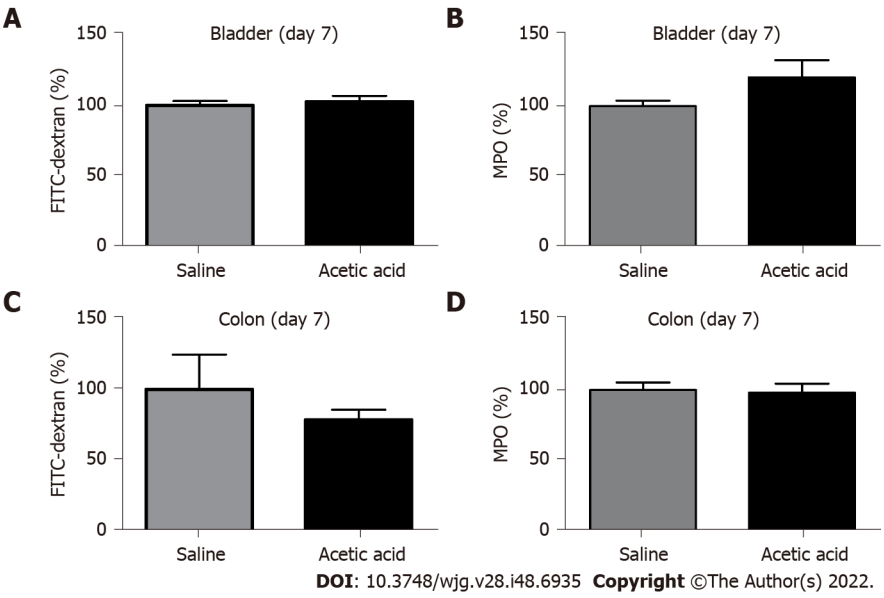

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Figure 2 Bladder and colorectal permeabilities and inflammation. A-D: Urinary bladder (A) and colorectal (B) permeabilities assessed by fluorescein isothiocyanate (FITC)-dextran, and urinary bladder (C) and colon (D) inflammatory levels assessed by myeloperoxidase (MPO) activities. They were not different between mice with intravesical injections of saline (NaCl 0.9%) or acetic acid (0.75%) at day 7. *n* = 4-5 mice per group. Results of mice treated by intravesical injections of acetic acid were normalized to those of mice treated with saline. Variability in the results is represented by the standard error of the mean.

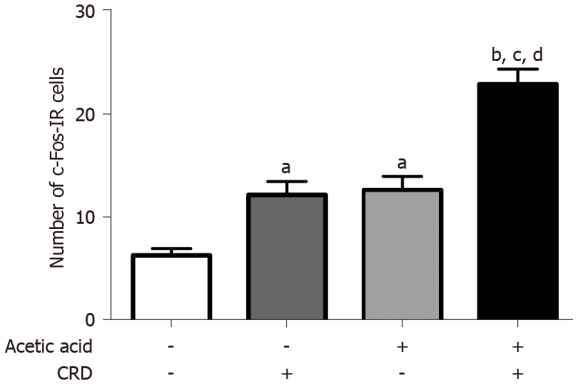

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Figure 3 Quantification of c-Fos immunoreactive cells. Quantification of c-Fos immunoreactive (IR) cells in lamina I and II of the dorsal horn per slice of the L6-S1 spinal cord on day 7 after intravesical injection of saline (NaCl 0.9%) or acetic acid 0.75%, with or without prior colorectal distensions (CRDs) at 45 mmHg for 120 min (*n* = 6-7 mice per group). [a]*P* < 0.05 and [b]*P* < 0.0001 (compared to mice treated with saline and no prior CRD); [c]*P* < 0.001 (compared to mice treated with saline and prior CRD); [d]*P* < 0.01 (compared to mice treated with acetic acid and no prior CRD). Results are expressed as the mean ± standard error of the mean.

Neural activation in the dorsal horn of the spinal cord L6-S1

In the absence of CRD, the number of c-Fos immunoreactive cells in lamina I and II of the L6-S1 level of the dorsal horn was 6.5 ± 0.7 per slice in mice treated with saline. The CRD performed on the 7th d in these mice treated with saline increased the number of c-Fos immunoreactive cells to 12.4 ± 1.3 (P < 0.05). In mice treated with 0.75% acetic acid, the number of c-Fos immunoreactive cells was 12.9 ± 1.3 in the absence of CRD and increased to 23.2 ± 1.5 (P < 0.05 *vs* all other groups) after CRD (Figure 3).

Implication of central microglia from the dorsal horn of the spinal cord L6-S1 in bladder-colon cross-sensitization

The number of microglial cells has been observed in a transgenic mouse model that specifically expresses green fluorescent protein associated with the fractalkine receptor (Figure 4A and B). Seven days after injection of a 0.75% acetic acid solution into the bladder, the number of microglial cells increased compared to mice injected with saline (113.4 ± 13.0 *vs* 102.9 ± 11.2 per field, respectively, P < 0.05) (Figure 4C).

We administered minocycline, a microglial inhibitor, at the central level 1 h before the intravesical injection of acetic acid or saline to demonstrate the involvement of microglia cells. Both intrathecal and

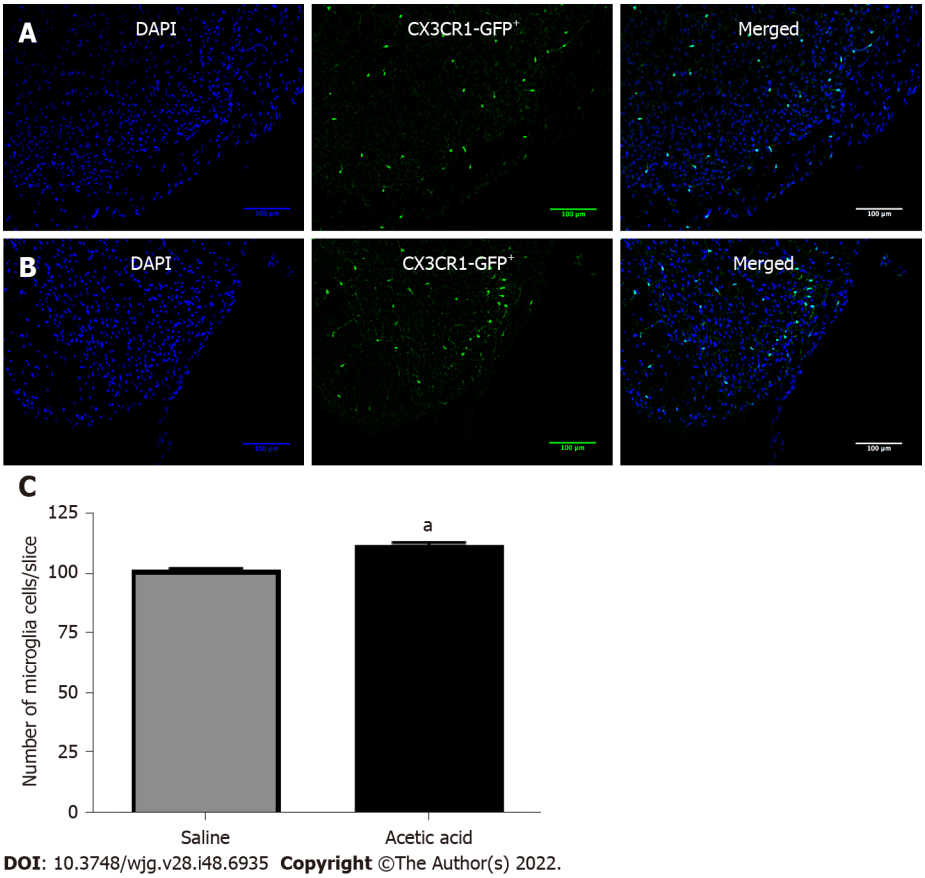

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Figure 4 Number of microglia cells in the spinal cord. A and B: Microglial cells in the dorsal horn of the L6-S1 level of the spinal cord were stained green [green fluorescent protein on the fractalkine receptor-positive (CX3CR1^{gfp+}) mice], while nuclei appear in blue (DAPI) at day 7 after intravesical injections of saline (NaCl 0.9%) (A) or acetic acid 0.75% (B); C: The number of microglial cells per field was compared between the two groups using a Mann-Whitney bilateral test (n = 5 per group, average of 23 slices analyzed per mouse). $^{a}P < 0.05$. Results are expressed as the mean ± standard error of the mean.

