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OPINION REVIEW

Risk factors for small intestinal adenocarcinomas that are common in the proximal small intestine

Shunji Fujimori, Ryohei Hamakubo, Aitoshi Hoshimoto, Takayoshi Nishimoto, Jun Omori, Naohiko Akimoto, Shu Tanaka, Atsushi Tatsuguchi, Katsuhiko Iwakiri

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Abstract

The frequency of primary small intestinal adenocarcinoma is increasing but is still low. Its frequency is approximately 3% of that of colorectal adenocarcinoma. Considering that the small intestine occupies 90% of the surface area of the gastrointestinal tract, small intestinal adenocarcinoma is very rare. The main site of small intestinal adenocarcinoma is the proximal small intestine. Based on this characteristic, dietary animal proteins/lipids and bile concentrations are implicated and reported to be involved in carcinogenesis. Since most nutrients are absorbed in the proximal small intestine, the effect of absorbable intestinal content is a suitable explanation for why small intestinal adenocarcinoma is more common in the proximal small intestine. The proportion of aerobic bacteria is high in the proximal small intestine, but the absolute number of bacteria is low. In addition, the length and density of villi are greater in the proximal small intestine. However, the involvement of villi is considered to be low because the number of small intestinal adenocarcinomas is much smaller than that of colorectal adenocarcinomas. On the other hand, the reason for the low incidence of small intestinal adenocarcinoma in the distal small intestine may be that immune organs reside there. Genetic and disease factors increase the likelihood of small intestinal adenocarcinoma. In carcinogenesis experiments in which the positions of the small and large intestines were exchanged, tumors still occurred in the large intestinal mucosa more often. In other words, the influence of the intestinal contents is small, and there is a large difference in epithelial properties between the small intestine and the large intestine. In conclusion, small intestinal adenocar-

cinoma is rare compared to large intestinal adenocarcinoma due to the nature of the epithelium. It is reasonable to assume that diet is a trigger for small intestinal adenocarcinoma.

Key Words: Small intestine; Large intestine; Adenocarcinoma; Risk factor; Carcinogenesis

Core Tip: When investigating the risk factors for small intestinal adenocarcinoma, an important point to note is that small intestinal adenocarcinoma is often found in the proximal small intestine. Intestinal contents remain in the ileum longer than in the jejunum, so poorly absorbed food is unlikely to be a carcinogenic factor. Animal proteins and lipids, bile concentrations, and aerobic bacteria, which are thought to be concentrated in the proximal small intestine, may be carcinogens in the small intestine. Since small intestinal adenocarcinoma is much rarer than colorectal adenocarcinoma, it is unlikely that small intestinal villi are involved in carcinogenesis.

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INTRODUCTION

Although the small intestine occupies 75% of the gastrointestinal tract length and 90% of the mucosal surface area, primary small intestinal cancer accounts for less than 5% of gastrointestinal cancers[1]. During the last century, enteroscopy was a difficult procedure to perform, and thus, the elucidation of small intestinal cancer was delayed compared to that of other gastrointestinal cancers. However, in this century, capsule endoscopy and balloon-assisted endoscopy have made it easier than ever to examine the small intestine. The frequency of small intestinal cancer has been increasing since 2000 or earlier and continues to rise with the addition of improved diagnostic power *via* new endoscopes[2]. Especially in patients with anemia of unknown cause, cases of small intestinal cancer diagnosed as a bleeding source are increasing[3]. In addition, many cases are diagnosed by positron emission tomography or computed tomography. The frequency of small intestinal adenocarcinoma is still on the rise, partly due to the widespread performance of small intestinal examinations[4].

According to a 2006 French report, the incidence of primary small intestinal malignancies in both men and women was approximately four times that 30 years ago (1.2 males and 0.8 females per 100000 in 2006)[5]. Primary small intestinal malignancies include neuroendocrine tumors, sarcomas, and lymphomas in addition to adenocarcinomas. A report of 10946 primary malignancies of the small intestine, mainly in Europe, showed that adenocarcinomas accounted for 37% of cases, carcinoid tumors accounted for 37% of cases, sarcomas accounted for 12% of cases, and malignant lymphomas accounted for 4% of cases[6]. In the United States, 40% of primary malignancies of the small intestine are reported to be adenocarcinomas, and 36% are carcinoid tumors[7].

According to the cancer statistics published yearly by the American Cancer Society, 5420 small intestinal malignancies and 145290 colorectal malignancies were predicted to develop in the United States in 2005[8]. The frequency of small intestinal malignancies was only 3.7% of that of colorectal malignancies. In 2019, the predicted number of colorectal malignancies was almost 145600 cases. However, the number of small bowel malignancies was 10590, which is 7.3% of the number of colorectal malignancies. Additionally, this percentage is twofold higher than that reported in 2005[9]. In the United States, colorectal adenocarcinomas account for 98% of colorectal malignancies, but small intestinal adenocarcinomas account for only approximately 30%-40% of small intestinal malignancies [10]. Therefore, regarding adenocarcinoma at present, the number of cases of small intestinal adenocarcinoma is approximately 3% of that of large intestinal adenocarcinoma cases. Considering that the small intestinal villi and circular folds have even been reported to occupy 98% of the intestinal surface area [11], the frequency of small intestinal adenocarcinoma per surface area is extremely low compared to that of colorectal adenocarcinoma.

In the 1970s, an experiment was performed in which azoxymethane, a carcinogen, was intravascularly administered to rats. The results showed that adenocarcinomas appeared in the proximal small intestine, which corresponds to the duodenum, and the large intestine. However, no adenocarcinoma appeared in the jejunum/ileum[12]. It is worth noting that in this experiment, in rats in which a part of the small intestine or large intestine had been replaced, tumors still appeared in the large intestine and

not the small intestine, regardless of the position in the digestive tract. In other words, the content of the intestinal tract did not significantly influence the development of tumors in the intestinal tract, and this experiment showed that the properties of the intestinal tract are involved in the development of tumors. This azoxymethane administration experiment was conducted again recently, and the results did not show carcinogenicity in the small intestine[13]. In the 1980s, an experiment was conducted in which dimethylhydrazine was administered to rats to examine the reproducibility of the above experiment, and the results were similar[14]. Based on this fact, many tumors can develop in the proximal small intestine (probably in the papilla of Vater) and in the large intestine, regardless of the position in the digestive tract. However, tumor development is less common in the jejunum and ileum. In other words, there is a decisive difference between the small and large intestines.

Differences between the small and large intestines are mainly the presence or absence of villi, intestinal contents, intestinal content retention time, intestinal flora, intestinal epithelial turnover, mucosal properties, and genetic factors. There are very few adenocarcinomas in the small intestine compared to the large intestine, but small intestinal adenocarcinomas are more common in the jejunum than in the ileum, as shown below. Here, we would like to consider the risk factors for small intestinal adenocarcinoma.

RISK FACTORS FOR SMALL INTESTINAL ADENOCARCINOMA IN THE PROXIMAL SMALL INTESTINE

Table 1 summarizes the reports of small intestinal adenocarcinoma according to site, namely, the duodenum, jejunum, and ileum. The majority of reports show that adenocarcinoma is the most common malignancy in the duodenum. The oral side of the duodenum can usually be explored endoscopically, which is why many malignancies are diagnosed in this area. However, these reports may include cancer of the papilla of Vater. The only Chinese report on this topic does not show a high cancer incidence rate in the duodenum. In this report, 160 cases of cancer of the papilla of Vater were excluded from 202 cases of all small intestinal adenocarcinomas, and adenocarcinomas were more common in the jejunum than in other parts of the small intestine[15]. When we examined the adenocarcinomas of the small intestine that were diagnosed at our institution only in patients in whom a normal papilla of Vater was observed, the jejunum was the most common site. This finding is in agreement with that reported in China. Because the duodenum is short, this result may be appropriate in assessments of the small intestine alone. In recent years, the papilla of Vater has been suggested to have characteristics different from those of the duodenum[16,17]. A mixture of bile and pancreatic juice passes through the papilla of Vater, and the bile and pancreatic ducts themselves have different carcinogenic properties. It is natural to think that the carcinogenic origin in the papilla of Vater is different from that in the small intestinal mucosa. We did not examine cancer of the papilla of Vater here.

When comparing the jejunum and the ileum and excluding the duodenum, adenocarcinoma was more common in the jejunum than in the ileum in all reports. Based on these results, it seems clear that there are more small intestinal adenocarcinomas in the proximal small intestine than in the distal small intestine. According to a report summarizing small intestinal gastrointestinal stromal tumors (GISTs), the jejunum has more GISTs than the ileum[18]. In addition, reports of small intestinal neuroendocrine tumors are often reported in the jejunum within 1 m from the ileocecal valve[19]. Differences in the site of occurrence are observed depending on the type of tumor. Here, since small intestinal adenocarcinoma is more common in the proximal small intestine, we would like to determine the risk factors for adenocarcinoma of the small intestine by considering the difference between the proximal side of the small intestine and the distal side.

Food

Most absorbable dietary components are absorbed in the duodenum and jejunum[20]. In other words, undegraded proteins and unabsorbed lipids flow in the proximal part of the small intestine. Diets containing high volumes of animal fat and protein have been reported to have a high risk of small intestinal adenocarcinoma, with correlation coefficients of 0.61 and 0.75, respectively[21]. Lipids and even small amounts of large peptides penetrate the cell membrane, which may be involved in carcinogenesis. Most proteins and lipids are absorbed in the proximal intestine and rarely reach the ileum, so there is no contradiction in this respect. Therefore, they may be involved in the carcinogenesis of the small intestine.