ICV injections of minocycline prevented the development of cross-sensitization induced by intravesical administration of acetic acid (Figure 5A and B). ICV injections were favored for the following experiments because they are less traumatic and cause less stress to the animals.

Involvement of central NK1R in bladder-colon cross-sensitization

The ICV injection of CP 99994, a NK1R antagonist, 1 h before the intravesical injection of acetic acid, prevented the development of bladder-colon cross-sensitization compared to mice treated intracerebroventricularly by saline (Figure 6A). The intravesical administration of acetic acid increased the phosphorylation of MAPK-p38, a microglial protein involved in chronic pain generation[21], compared to mice injected intravesically with saline, but the ICV injection of CP 99994 prevented this phosphorylation, suggesting that microglial activation depends on the activation of NK1R (Figure 6B).

The ICV injection of SB203580, a MAPK-p38 inhibitor, 1 h before the intravesical injection of acetic acid prevented the occurrence of bladder-colon cross-sensitization (Figure 7A). The intravesical administration of acetic acid induced an increase in NK1R expression in the posterior horn of the L6-S1 level of the spinal cord compared to the administration of saline (Figure 7B). Acetic acid-induced spinal overexpression of NK1R was blocked by prior ICV administration of SB203580 (Figure 7B), suggesting that the MAPK-p38 pathway is involved in the development of bladder-colon cross-sensitization.

Assessment of the expression of spinal pro- and anti-inflammatory transcripts

Expression levels of *IL-1β*, *TNF-α* and *IL-10* mRNA did not differ between control and acetic acid groups (P = 0.22, 0.47 and 0.19, respectively) (Figure 8).

DISCUSSION

A mouse model of bladder to colon cross-sensitization with persistent visceral hypersensitivity in the colorectum has not been developed until now to our knowledge. Our study showed that pelvic visceral cross-sensitization involves central sensitization with microglia modulation following a peripheral

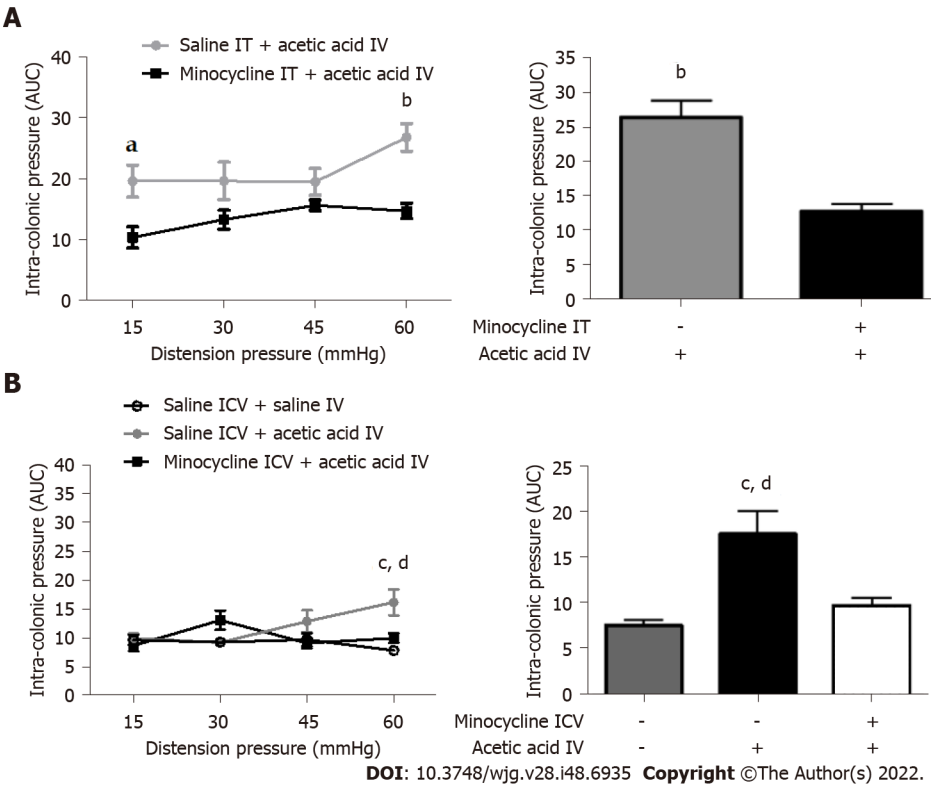

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Figure 5 Effect of minocycline injection at the central level on visceral sensitivity. A and B: Changes in intracolonic pressure in response to isobaric colorectal distensions (15, 30, 45 and 60 mmHg) intrathecal (IT) injections of saline (NaCl 0.9%) (A) or minocycline (1.25 mg/kg) prior to intravesical (IV) injections of acetic acid 0.75% and after intracerebroventricular (ICV) injections of saline or minocycline (1.25 mg/kg) (B) prior to IV injections of saline or acetic acid 0.75% in mice at day 7, both with details of the comparison at 60 mmHg of distension on the right. n = 6-8 mice per group. $^{a}P < 0.05$ and $^{b}P < 0.001$ (compared to the minocycline IT + acetic acid IV group); $^{c}P < 0.0001$ (compared to the saline ICV + saline IV group); $^{d}P < 0.05$ (compared to the minocycline ICV + acetic acid IV group). Variability in the results is represented by the standard error of the mean (area under the curve ± standard error of the mean). AUC: Area under the curve.

inflammatory event and is mediated by the NK1 and MAPK-p38 pathways.

The pathophysiology of colon-bladder cross-sensitization is complex and probably involves both peripheral and central mechanisms including: the sensitization of sensory nerve terminals in both organs; cross-sensitization of adjacent sensory primary afferent neurons within dorsal root ganglia (involving satellite glia cells and macrophages); axon reflexes *via* primary sensory afferent neurons with a dichotomizing axon explaining neurogenic inflammation if there is a convergence of sensory information from distinct organs to a single neuron and antidromic release of inflammatory mediators in the unaffected organ (pre-spinal convergence); the sensitization of second order spinal neurons in which there is a convergence of inputs from both colon and bladder, involving spinal interneurons (convergence-projection theory or dorsal root reflex); and supraspinal mechanisms (modified central processing of visceral stimuli in the amygdala, *etc.*)[10]. Our work adds to the current understanding that central sensitization in the spinal cord involves microglia modulation and is mediated by the NK1 and MAPK-p38 pathways.

It is already known that an increase in proinflammatory factors within the bladder tissue can sensitize the colon[22-24]. Several mechanisms have been proposed to explain this inter-organ sensitization. These mechanisms include the increased mechanical sensitivity of visceral muscle afferents, a higher proportion of chemosensitive visceral afferents[23], brainstem neurons integrating somatovisceral messages after bladder irritation[25], and central sensitization resulting from stressful life events[26].

In our study, we used a mouse model of colonic visceral hypersensitivity induced by cross-organ sensitization to demonstrate that microglia play a central role in the development of this hypersensitivity. Indeed, we showed that the ICV injection of minocycline, which is known to be an inhibitor of microglial cells, blocks the cross-sensitization process. It has already been shown that minocycline also induces a decrease in neuronal excitability by preventing phosphorylation of the ERK protein and MAPK, which are expressed in the spinal cord[27]. The analgesic effect of minocycline is mediated by its action in both microglial activation and neuronal activation. Microglial cells have already been shown to be recruited during colonic sensitization induced by chronic stress in rats[12], but our study is the first to identify the crucial role of microglia in bladder to colon cross-sensitization and in a mouse model.