Bile and pancreatic juice

The proximal part of the small intestine has higher levels of bile and pancreatic juice than the distal part. A review of the literature on the effects of bile and pancreatic juice reveals that bile may be converted to carcinogenic deoxycholic acid by bacteria and that cholecystectomy reduces the incidence of small intestinal cancer[22,23]. In other words, bile may be involved in small intestinal carcinogenesis. However, it is difficult to judge the validity of the results because there are few reports on the effects of

Table 1 Regions where small intestinal adenocarcinoma was reported

Country	Year	Number[1]	Duodenum		Jejunum		Ileum		Ref.
France	2020	347	210	60.6%	72	20.7%	65	18.7%	[31]
China	2020	42	11[2]	26.2%	29	69.0%	2	4.8%	[15]
Japan	2015	47	14	29.8%	21	44.7%	12	25.5%	[32]
United States	2010	421	230	54.6%	142	33.7%	49	11.7%	[33]
China	2010	197	108	54.8%	59	29.9%	30	15.3%	[34]
United States	2006	460	272	59.1%	98	21.3%	90	19.6%	[35]
United States	2005	195	113	57.9%	54	27.7%	28	14.4%	[7]
United States	1996	1404	777	55.3%	376	26.8%	251	17.9%	[2]
Japan	Our cases	50[3]	20	40.0%	26	52.0%	4	8.0%	N/A

[1]Number excludes cases of unknown site.
[2]A total of 160 cases of primary cancer in the papilla of Vater were excluded.
[3]Only patients with a normal papilla of Vater were included.
N/A: Not applicable.

bile on the small intestine.

Intestinal chemicals

The contents of the intestinal tract include chemicals contained in the diet and various chemical substances produced by bacteria. As mentioned above, chemical carcinogenesis occurs when the large intestine comes into contact with chemical substances. However, since the transit time of the intestinal contents into the small intestine is approximately 4 h, which is considerably shorter than that of the large intestine, the effect of chemical substances in the intestine is thought to be smaller than that of the large intestine. In addition, if carcinogenesis due to dietary or bacterial chemical substances is the main cause of adenocarcinoma, more adenocarcinomas would be likely to develop in the ileum, where the intestinal contents stagnate longer than in the jejunum. However, if the carcinogen is absorbed in the oral side of the small intestine, this is not inconceivable.

Intestinal flora

Intestinal bacteria influence the intestinal tract through various means. Among the various chemicals produced by intestinal bacteria, those that not only cause inflammation but also have a direct carcinogenic effect and those that delay or prevent cell division of the intestinal epithelium have been reported[24]. Delaying epithelial turnover may be beneficial for bacteria directly involved in the epithelium. This delay in turnover is considered to be a factor that increases the possibility of cancer cell engraftment. There is a high possibility that intestinal bacteria are involved in carcinogenesis in the large intestine. However, it is difficult to explain why the number of jejunal adenocarcinomas is larger than that of ilial adenocarcinomas if intestinal bacteria are strongly associated with carcinogenesis in the small intestine. This is because it is difficult to explain why there are few bacteria in the proximal small intestine but many small intestinal adenocarcinomas in that area. However, the proximal small intestine is characterized by a relatively large number of aerobic bacteria, although the absolute number of bacteria is small. Therefore, given that small intestinal adenocarcinoma predominantly occurs in the proximal small intestine and aerobic bacteria are abundant in the proximal small intestine, the role of aerobic bacteria in small intestinal adenocarcinoma must be considered.

Immunity

To absorb and excrete various substances, the cell membrane of the small intestine needs to have direct contact with the outside environment of the body. Therefore, the intestinal lumen needs to protect itself against bacteria, viruses, and many substances that invade the body using various types of immune mechanisms. The small intestine has the highest levels of immunity in the body. Additionally, the lymphatic system within the small intestine is stronger in the distal small intestine, where cancer may be strongly eliminated by immune mechanisms. Benzopyrene, for example, has been reported to suppress mouse immunity and thus, in the presence of carcinogens, lead to adenocarcinoma development in the proximal small intestine[25]. However, it remains unclear whether immunity can explain why cancer is overwhelmingly less common in the small intestine than in the large intestine because there are few reports on this topic.

Table 2 Risk factors for small intestinal adenocarcinoma

Factor	Disease	Risks of small intestinal adenocarcinoma	Ref.
Animal fat		Correlation coefficient of 0.61	[21]
Animal protein		Correlation coefficient of 0.75	[21]
		2–3-fold higher risk	[36]
Bile salts		Bile salts may transform into carcinogenic deoxycholic acid	[22]
Hereditary polyposis	FAP	APC mutation; 5% is small bowel adenocarcinoma and half is duodenal adenocarcinoma	[27]
		The incidence is 330 times higher than that in the general population	[28]
	HNPCC	MMR gene mutation; lifetime risk is at approximately 1% in a French registry	[29]
	PJS	STK11 mutation; incidence is 520 times higher than that in the general population	[30]
Disease	Crohn's disease	The incidence is 17.4 times higher than that in the general population	[37]
		The incidence is almost 3 times higher than that in the average American	[38]
	Celiac disease	It is implicated in 8%-13% of small bowel adenocarcinoma cases	[39,40]

FAP: Familial adenomatous polyposis; APC: Adenoma polyposis coli; HNPCC: Hereditary nonpolyposis colorectal cancer; MMR: Mismatch repair: PJS: Peutz-Jeghers syndrome; STK 11: Serine/threonine kinase 1.

Villus length

The small intestine has villi and crypts, and the large intestine has only crypts and no villi. The lifespan of cells that have migrated to the villi is short, and the small intestinal epithelium is thought to be renewed every 3-5 d[26]. The proximal small intestine has longer and denser villi than the distal part. Therefore, the rate of epithelial turnover in the proximal small intestine may be longer than that in the distal part. This may be the reason why small intestinal adenocarcinoma is more common in the proximal small intestine. However, considering that many cancers develop in the large intestine, which has only crypts, it is unlikely that villi are significantly involved in cancer development.

SUMMARY OF SMALL INTESTINAL ADENOCARCINOMA RISK FACTORS

Based on the above rationale and considering that the number of small intestinal adenocarcinomas in the proximal small intestine is larger than that in the distal small intestine, the possible causes of carcinogenesis are the effects of diet, bile concentration, aerobic bacteria, and intestinal immunity, in the order described. However, none of the above causes is definitive. Table 2 briefly summarizes the reported risk factors for small intestinal adenocarcinoma. In addition to the above causes, genetic factors and inflammatory diseases have been added to the table. Papillary carcinoma is increased in patients with familial adenomatous polyposis due to a mutation in adenoma polyposis coli, but it is less associated with the jejunum and ileum[27,28]. Small intestinal adenocarcinoma also occurs in hereditary nonpolyposis colorectal cancer patients due to mismatch repair mutations, but it is much less frequent than colorectal adenocarcinoma[29]. Small intestinal adenocarcinoma occurs 520-fold more often in patients with Peutz-Jeghers syndrome than in healthy individuals, but the population with small intestinal adenocarcinoma was originally small[30]. An increase in small intestinal adenocarcinoma is also observed in patients with Crohn's disease and celiac disease, which are thought to be related to inflammatory carcinogenesis. Therefore, it is understandable that genetic factors and disease factors are involved in small intestinal adenocarcinoma.

CONCLUSION

Small intestinal adenocarcinoma is characterized by its predominance in the proximal small intestine. Animal proteins and lipids, bile concentrations, aerobic bacteria, and intestinal immunity were discussed as factors playing a role in small intestinal adenocarcinoma. Of these, it is highly possible that the dietary content absorbed in the proximal part of the small intestine is a risk factor for small intestinal adenocarcinoma. However, since the number of small intestinal adenocarcinomas is small, there are few reports, and none of the results are definitive. Moreover, in small intestine/large intestine replacement

experiments, at least in rats, the results show that the contents of the intestine are rarely involved in carcinogenesis. It seems that the nature of the organs is strongly related to susceptibility to carcinogenesis. Future studies are expected.

FOOTNOTES

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REVIEW

COVID-19 and hepatorenal syndrome

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Abstract

Coronavirus disease 2019 (COVID-19) is a highly infectious disease which emerged into a global pandemic. Although it primarily causes respiratory symptoms for affected patients, COVID-19 was shown to have multi-organ manifestations. Elevated liver enzymes appear to be commonly observed during the course of COVID-19, and there have been numerous reports of liver injury secondary to COVID-19 infection. It has been established that patients with pre-existing chronic liver disease (CLD) are more likely to have poorer outcomes following COVID-19 infection compared to those without CLD. Co-morbidities such as diabetes, hypertension, obesity, cardiovascular and chronic kidney disease frequently co-exist in individuals living with CLD, and a substantial population may also live with some degree of frailty. The mechanisms of how COVID-19 induces liver injury have been postulated. Hepatorenal syndrome (HRS) is the occurrence of kidney dysfunction in patients with severe CLD/fulminant liver failure in the absence of another identifiable cause, and is usually a marker of severe decompensated liver disease. Select reports of HRS following acute COVID-19 infection have been presented, although the risk factors and pathophysiological mechanisms leading to HRS in COVID-19 infection or following COVID-19 treatment remain largely unestablished due to the relative lack and novelty of published data. Evidence discussing the management of HRS in high-dependency care and intensive care contexts is only emerging. In this article, we provide an overview on the speculative pathophysiological me-chanisms of COVID-19 induced HRS and propose strategies for clinical diagnosis and management to optimize outcomes in this scenario.

Key Words: COVID-19; Hepatorenal syndrome; Pathophysiology; Clinical assessment;

Management; Prognosis

Core Tip: There have been numerous reviews evaluating the causative relationship between coronavirus disease 2019 (COVID-19) and liver pathology, given an emerging number of cases reporting COVID-19 induced liver injury. There are few reports noting the onset of hepatorenal syndrome (HRS) in the face of COVID-19 infection. Occurrence of HRS in any circumstance is typically an indicator of severe and perhaps life-threatening disease, potentially requiring liver transplantation. With a paucity in literature compilation on the associations between COVID-19 and HRS, we provide a review which discusses the purported pathophysiological mechanisms of COVID-19 induced HRS, and propose clinical assessment and management approaches in this scenario.