We showed that the role of microglia in the development of cross-sensitization from the bladder to the colon is mediated by the tachykininergic pathway, particularly involving NK1R. This receptor activates, directly or indirectly, the MAPK-p38 protein *via* its phosphorylation. Several studies have

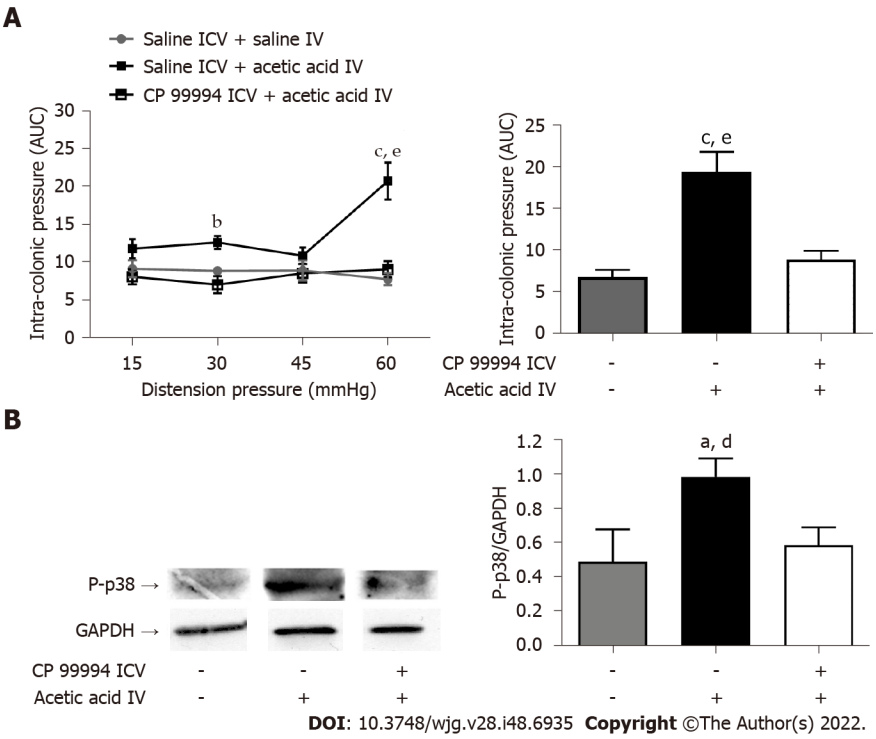

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Figure 6 Effect of CP 99994 injection at the central level on visceral sensitivity and the expression of phosphorylated MAPK-p38. A: Changes in intracolonic pressure in response to isobaric colorectal distensions (15, 30, 45 and 60 mmHg) after intracerebroventricular (ICV) injections of saline (NaCl 0.9%) or CP 99994 (7.5 mg/kg) prior to intravesical (IV) injection of saline or acetic acid 0.75% in mice at day 7, with details of the comparison at 60 mmHg of distension on the right. *n* = 8 mice per group; B: Expression of phosphorylated MAPK-p38 (P-p38) at the L6-S1 level of the spinal cord, with quantitative analysis (ratio to the reference protein GAPDH) on the right. Samples were run on separate gels and compiled for the figure. *n* = 5-8 mice per group. [a]*P* < 0.05, [b]*P* < 0.01 and [c]*P* < 0.001 (compared to the saline ICV + saline IV group); [d]*P* < 0.05 and [e]*P* < 0.001 (compared to the CP 99994 ICV + acetic acid IV group). Variability in the results is represented by the standard error of the mean (area under the curve ± standard error of the mean). AUC: Area under the curve.

confirmed that MAPK-p38 is expressed only by microglial cells[21,28,29]. Its involvement in the course of chronic stress-induced colonic sensitization has already been demonstrated in rats[12]. Another study suggested that the activation of microglial MAPK-p38 protein at the ventromedial nucleus of the spinal cord (rostral ventromedial medulla) was responsible for uterocolonic cross-sensitization in an acute model[30]. In our work, we found that visceral hypersensitivity induced by chronic stress was associated with the phosphorylation of MAPK-p38 at the microglial cell level in mice and that this effect was inhibited by the ICV injection of a MAPK-p38 inhibitor (SB203580).

Bradesi *et al*[12] also showed that the fractalkine receptor potentiates the development of visceral hypersensitivity *via* a chemokine function on NK1R. The activation of MAPK-p38 is known to be associated with the increased synthesis and secretion of several neurotransmitters of inflammation, such as COX2, IL-1β, inducible nitric oxide synthase, PLA2 and PGE2[31]. The mediators of inflammation involved in our model are probably different from those involved in the trinitrobenzenesulfonic acid-induced colitis models, in which expression of IL-1β is upregulated in the spinal cord[32] since we did not demonstrate any changes at the *TNF-α*, *IL-1β* and *IL-10* mRNA level.

We therefore propose a mechanistic view of the molecular and cellular mechanisms underlying the development of colonic hypersensitivity by bladder-to-colon cross-sensitization in which the inflammatory reaction following irritation of the urothelium induces the activation of extrinsic primary afferent neurons, some of them co-innervating the bladder and the colon and giving rise to axon reflexes, while others innervating the colon are activated by paracrine interactions. Convergent neurons in the dorsal root ganglia and the spinal cord, and those innervating the colon secondarily sensitize the colon. Some activated extrinsic primary afferent neurons relaying to other neurons in the dorsal horn of the spinal cord specifically secrete substance P, a member of the tachykinin family, which binds to NK1R activating the MAPK-p38 by phosphorylation. This in turn could either directly or indirectly induce the synthesis of mediators involved in neuroplasticity and neuroinflammation in the spinal cord.

A mini-invasive approach that specifically targets the bladder without inducing structural lesions in neighboring areas (especially the colon and peritoneum) is a prerequisite for cross-sensitization. Indeed, abdominal surgery is known to induce stress, involving a hormonal stress response at the peripheral and central levels, and implicating the corticotropin-releasing factor pathways[33]. It can induce visceral sensitization in the absence of signs of overt inflammation in mice[16]. Similarly, we decided to use measurements of the intracolonic pressure as a surrogate marker of visceromotor responses induced by CRD to assess visceral sensitivity in our study. This technique was an alternative to the measurement of