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INTRODUCTION

The impact of coronavirus disease 2019 (COVID-19) has been tremendous since the initial case was reported in December 2019, and COVID-19 subsequently spiraled into a global pandemic which is affecting populations and societies significantly up to this day[1-3]. The severity of COVID-19 could be wide ranging from mild to severe disease[4]. This depends on various intrinsic and environmental factors for each individual[4,5]. The manifestations of COVID-19 are thought to be primarily respiratory, with severe COVID-19 infection leading to acute respiratory distress syndrome potentially progressing towards life-threatening septic shock and multi-organ failure[6,7]. There is emerging evidence on the multi-systemic effects of COVID-19 outside of the respiratory system. It is suggested that COVID-19 has direct associations with acute disease processes across the neurological, cardiovascular, renal and gastroenterological systems amongst other organ systems, but the pathophysiology of how COVID-19 affects these organs has not been fully established in most instances[8-12].

Liver injury secondary to COVID-19 has been investigated, with its incidence ranging between 15% and 53%[13]. In most patients, the effect of COVID-19 on the liver is a transient reaction with elevation of transaminases which resolves and most patients achieve recovery back to their normal baseline[14]. However, individuals with underlying cirrhosis and chronic liver disease (CLD) were found to have significantly greater 30-day mortality and lengthier hospitalization, and poorer prognosis following acute recovery from COVID-19 induced liver injury[15]. Histological damage to hepatocytes and bile duct cells was found in patients testing positive for COVID-19[16].

Hepatorenal syndrome (HRS) is a state of kidney function deterioration (usually profound oliguria and sodium retention) in patients with advanced cirrhosis or acute liver failure[17,18]. The decline in kidney function could be rapid (Type I) or gradual (Type II), dependent on the etiology of HRS[18]. In cirrhosis, Type 1 and Type 2 HRS has been replaced with newer terminology and HRS is now defined as either acute (HRS-AKI), sub-acute (HRS-AKD) or chronic (HRS-CKD)[19]. HRS is a diagnosis of exclusion in which other causes of kidney dysfunction are not identified, and where the kidneys were not found to be structurally damaged[17,18]. The dominant theory to explain for the pathophysiology of HRS is significant constriction of blood vessels which perfuse the kidneys, most likely mediated by splanchnic vasodilation (leading to central hypovolemia) and hepatorenal reflex mechanisms as a result of portal hypertension[17,18]. HRS is most likely seen in the context of advanced stage cirrhosis. The most common etiologies of cirrhosis include alcohol, non-alcoholic steatohepatitis and chronic viral hepatitis[20]. Multiple triggers of HRS have been recognized and spontaneous bacterial peritonitis (SBP) in patients with ascites from decompensated cirrhosis is a leading cause[21]. HRS is a marker of poor prognosis in hepatology, and the risk of death is very high unless prompt liver transplantation or acute dialysis can be provided[22].

There were select case reports of liver injury following COVID-19 infection where acute kidney dysfunction was found, suggesting the potential manifestation of COVID-19 induced HRS[23,24]. Evidence of the pathophysiology, optimal strategies for clinical assessment and management of COVID-19 induced HRS is seldom discussed at present due to a relative lack of cases. In this review, we will explore the speculative pathophysiological mechanisms of HRS following COVID-19 infection based on early evidence, and propose potential clinical assessment and management strategies to optimize HRS outcomes in this scenario.

POTENTIAL PATHOPHYSIOLOGICAL MECHANISMS OF COVID-19 INDUCED HRS

Current perspectives on the potential pathophysiological mechanisms of COVID-19 induced HRS are that of a multifactorial process (Figure 1). COVID-19 induced liver injury can be the result of direct viral cytopathic hepatocyte injury, systemic inflammatory cytokine storms causing hepatocyte cell death, endothelitis and dysfunction of the liver vasculature leading to widespread cell damage and ischemia, and drug-associated exacerbations in COVID-19 induced liver injury[25,26]. Subsequently, these multiple pathways of liver injury may result in circulatory dysfunction, progressing to HRS with vasoconstriction and hypoperfusion of the kidneys amongst other mechanisms[17-19].

Direct COVID-19 infection of hepatocytes

Direct viral cytopathic injury to the liver, similar to how various organs are affected by COVID-19 infection, should be considered as the major pathophysiological mechanism. Ultrastructural histological examination identified severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) particles in the cytoplasm of hepatocytes[27]. Features of cellular viral invasion such as conspicuous mitochondrial swelling, decreased glycogen granules, and endoplasmic reticulum dilatation were observed in SARS-CoV-2-infected hepatocytes[27]. The presence of binuclear hepatocytes, central lobular necrosis and hepatocyte apoptosis were observed features of liver damage following COVID-19 infection[27]. Induction of hepatocyte apoptosis from COVID-19 infection could be the result of p7a overexpression [26,28]. p7a is a protein which can be expressed in cells infected by SARS-CoV-2, and induces apoptosis in cell lines derived from organs including the lungs, liver and kidneys *via* a caspase-dependent pathway[29]. This mechanism confirms the pathway of how SARS-CoV-2 can directly attack liver tissues and cause damage.