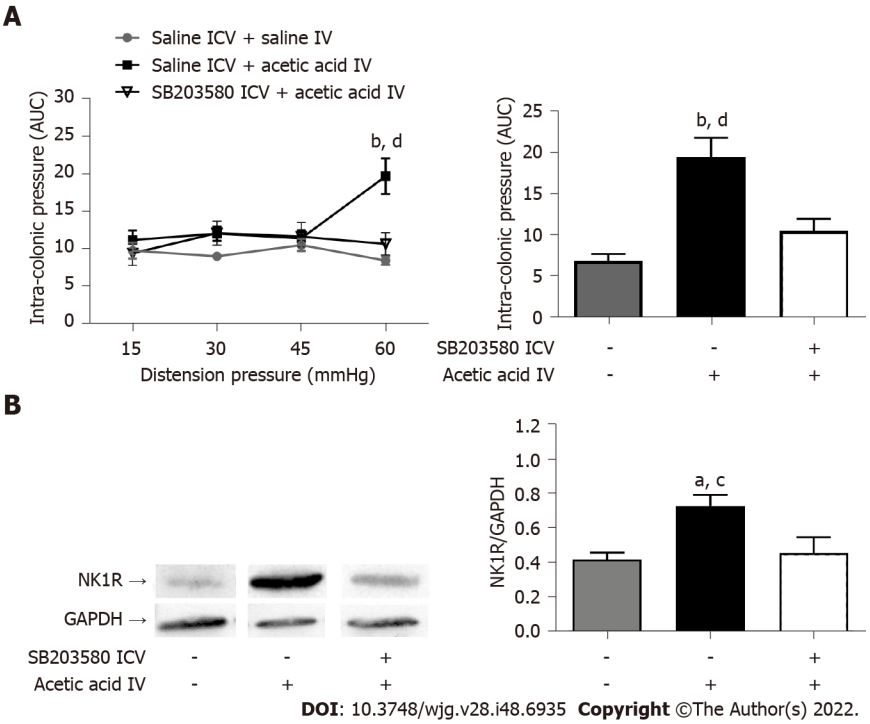

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Figure 7 Effect of SB203580 injection at the central level on visceral sensitivity and the expression of NK1 receptors. A: Changes in intracolonic pressure in response to isobaric colorectal distensions (15, 30, 45 and 60 mmHg) after intracerebroventricular (ICV) injections of saline (NaCl 0.9%) or SB203580 (2.5 mg/kg) prior to intravesical (IV) injection of saline or acetic acid 0.75% in mice at day 7, with details of the comparison at 60 mmHg of distension on the right. *n* = 8 mice per group; B: Expression of NK1R at the L6-S1 level of the spinal cord, with quantitative analysis (ratio to the reference protein GAPDH) on the right. Samples were run on separate gels and compiled for the figure. *n* = 5-8 mice per group. [a]*P* < 0.05 and [b]*P* < 0.001 (compared to the saline ICV + saline IV group); [c]*P* < 0.05 and [d]*P* < 0.01 (compared to the SB203580 ICV + acetic acid IV group). Variability in the results is represented by the standard error of the mean (area under the curve ± standard error of the mean). AUC: Area under the curve.

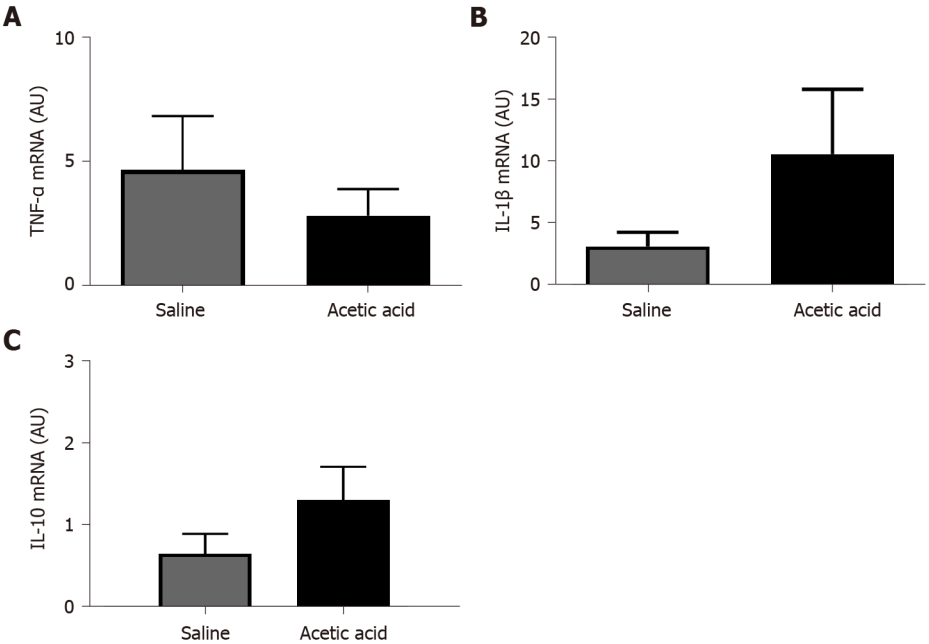

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Figure 8 Expression of inflammatory cytokines in the spinal cord. A-C: Expression levels of *TNF-α* (A), *IL-1β* (B) and *IL-10* (C) mRNA in the L6-S1 level of the spinal cord on day 7 were not different between mice with intravesical injections of acetic acid 0.75% or saline (NaCl 0.9%) on day 7. *n* = 7 per group. Results were expressed as the mean ± standard error of the mean. AU: Arbitrary unit.

the electromyographic activity of the abdominal muscles[34] and had the advantage of being minimally invasive.

When we planned our study, the available animal models for the study of bladder-bowel interactions included acute[11] and chronic[35,36] colon irritation with 2,4,6-trinitrobenzenesulfonic acid in mice[36] and in rats[11] and acute bladder irritation by cyclophosphamide, an antitumoral drug known to induce hemorrhagic cystitis in mice[35] and by infusing protamine sulphate and potassium chloride in rats[11]. In those studies, colonic sensitivity was assessed on day 5 after bladder irritation in mice and on the day of the irritation in rats.

The main limitation of our model is that the chronicity of the cross-organ sensitization was considered at day 7 following bladder irritation in mice. This period may seem short in view of clinical situations that evolve over several years. However, no other inflammatory model of cross-sensitization from the bladder to the colon published in the literature exceeds 48 h[35]. Furthermore, stress-induced chronic colonic sensitization models with 11 d of homotypic stress[12] were shown to be sufficient to induce changes in colonic tenderness and spinal microglia modulation. Our 7-d period in mice therefore seems to be long enough to attest to the chronicity of the process. Further studies with a longer longitudinal assessment of visceral sensitivity are necessary to confirm our results. Our results can only be extrapolated to males because sex-dependent differences in the responses to the sensitization process[37,38] and microglia[39] have been reported. Since pelvic neuroanatomy is similar in other rodents and in humans, we could expect similar results in those species, which could explain in part the common overlap between BPS and IBS in the clinic.

CONCLUSION

In conclusion, we have developed the first model of cross-organ chronic visceral sensitization between bladder and colon in mice. Pelvic cross-sensitization involves central sensitization with microglia modulation and is mediated by the NK1 receptor pathway and MAPK-p38 activation.

ARTICLE HIGHLIGHTS

Research background

Limited mechanistic data is available in the context of overlap between bladder pain syndrome and irritable bowel syndrome. Based on the fact that pelvic organs share common sensory pathways, a few studies have offered evidence that cross-sensitization between the bladder and the colon may explain sensitization of both organs. This may involve primary extrinsic afferents or central sensitization, both at the spinal and supraspinal levels.

Research motivation

The precise pathophysiology involved in cross-sensitization of the bladder and the colon remains poorly understood.

Research objectives

The objectives of this study were to develop a model of chronic bladder-colon cross-sensitization and to investigate the mechanisms involved.

Research methods

Chronic cross-organ visceral sensitization was obtained in C57BL/6 mice using ultrasound-guided intravesical injections of acetic acid under brief isoflurane anesthesia. Colorectal sensitivity was assessed in conscious mice by measuring intracolonic pressure during isobaric colorectal distension. Three different inhibitors/antagonists were assessed to gain a better understanding of which pathway could be involved in the cross-sensitization process.

Research results

Visceral hypersensitivity to colorectal distension was observed after the intravesical injection of acetic acid. This effect started 1 h post-injection and lasted up to 7 d post-injection. Colorectal sensitization was prevented by intrathecal or intracerebroventricular injections of minocycline, a microglia inhibitor, by intracerebroventricular injection of CP-99994 dihydrochloride, a NK1 antagonist, and by intracerebroventricular injection of SB203580, a MAPK-p38 inhibitor.

Research conclusions

We described a new model of cross-organ visceral sensitization between the bladder and the colon in mice lasting up to 7 d. Intravesical injections of acetic acid induced colorectal hypersensitivity to

distension, mediated by neuroglial interactions, MAPK-p38 phosphorylation and the NK1 receptor.

Research perspectives

A bladder-colon chronic cross-sensitization mouse model using intravesical injections of acetic acid can be used as a preclinical model of overlap between bladder pain syndrome and irritable bowel syndrome.

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FOOTNOTES

Author contributions: Gourcerol G and Leroi AM designed the research study; Atmani K, Meleine Mathieu, Baron M, Bouleté I, Guérin C, Vaudry D, do Rego JC and Bahlouli W performed the research; Atmani K, Wuestenberghs F, Meleine M and Gourcerol G analyzed the data; Atmani K, Wuestenberghs F and Gourcerol G drafted the manuscript; Atmani K, Wuestenberghs F, Baron M, Bouleté I, Guérin C, Bahlouli W, Vaudry D, do Rego JC, Cornu JN, Leroi AM, Coëffier M, Meleine M and Gourcerol G critically reviewed the manuscript for important intellectual content; All authors approved the final version of the article, including the authorship list; Gourcerol G accepts official responsibility for the overall integrity of the manuscript.

Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals, and the protocol was approved by the local Ethics Committee for Animal Experiments (CENOMEXA No: N/02-01-13/02/01-16).

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Retrospective Study

Clinical features and long-term outcomes of patients with colonic oligopolyposis of unknown etiology

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Abstract

BACKGROUND

Colonic adenomatous polyposis of unknown etiology (CPUE) is an adenomatous polyposis phenotype that resembles Familial Adenomatous Polyposis (FAP) even though no germline pathogenic variant is identified.

AIM

We sought to better characterize the clinical features and outcomes in a cohort of CPUE patients.

METHODS

This is a retrospective case series of patients 18 years old or older with adenomatous oligopolyposis (between 10-100 adenomas) and negative genetic testing, identified through the Hereditary Gastrointestinal Cancer Database at Massachusetts General Hospital, a tertiary academic referral center. A retrospective chart review was performed with a focus on demographics, alcohol and tobacco use, medication use, familial malignancy and polyp burden, genetic testing information, endoscopic surveillance data including the corresponding histopathology, colonic and extracolonic malignancies, mortality events, and their etiology. Spearman correlation and Pearson Chi-square test (or Fisher's exact test) were used for continuous and categorical variables respectively.

RESULTS

CPUE patients were primarily male (69%) and presented for genetic counseling at

63.7 years. Only 2 patients (2.9%) reported a first-degree relative with polyposis. During an average surveillance period of 12.3 years, 0.5 colonoscopies *per* year were performed. Patients developed 2.3 new adenomas *per* year. 4 (5.7%) were diagnosed with colorectal cancer (CRC) at a mean age of 66 years, and 3 were diagnosed prior to the onset of oligopolyposis. 7 (10%) required colectomy due to advanced dysplasia or polyp burden. With respect to upper gastrointestinal manifestations, 1 patient had a gastric adenoma, but there were no cases of gastric or small bowel polyposis. During surveillance, 10 (14%) patients died at a mean age of 72, and none were due to CRC.

CONCLUSION

CPUE is distinct from familial adenomatous polyposis (FAP) syndrome and the use of FAP surveillance guidelines may result in unnecessarily frequent upper and lower endoscopies.

Key Words: Colonic polyposis of unknown etiology; Multigene cancer panel; Colorectal cancer; Colectomy; Surveillance; Mortality

Core Tip: Colonic adenomatous polyposis of unknown etiology (CPUE) resembles familial adenomatous polyposis (FAP) syndrome, but no genetic alterations are identified. The optimal management of CPUE is uncertain. Patients with CPUE are typically older males that exhibit a low rate of new adenoma formation without upper gastrointestinal polyposis during long-term surveillance. 10% required colectomy for polyposis, and none died from colon cancer. The clinical behavior of CPUE is distinct from FAP, and the current application of FAP surveillance guidelines for CPUE may result in unnecessarily frequent upper and lower endoscopies.

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INTRODUCTION

Colorectal cancer (CRC) is the 4th most frequently diagnosed cancer and the second leading cause of cancer death in the United States[1,2]. Most CRCs are considered sporadic and the lifetime cumulative risk for CRC in the general risk population is estimated as 5%[3]. Approximately 5% of CRCs are attributable to a hereditary CRC syndrome. These are broadly classified into polyposis and nonpolyposis syndromes.

Current clinical guidelines suggest a minimal set of genes that should be tested in all patients suspected of hereditary CRC or polyposis, preferably by using a multigene panel because of overlapping clinical phenotypes, inconsistent definitions for oligopolyposis, challenges with accurately classifying polyp histology, and variable modes of inheritance[4,5]. Adenomatous polyposis syndromes are the most common polyposis syndromes and typically result from a germline mutation in *APC* or biallelic variants in *MUTYH*. Rarely, adenomatous polyposis that may be phenotypically indistinguishable from *APC/MUTYH*-related polyposis, can be observed secondary to germline mutations in *AXIN2, GREM1, NTHL1, POLE, POLD1*, or *MSH3*[5-7]. Germline mutations in one of these genes are not always identified, and the term "colonic adenomatous polyposis of unknown etiology (CPUE)" has been coined to describe cases of adenomatous polyposis in which no pathogenic variant is found in a polyposis gene. This occurs in as many as 30-50% of all polyposis cases[8,9]. *APC* promoter 1B mutations[10] and somatic mosaicism for *APC*[11] are possible mechanisms, but this is likely to account for only a small fraction of these cases. Recent data also suggested that missed germline mutations can be potentially revealed by retesting[12].

CPUE appears to be more common in those with lower polyp numbers. 48% of cases of polyposis with over 100 adenomas were explained by a germline mutation, most commonly in *APC* or *MUTYH*. However, only 13.6% of individuals with 20-99 adenomas and 6.4% of those with 10-19 adenomas exhibited a germline mutation in a polyposis gene. Thus, a diagnosis of CPUE is more common in individuals who exhibit oligopolyposis (10-99 adenomas)[13].

The clinical features of individuals with adenomatous oligopolyposis of unknown etiology are not well-defined, and management recommendations are largely extrapolated from guidelines for familial

adenomatous polyposis (FAP) syndrome[14]. Previous reports of CPUE are mostly small and heterogeneous. While some have described a benign course[6], others found duodenal adenomas and fundic gastric polyposis in addition to an approximately 30% risk of extracolonic malignancies including skin cancer, leukemia, breast, bladder, and prostate cancer[9,15,16]. We sought to better describe the clinical characteristics and outcomes in a large cohort of CPUE patients.

MATERIALS AND METHODS

Study population

The study population of CPUE patients was identified through the Hereditary Gastrointestinal Cancer Database at Massachusetts General Hospital. Patients 18 years old or older who were documented to have at least 10 cumulative adenomas and less than 100 adenomas and completed sequencing of at least *APC* and the 2 common mutations (*Y179C* and *G396D*) of *MUTYH* without evidence of a pathogenic germline mutation were included.

Data collection

Patient charts were reviewed utilizing the EPIC Electronic Health Record from the first available endoscopic surveillance documentation through November, 2021. Data were retrospectively collected including demographics (age, gender, race maternal and paternal ethnicities), self-reported tobacco and alcohol use, metabolic comorbidities (diabetes mellitus, obesity, metabolic syndrome), and medication usage (as documented at the time of genetic counseling) associated with possible chemoprevention effect (Statins, Aspirin, and Glucophage). Genetic data and relevant family history were collected and included the date and age at the time of genetic consultation, results of genetic testing, and family history of polyps and malignancy up to 3rd degree relatives.

Data from colonoscopies, sigmoidoscopies, esophagogastroduodenoscopies, and video capsule endoscopies (VCE) including indication, quality of bowel preparation, polyps, and other significant findings were recorded. Histopathology reports were reviewed. Colonic and extra-colonic malignancies, mortality, and causes of death were also documented.

A Research Electronic Data Capture platform, a secure, password-protected database, was utilized to store data, and these data were later exported as Excel sheath files (saved on encrypted drives) for analysis.

Data analysis

Categorical variables were described as frequencies and percentages. Pearson Chi-square test (or Fisher's exact test if > 20% of cells had expected count < 5) were used to test correlations of dichotomous and categorical variables. Continuous variables were described as a mean ± standard deviation (SD), median, and range. Student's *t*-test and non-parametric Mann-Whitney *U* test were used to compare means or mean ranks across scale variables of two independent samples. A univariate logistic regression analysis was performed to assess the impact of a set of predictors on extra colonic malignancies (dependent variable). Limited by the low number of cases observed we did not have the power to estimate their confounding effect using multiple regression. All statistical tests were two-sided and $P < 0.05$ was considered as statistically significant. SPSS software [IBM SPSS Statistics for Windows, ver. 28.0.1.0(142)] was utilized for statistical analysis. A statistical review of the study was performed by a biomedical statistician.

This study was approved by the institutional review board and was carried out in accordance with the ethical principles described in the Helsinki Declaration.

RESULTS

Patient demographics

70 patients met the inclusion criteria and comprised our cohort of CPUE patients with oligopolyposis. The last clinical surveillance was documented at a mean age of 69.3 (range 30.6 - 85.5. median 70.6). 48 (69%) patients were male and 62 (89%) were Caucasian, predominantly represented by Irish and English ancestry. 29 patients (41.4%) were diagnosed with any metabolic comorbidity. 34 patients (49%) reported any history of alcohol usage. 7 patients (21.2%) were documented to consume more than 1 alcoholic beverage *per* day. 14 of these patients (42.4%) consumed between 1-7 drinks *per* week. 41 patients (58.6%) reported any smoking history. Data concerning current use of tobacco could be retrieved in 39 patients, and 15 of these patients (38.4%) were reported as active smokers. 17/41 (41.4%) patients who smoked had reported a mean of 30.1 packs/year (range 0.5 - 100 packs/year, median 25) (Table 1).

Table 1 Colonic adenomatous polyposis of unknown etiology cohort characteristics, *n* (%)

Cohort characteristics	Number (% of cohort)
Male	48 (69%)
Ethnicity	
White non-Hispanic	62 (88.5%)
Hispanic or Latino	5 (7.1%)
Black or African American	2 (2.9%)
Asian	1 (1.4%)
Paternal lineage	
Irish	25 (24%)
English	14 (13%)
French	12 (11%)
Scottish	11 (10%)
Italian	9 (9%)
Maternal lineage	
Irish	20 (23%)
English	14 (16%)
Italian	14 (16%)
Canadian	6 (7%)
Scottish	6 (7%)
German	5 (6%)
Any metabolic comorbidity reported (Obesity/diabetes mellitus)	29 (41.4%)
Any alcohol usage	34 (49%)
1-7 drinks/week	14 (20%)
More than 1 drink/day	7 (10%).
Any smoking history	41 (58.5%)
Active smokers	15 (21.4)
Mean pack years	17 (30.1)

Data include demographic and clinical features for all 70 patients in the cohort. Leading five paternal and maternal lineages are presented (Full data in supplementary table). Presence of metabolic comorbidities were combined. Data concerning alcohol consumption and smoking were available for 69 patients. Detailed data concerning smoking burden (Pack/years) were available for 17 patients.

Genetic test results

All 70 CPUE patients had genetic counseling and testing at a mean age of 63.7 years (range 27 - 83, median 65.5). Each patient had documentation of at least 10 adenomas as an indication for counseling, and most presented with a cumulative polyp burden of 10-20 polyps (36 patients; 51.4%), followed by 21-30 polyps (18 patients; 25.7%), 31-50 polyps (12 patients; 17.1%) and 4 patients (5.7%) with 51-100 polyps.

All patients had sequencing of the *APC* (full) and *MUTYH* genes (24% had sequencing of the 2 common mutations *Y179C* and *G396D*, 76% had full sequencing). 26 patients (37%) had sequencing of only these 2 genes, 5 patients (7%) had 4-7 additional genes analyzed (including *BRCA1, BRCA2*, *MLH1, MSH2, MSH6, PMS2,* and *EPCAM*), and the majority (39 patients, 56%) had multi-gene panels with a mean of 35.2 (3.6) genes tested (range 12 - 91; Median 28). The CRC-related genes that were sequenced are described in Table 2 (full list in Supplementary Table 1).

None of the patients carried a pathogenic *APC* or *MUTYH* mutation, and no other polyposis-related gene mutations were identified when tested as part of a multi-gene panel. 2 patients were identified as carriers of a non-polyposis associated pathogenic variant [*APC* I1307K and *CFTR* (TG)11-5T]. 5 patients (7.1%) carried a *VUS* (in *RAD 50*, *ATM*, *BARD1* or *APC*).

Table 2 Genes sequenced in the colonic adenomatous polyposis of unknown etiology cohort

Gene name	Number of patients tested	Percentage (%)
APC	70	100
MUTYH (full seq.)	53	75.7
MLH1	43	61.4
MSH2	43	61.4
MSH6	42	60.0
PMS2	42	60.0
EPCAM	41	58.6
CHEK2	39	55.7
TP53	39	55.7
BMPR1A	38	54.3
CDH1	38	54.3
PTEN	38	54.3
SMAD4	38	54.3
STK11	38	54.3
GREM1	36	51.4
POLD1	36	51.4
POLE	36	51.4
ATM	33	47.1
AXIN2	28	40.0
MUTYH (*Y179C* and *G396D* mutations only)	17	24.3
NTHL1	15	21.4
MSH3	13	18.6
BLM	7	10.0
GALNT12	7	10.0
RPS20	4	5.7
MLH3	3	4.3
RNF43	1	1.4

Colorectal cancer related genes sequenced in the colonic adenomatous polyposis of unknown etiology cohort. All had at least *APC* and *MUYTH* sequenced. Full list of genes tested in Supplementary Table 1.

Family history of cancer and polyps

At the time of initial genetic consultation, 54 patients (77.1%) reported a family history of any malignancy in a first-degree relative (FDR). There was a total of 113 cases of malignancy in FDRs in our cohort. Of these, the most prevalent were CRC (25 cases, 22.1%), breast (20 cases, 17.7%), and prostate (14 cases, 12.4%) cancer (Table 3). 14 patients (20%) had at least 1 FDR with CRC and 6 patients (8.6%) had at least 1 FDR and 1 second-degree relative (SDR) with CRC. The mean age of CRC diagnosis in a FDR was 66 (range 44 - 97, median 66). Most of these patients (30) had only 1 FDR (55.6%) with any cancer. With respect to SDRs, 94 cases of any malignancy were found in our cohort, mostly represented by 17 cases of CRC (18%) and 11 cases (11%) of breast cancer.

22 patients (31.4%) were reported to have a FDR with any colon polyp, and most of these patients were reported to have 1 or 2 FDR (36% and 41%, respectively) with any colonic polyp. Only 2 patients (2.9%) were reported to have a family history of multiple polyps in a FDR. In one patient, the father required a partial colectomy for multiple polyps.

Polyp and adenoma burden during colonoscopy surveillance

Among the 70 patients, 430 colonoscopy reports were reviewed, resulting in a calculated mean of 6.1 ±

Table 3 Malignancies in first-degree relatives of patients with colonic adenomatous polyposis of unknown etiology

Type of cancer in FDR	Number of cases (Total = 113)	Percentage (%)
CRC	25	22.1
Breast	20	17.6
Prostate	14	12.3
Non-melanoma skin cancer	10	8.8
Lung	8	7.0
Gastric	4	3.5
Bladder/Ureter	4	3.5
Brain	4	3.5
Renal	3	2.6
Melanoma	3	2.6
Cervical	3	2.6
Pancreas	2	1.7
Unknown type	2	1.7
Leukemia	2	1.7
Ovarian	2	1.7
Thyroid	2	1.7
Lymphoma	1	0.8
Liver	1	0.8
Malignant meningioma	1	0.8
Esophageal	1	0.8
Liposarcoma	1	0.8

54 patients reported a family history of cancer. Highest frequencies were noted for CRC (22.1%), Breast (17.6%) and Prostate (12.3%). 10 patients (18.5%) had 2 FDRs, 8 patients (14.8%) had 3 FDRs, 1 patient (1.9%) had 4 FDRs, 4 patients (7.4%) had 5 FDRs and 1 patient (1.9%) had 7 FDRs with any malignancy. FDR: First-degree relative; CRC: Colorectal cancer.