The extent of viral tropism is typically dependent on the availability of viral receptors at the surface of host cells in specific tissues[30]. The spike (S) protein of SARS-CoV-2 mediates cellular entry of SARS-CoV-2. S protein is cleaved by transmembrane serine protease 2/transmembrane serine protease 4 and interacts with the angiotensin converting enzyme 2 (ACE2) protein in host cells specifically[30]. In normal circumstances, ACE2 would only be expressed in bile duct epithelial cells, central hepatic vein and portal vein endothelial cells within the hepatobiliary system, being almost absent in hepatocytes [31]. The level of ACE2 expression in bile duct epithelial cells is comparable to that of alveolar epithelial cells in the lungs (commonly recognized to have the greatest expression of ACE2 in the body)[31]. The compensatory differentiation and proliferation of liver parenchymal cells derived from bile duct cells during diseased states may explain the underlying pathophysiological mechanisms of how SARS-CoV-2 induces liver injury[32]. The degree of ACE2 expression in hepatocytes is regulated by multiple factors. Histological studies in mice and humans identified greater ACE2 expression in subjects with cirrhosis [33]. The degree of hypoxia is also shown to correlate with ACE2 expression in hepatocytes, and notably the affinity of S protein towards the ACE2 receptor is increased with hypoxia due to trypsin activation, trypsin being a protein commonly expressed in liver epithelial cells[34,35]. These findings may explain why the effects of COVID-19 infection would tend to be more severe in patients living with underlying CLD and other hypoxic conditions.

Systemic inflammatory cytokine storms in COVID-19 induced liver injury

Another purported pathway of COVID-19 induced liver injury is an excessive immune response triggered by the virus causing a systemic inflammatory cytokine storm[36]. During a systemic inflammatory cytokine storm stimulated by COVID-19 induced liver injury, complement and interleukin-23 (IL-23) are released into the bloodstream, activating kupffer cells and inducing their production of tumor necrosis factor α (TNF-α)[37,38]. As an inflammatory cytokine, TNF-α aggravates the responses of inflammation by upregulating the expression of endothelial cell adhesion molecules and inducing hepatocytes to secrete chemokines[39]. Under the induction of chemokines, $CD4^+$ T cells and neutrophils are rapidly recruited to the liver, in which $CD4^+$ T cells assist mucosal molecules to promote neutrophil entry into the liver parenchyma[40,41]. Neutrophils directly damage hepatocytes by releasing oxidants and proteases, resulting in cell necrosis[41]. In patients who are severely affected or critically ill following COVID-19 infection, much higher plasma levels of inflammatory cytokines and lower lymphocyte counts were observed[42]. Previous studies note that an increase in IL-6 and IL-10 and a decrease in $CD4^+$ T cells were independent risk factors related to severe liver damage, and lymphopenia and C-reactive protein levels were found to be independently associated with the degree of liver injury [43].

The inflammatory storm response from COVID-19 infection is usually mild in the early stages of COVID-19 infection, but patients may clinically deteriorate rapidly if appropriate management of COVID-19 is not administered in a timely manner, with the inflammatory storm response occurring more strongly during the post-viral inflammatory phase[6]. For patients living with decompensated CLD complications, notably ascites, their threshold to develop systemic inflammatory storm responses following COVID-19 infection would even be lower than those without CLD due to underlying pro-inflammatory risks with SBP[44,45].