3.4 colonoscopies (range 1 - 15; median 6) performed per patient. The first documented colonoscopy was in March 1992 and the last one was documented in November 2021. Among the 63 patients who had more than one colonoscopy performed over more than one year of surveillance, 418 colonoscopies were documented, with a calculated mean of 6.6 ± 3.2 colonoscopies (range 2 - 16, median 6) *per* patient, during a mean surveillance period of 12.3 ± 6.2 years (range 1.3 - 24.8, median 11.8 years). The calculated average frequency of colonoscopy surveillance among this group was 0.5 colonoscopies *per* year.

For these 63 patients, there was a total of 2547 documented polyps in 408 colonoscopies and 1826 documented adenomas in 394 colonoscopies, with a mean total cumulative burden of 39 ± 24.7 polyps (range 10 - 111; median 29) and 29.0 ± 18.9 adenomas (range 10 - 102; median 24). Over the entire surveillance period, this translates to a mean of 3.2 polyps diagnosed per year and 2.3 adenomas *per* year.

With respect to the distribution of polyps and adenomas in the colon, the right colon was the most prevalent location (75% and 54%, respectively), followed next by the transverse colon (49.7% and 38.8%, respectively). For polyps in general, the next most prevalent locations were the sigmoid colon (41.6%), left colon (39.0%), and rectum (26.6%), while for adenomatous polyps the locations and prevalence were left colon (31.1%), sigmoid colon (22.4%) and the rectum (10.5%).

The most prevalent adenoma histology found was tubular adenoma with low-grade dysplasia (90%). High-grade dysplasia in a tubular adenoma or tubulovillous adenoma was seen in 11 exams (3.2%) (Supplementary Table 2). In 142 of the 428 colonoscopies (33%), at least one non-adenomatous polyp was reported. Except for one colonoscopy in which only an inflammatory polyp was described, serrated polyps were reported in 141 of these 142 colonoscopies (99%), and these included hyperplastic polyps (74%), sessile serrated polyps/adenomas (24%) and traditional serrated adenoma (1.3%). Most of the serrated polyps were located in the sigmoid colon (33.3%) followed by the right colon (22.5%), rectum (20%), transverse colon (13.3%), and left colon (10.7%). No hamartomas were reported.

Incidence of invasive CRC

Four patients were diagnosed with invasive CRC (5.7%; mean age 66). Three underwent colectomy and one had a malignant polyp that was resected endoscopically. Among these four cases, three patients were diagnosed with CRC prior to the development of oligopolyposis. The first was a male diagnosed with rectal carcinoma at the age of 63 along with 4 adenomas; this patient later developed 23 more adenomas over 17 years of surveillance. The second patient was a male diagnosed at the age of 70 with a malignant polyp (T1N0M0, well-differentiated adenocarcinoma) in the sigmoid colon that was completely resected at colonoscopy. He presented with a polyp burden of only 6 adenomas over a 15-year period prior to the diagnosis of CRC. The third patient was a female diagnosed with a sigmoid colon CRC at the age of 71 at her first colonoscopy with a polyp burden at that time of 5 adenomas. This patient later developed 33 adenomas over 4.5 years of surveillance. The fourth patient was a female diagnosed with transverse colon CRC at the age of 69, one year after her first colonoscopy with a cumulative burden of approximately 30 adenomas. Immunohistochemical stains for DNA mismatch repair proteins were available for two of these patients, and both demonstrated preserved expression of all proteins (hMLH1, hMSH2, hMSH6, and hPMS2).

Rates of colectomy for high polyp burden or advanced dysplasia

Four additional patients (5.7%, mean age 64) underwent colectomy due to a high polyp burden without cancer. Two had a subtotal colectomy for multiple tubular and tubulovillous adenomas, some of which were large and unresectable endoscopically. Another had a subtotal colectomy due to a cumulative burden of approximately 50 adenomas as well as recurrent diverticulitis. One had a total proctocolectomy due to a cumulative burden of more than 80 adenomas in addition to adenomatous polyps with high-grade dysplasia. These patient characteristics are summarized in Table 4. Three patients (4.3%, mean age 52) were diagnosed with intramucosal carcinoma during colonoscopy, and all underwent colectomy.

When comparing these 11 patients who had a significant clinical outcome (intramucosal cancer, invasive cancer, or risk-reducing colectomy for polyposis) to the rest of the cohort (59 patients), no difference was found in any clinical parameters including gender, tobacco use, metabolic comorbidities, familial malignancy burden, personal malignancy burden or duration of colonoscopy surveillance (Supplementary Table 3).

Extracolonic findings

With respect to upper gastrointestinal findings, 39 patients (55.7%) had at least 1 upper endoscopy performed (first exam at mean age 62.3, range 22 – 83, median 65). 10 patients (14%) were found to have any gastric polyp. In 11 gastroscopies, there were up to 5 polyps documented and none was above 1 cm. Among cases in which histologic sampling was performed, the most common histology was fundic gland polyp without dysplasia (72%) followed by hyperplastic polyp (22%), and there was one case of 1 gastric adenoma that also exhibited high-grade dysplasia (6%). This patient also had low-grade dysplasia that arose in a background of chronic gastritis and intestinal metaplasia secondary to H. pylori.

No duodenal adenomas were detected. 4 patients (5.7%) had a formal small bowel evaluation with VCE, and no small bowel polyps were identified.

A total of 49 extra colonic malignancies (ECM) were documented in 35 patients (50%), and 9 patients (20%) had more than 1 ECM. The mean age of first ECM diagnosis was 60 (range 23- 82, median 62) with non-melanoma skin cancer (51%) and prostate cancer (12%) as the most common. (Table 5). Gender, age, and cumulative adenoma burden were evaluated by univariate logistic regression analysis for their potential contribution to the development of an ECM. Age was found to have a correlation with breast cancer and melanoma occurrence with an odds ratio of 0.8 (P = 0.01) and 0.9 (P =0.01). Otherwise, cumulative adenoma burden was not found to be a predictor. (Supplementary Table 4). No correlation was found between the cumulative adenoma burden and the total number of extra colonic malignancies reported (P = 0.18).

Mortality

10 patients (14%) died during follow-up. The mean age of death was 72 (range 61 - 78, median 73.5). 5 patients (50%) died of malignancy, but none was from CRC. The mean age of death from cancer was 74.4 (range 71 - 77, median 75) and 5 patients (50%) died from non-malignancy causes at a mean age of 69.6 (range 61 - 78, median 69). None of these causes were directly related to the underlying polyposis (*i.e.*, complications from colonoscopy or colectomy) (Table 6).

DISCUSSION

CPUE is a colonic adenomatous polyposis syndrome in which no germline mutation is detected. Our relatively large CPUE cohort is comprised primarily of older white males without a family history of polyposis but a modest family history of colon cancer and personal history of tobacco use. The adenoma

Table 4 Clinical features of 11 patients with a significant clinical outcome (cancer, advanced dysplasia, or colectomy)

ID (Gender)	Colectomy (age) (yr)	Colectomy – indication	Surveillance (No. yr to colectomy; Total yr)	Total adenoma burden	No. FDR with CRC	No. SDR with CRC
26 (F)	RHC (62)	IMC	12; 16	Multiple (at least 34)	0	0
29(M)	SIG (52)	IMC	0; 12	Multiple at least 15	0	0
76(F)	IPAA (42)	IMC	0; 14	Multiple (at least 15)	0	0
32(M)	IRA (64)	Polyp burden	0; 1	Multiple (>30, many > 1 cm)	0	0
46(M)	IRA (65)	Polyp burden + recurrent diverticulitis	15; 24	Multiple (at least 47)	0	0
72(M)	IRA (69)	Polyp burden	12; No data post colectomy	Multiple (at least 31)	0	0
62(F)	IPAA (58)	Polyp burden	3; 8.5	Multiple (approx. 83)	0	1 (65)
37(M)	APR (63)	Rectal CRC a	0; 17	27	1 (66)	0
56(F)	SIG (71)	Sigmoid CRC[1]	0; 4.5	38	1 (68)	0
68(F)	Colectom[3](69)	Transverse CRC[2]	1; No data post colectomy	Multiple (at least 28)	1 (70)	0
82(M)	None(70)	SigmoidMP[4]	15; 16	16	0	0

[1]CRC diagnosed at 1st colonoscopy.
[2]CRC diagnosed at 3rd colonoscopy.
[3]No data about the type of colectomy.
[4]Resected endoscopically.
Adenoma burden, surgical data and familial burden of CRC (1st and 2nd degree relatives) of 11 patients who had a significant clinical outcome. CRC: Colorectal cancer; F: Female; M: Male; MP: Malignant polyp; IMC: Intramucosal carcinoma; RHC: Right hemicolectomy; IRA: Subtotal colectomy with ileorectal anastomosis; IPAA: total proctocolectomy with ileal pouch anal anastomosis; APR: abdominoperineal resection; SIG: sigmoidectomy; FDR: First-degree relative.