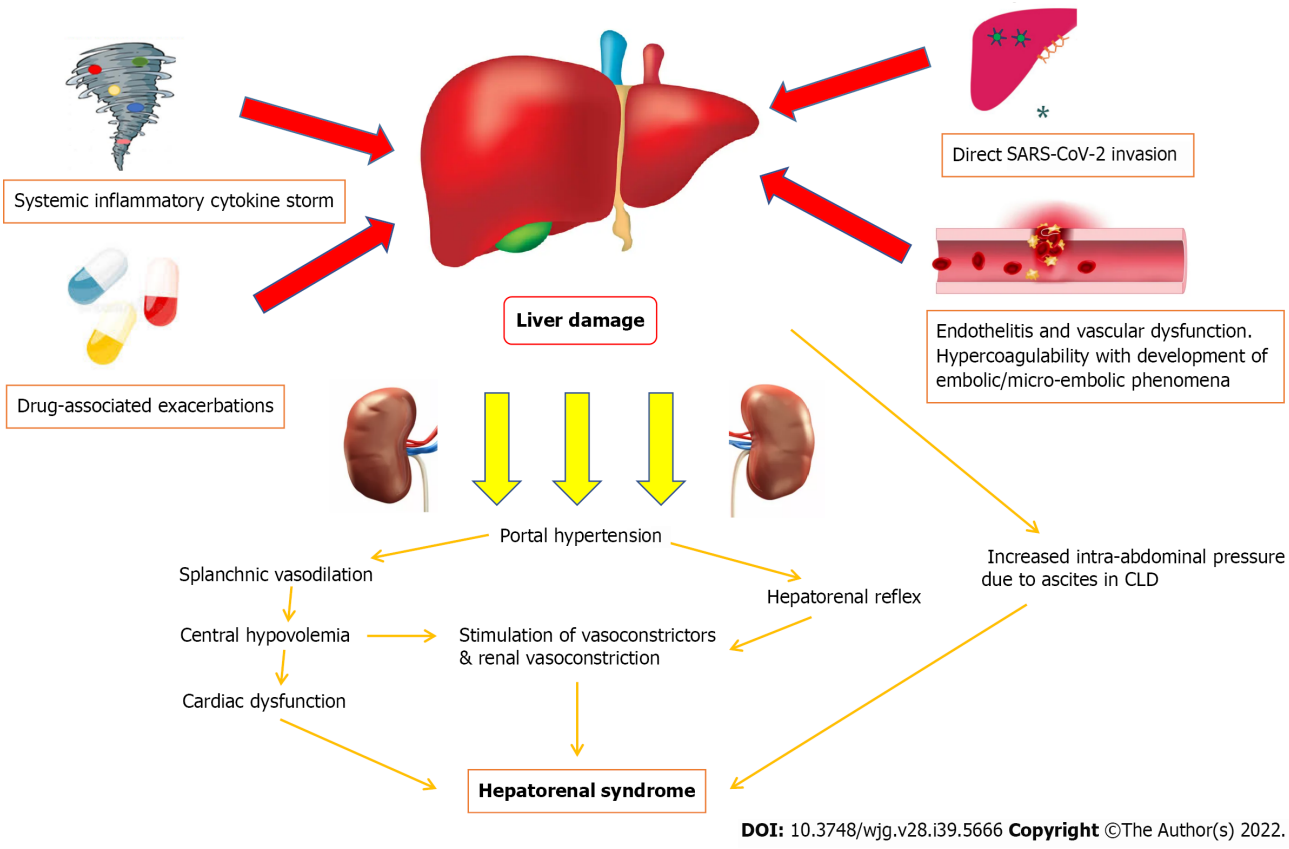

Figure 1 Potential pathophysiological mechanisms of COVID-19 induced hepatorenal syndrome. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

Endothelitis and vascular dysfunction in COVID-19 induced liver injury

The impact of COVID-19 towards thrombo-inflammation in endothelial tissues is significant. The ACE2 protein, which is present in many organs across the body, facilitates SARS-CoV-2 entry into endothelial cells *via* endocytosis with its binding to the ACE2 protein[46]. Viral infection and immune-mediated inflammatory responses occur within endothelial cells, leading to vascular dysfunction, especially in capillaries[47-49]. Subsequently, vascular dysfunction progresses to a hypercoagulable state and the development of embolic/micro-embolic phenomena, tissue edema, and organ ischemia[47-49]. In the liver, ischemia reperfusion injury which occurs typically after rapid recovery of blood circulation following events of vascular dysfunction leading to ischemia, has been touted as an underlying pathophysiological mechanism of COVID-19 induced liver injury[48,49]. Reperfusion following ischemia activates neutrophils, kupffer cells, and platelets within the cellular surroundings, leading to a series of destructive cellular reactions such as reactive oxygen species and calcium overload, which manifests towards widespread inflammatory response and cell damage[48,49]. Eventually, increased anaerobic glycolysis leads to reduced adenosine triphosphate production, which ultimately results in hepatocyte cell death from inhibition of hepatocyte signal transduction[42,48,49].

Drug-associated exacerbations in COVID-19 induced liver injury

In addition to the direct viral and systemic inflammatory mechanisms of liver injury following COVID-19 infection, the impact of various drugs received during acute hospitalization in patients with COVID-19 associated liver injury has been discussed. There has been debate whether these drugs (drugs trialed/used for COVID-19 treatment-antivirals such as Lopinavir/Ritonavir, Remdesivir, Favipiravir, Arbidol, Oseltamivir and others; antibiotics such as Doxycycline and Azithromycin; chloroquines; steroids; non-steroidal anti-inflammatory drugs) play a greater pathophysiological role in causing liver injury compared to COVID-19 infection itself[50].

Several studies have evaluated antivirals targeting COVID-19 infection in the midst of liver injury. For example, Lopinavir is a protease inhibitor conventionally used to treat human immunodeficiency virus infection in combination with a low dose of Ritonavir, another protease inhibitor, which enhances its biological half-life[51]. If high doses of Ritonavir (> 1 g daily) are taken, severe hepatotoxicity may ensue[52]. Recent studies show that Lopinavir/Ritonavir, when prescribed with or without ribavirin, interferon beta, and/or corticosteroids, was independently associated with increased levels of serum alanine transaminase (ALT) and aspartate transaminase (AST) in patients with positive COVID-19

status[53]. It was demonstrated from a retrospective observational study by Jiang and colleagues that Lopinavir/Ritonavir use in COVID-19 patients is associated with liver injury and abnormal liver function, particularly for patients in a non-critical state[54]. Ultimately, considering the fact that there is yet to be a completely effective antiviral therapy for COVID-19 and that antiviral drugs may cause abnormal liver function, there should be careful consideration of whether to prescribe antivirals, in particular for patients with CLD and/or metabolic diseases[52].