Table 5 Extra colonic malignancies in the colonic adenomatous polyposis of unknown etiology cohort

Type of malignancy	Number	%	Incidence rate (*Per* 1000 person-years)
Non-melanoma skin cancer	25	51	5.1
Prostate	6	12.2	1.2
Melanoma	5	10.2	1
Breast	4	8.2	0.8
Lung	2	4.1	0.4
Uterine	1	2	0.2
Non-Hodgkin's Lymphoma	1	2	0.2
Gallbladder	1	2	0.2
Ovary	1	2	0.2
Bladder	1	2	0.2
Pancreas	1	2	0.2
Merkel cell tumor	1	2	0.2

35 patients reported an extra-colonic malignancy. Highest frequencies were noted for Non-melanoma skin cancer (51%), Prostate (12.2%) and Melanoma (10.2%)

burden is modest and is characterized by a relatively low rate of adenoma growth (average of 2.3 adenomas *per* year). However, 15.7% were considered to have a significant outcome, which included colectomy due to polyp burden, advanced polyp histology of intramucosal carcinoma, or a diagnosis of CRC. There were no deaths related to CRC or polyposis.

Table 6 Causes of death in the colonic adenomatous polyposis of unknown etiology cohort

Malignancy causes (n = 5)	Age of death (yr)
Lung	71
Unknown (suspected) malignancy	72
Metastatic Merkle cell carcinoma	75
Gallbladder	77
Pancreas	77
Non- malignancy causes (n = 5)	
Pulmonary failure.	61
Ruptured aortic aneurysm	78
Unknown	65
Shock and multi-system organ failure.	69
Cardiac arrest	75

There were 10 deaths among CPUE patients. 5 (50%) were secondary to malignancy. CRC was not reported as a cause of death. CPUE: Colonic adenomatous polyposis of unknown etiology cohort; CRC: Colorectal cancer.

Interestingly, three patients had a CRC diagnosis before developing at least ten cumulative adenomas, However, all did exhibit colonic adenomas either prior to or at the time of CRC diagnosis, demonstrating that a predisposition to polyp formation was present at the same time. These findings reveal the heterogeneity of disease presentation associated with CPUE.

Although CPUE is often considered an attenuated variant of FAP, our findings in a large CPUE cohort over an extended period of surveillance (12.3 +/-6.2 years) suggest that CPUE is quite dissimilar from FAP given the gender distribution, age of onset, absence of family history, low rate of colon adenoma growth, and absence of upper gastrointestinal (GI) and other extra-colonic manifestations. Others have described CPUE cohorts to have higher rates of colon polyp formation, family history of polyposis and CRC, and upper GI polyposis. For example, CRC was observed in 19.3% of a different CPUE cohort. However, more comprehensive multi-gene panel testing was not performed. A low rate of upper GI findings in CPUE was also observed[17], consistent with our findings. The relatively high rates of metabolic co-morbidities as well as alcohol and tobacco use in our cohort suggest that there may be significant environmental and lifestyle contributors in patients with CPUE.

Because data are limited with respect to clinical features and outcomes in CPUE, it has been difficult to formulate definitive management recommendations. The National Comprehensive Cancer Network describes CPUE as a potential attenuated subgroup of familial adenomatous polyposis with possible FAP-related extra colonic manifestations. In addition to recommending short colonoscopy intervals (every 1-2 years), consideration is given to the evaluation of the upper GI tract, with specific attention to the duodenum and the ampullary area[5]. Our findings suggest that most with CPUE do not exhibit features suggestive of a FAP-related syndrome. Annual colonoscopy and routine upper GI surveillance may therefore not be required.

Our study has some limitations. Due to its retrospective nature, complete endoscopic and pathology data in some patients could not be retrieved, and not all endoscopy reports reliably quantified polyp burden. Thus, our results might reflect an underestimation of the cumulative polyp burden. In addition, approximately 40% had only *APC* and *MUTYH* genes sequenced, so alternative genetic etiologies for polyposis may not have been recognized. However, the frequency with which mutations in these other novel intermediate-risk genes are identified is very low[13,18], and it is unlikely that a significant number of these cases would be explained by one of these mutations. Finally, our cohort was comprised mostly of Caucasian men of Irish and English descent and may not be representative of the broader CPUE population. This may result in a selection bias that could be attributable to lower rates of referrals for genetic counseling and testing in non-white populations[19-20].

CONCLUSION

Most individuals with CPUE in our cohort exhibited a relatively benign course, characterized by a generally modest colonic adenoma burden, dominance of non-advanced histology, low rates of CRC during surveillance, negligible upper GI involvement, and low rates of mortality due to polyposis or CRC. We suggest that colonoscopy surveillance intervals could be extended, and that routine upper GI

screening may not be required.

ARTICLE HIGHLIGHTS

Research background

Colonic polyposis syndromes typically result from germline mutations in the *APC* or *MUTYH* genes and less commonly from other low/intermediate-risk genes. When no pathogenic variant is identified, a diagnosis of colonic polyposis of unknown etiology (CPUE) is made.

Research motivation

The existing literature on CPUE is limited, and the precise clinical features and long-term outcomes are not well-defined.

Research objectives

To characterize the natural history of CPUE by defining the malignancy risk, long-term colonic adenoma burden, and risk of extra-colonic tumors over an extended period of surveillance.

Research methods

We performed a retrospective detailed chart review of demographic, lifestyle habits, endoscopic, genetic, and clinical data of patients aged 18 years old or older meeting the criteria for CPUE in the Hereditary Gastrointestinal Cancer Database at Massachusetts General Hospital.

Research results

70 patients met the inclusion criteria and were predominantly Caucasian males. During an extended surveillance period, a very low cumulative colonic adenoma burden was observed, with no evidence for duodenal adenomas. 4 patients were diagnosed with colorectal cancer (CRC), but none had extra-colonic malignancies that are typically seen in familial adenomatous polyposis (FAP) syndrome (*i.e.*, gastric, duodenal, or thyroid cancer). There was no mortality attributable to CRC.

Research conclusions

Individuals with CPUE exhibited a relatively mild course with respect to polyp burden and cancer risk, which differs significantly from the FAP syndrome. The modest colonic burden implies colonoscopy surveillance intervals could be extended, and regular gastroscopic exams may not be necessary.

Research perspectives

CPUE is an underdiagnosed and heterogeneous clinical entity. The current findings should be validated in large-scale multi-center prospective studies, with greater representation of non-Caucasian populations in order to better define this unique condition in an evidence-based approach.

FOOTNOTES

Author contributions: Feldman D contributed to conceptualization and design, formal analysis and interpretation, investigation, resources and acquisition of data, methodology, visualization, writing, revising, and editing the draft critically for important intellectual content; Rodgers-Fouche L contributed to conceptualization, resources and acquisition of data, writing, revising, and editing the draft critically for important intellectual content; Hicks S contributed to conceptualization, resources and acquisition of data, writing, revising, and editing the draft critically for important intellectual content; Chung DC contributed to conceptualization and design, formal analysis and interpretation, investigation, methodology, resources and acquisition of data, supervision, visualization, writing - original draft, writing, revising, and editing the draft critically for important intellectual content; All authors have read and approve the final manuscript.

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