Antibiotics, particularly those of tetracycline-class and Azithromycin, have been shown to exacerbate liver damage in the context of COVID-19 induced liver injury[55]. For example, Doxycycline chelates zinc, which is required by the matrix metalloproteinases involved in COVID-19 infection, and inhibits SARS-CoV-2 RNA polymerase activity and direct viral entry[56,57]. Whilst generally safe to use with its anti-inflammatory effects, Doxycycline use may contribute towards hepatotoxicity and has been linked to occasional bile duct injuries[55]. There should be caution when prescribing Doxycycline alongside other potential hepatotoxic drugs, given reports of fulminant liver failure and hepatocellular necrosis occurring following the prescription of Doxycycline in these scenarios[55].

At a molecular level, there has been focused discussion on cytochrome P450 (CYP450) in the context of drug-associated exacerbations in COVID-19 induced liver injury. CYP450 is a superfamily of monooxygenase enzymes that mediate drug interactions during various pathological conditions[58]. It is presumed the metabolic activity of CYP450 would be altered by the effects of acute COVID-19 infection[57]. Liver injury in the context of COVID-19 infection complicates our understanding of how and to what extent CYP450 would be affected, and further work is needed in this area. Nevertheless, it is suggested that there would be clearance-associated pharmacokinetic interactions with antivirals and other drugs that are administered in this situation[57-61]. Common drugs affected by alterations in the CYP450 pathway could include Remdesivir, which is extensively metabolized by CYP450s, particularly CYP3A4, as well as Chloroquine and Colchicine which are both included in clinical trials researching COVID-19 treatment regimes[57-61].

Pathogenesis of HRS in COVID-19 induced liver injury

The multifactorial components of COVID-19 induced liver injury leads to the development of splanchnic vasodilation, with or without portal hypertension[62]. Splanchnic vasodilation is recognized as one of the major causative factors of HRS and occurs as the result of a plethora of vasodilatory responses[63]. With increased severities of hepatic damage, there is increased production of nitrous oxide in the splanchnic bed with reduced production in liver sinusoidal cells, which lead to increased portal gradient pressures[64]. Greater levels of other vasodilating peptides such as calcitonin gene-related peptide and adrenomedullin are also observed, as a result of increased production and reduced hepatic clearance[65].

Splanchnic vasodilation creates a state of hypovolemia in the central circulation, as splanchnic vasodilation combined with restricted portal blood flow causes blood to pool in the splanchnic circulation[66,67]. The body's physiological response to central hypovolemia will eventually lead to dysregulation of blood pressure, due to abnormalities in the baroreflex and cardiovascular responses to angiotensin II, norepinephrine, and vasopressin[68]. Central circulatory dysfunction can cause cardiomyopathy affecting both systolic and diastolic heart function. There will be electrophysiological alterations, which includes QT interval prolongation and electromechanical dyssynchrony[63,64]. The ability of the heart to respond to inotropic and chronotropic stimuli is reduced. Because of decreased systemic vascular resistance, cardiac output (in absolute terms) would be maintained at a high level initially[63,64]. However, the impaired cardiac function due to the aforementioned cardiac physiological changes will become clinically apparent with normalization of systemic vascular resistance and when there are further stress stimuluses[62-64].

Ultimately, renal perfusion pressure and blood flow to the kidneys will be reduced as a result of the various mechanisms which cause central circulatory dysfunction[68,69]. Overactivation of the sympathetic system as a homeostatic response could initially increase the kidney's reliance on blood pressure levels to maintain its perfusion[68]. Reduced blood flow to the kidneys would lead to more active stimulation of both β-adrenergic and subsequently α-adrenergic receptors, which results in afferent and efferent arteriole constriction[69]. The pathophysiological process of kidney damage is exacerbated by an inability of the liver in HRS to degrade renin, which will lead to persistent stimulation of the renin-angiotensin-aldosterone axis[70].

There are other theories of how HRS may manifest following severe liver injury from COVID-19 infection. One relates to the impact of reduced hepatic blood flow to kidneys *via* the hepatorenal reflex and exacerbated further by cytokine-induced vasoconstriction, which alters kidney hemodynamics[62,71]. Animal studies have highlighted that an acute increase in portal vein pressure results in increased renal nerve activity, although this phenomenon does not occur when the liver is denervated[71]. In studies assessing empirical treatment of HRS, a lumbar sympathetic block has been shown to improve kidney function [sodium excretion, blood flow and estimated glomerular filtration rate (eGFR) were shown to be improved][72]. This may explain why medical procedures such as transjugular intrahepatic portosystemic shunt (TIPS) improve HRS in many patients *via* reduction of portal pressure gradients. Another theory explaining the development of HRS in this context may relate to the direct effects of intra-abdominal ascitic pressure, in patients with underlying decompensated CLD where the ascites

may be exacerbated following COVID-19 infection[73]. Increased intra-abdominal ascitic pressure can lead to venous congestion and stimulation of the renin-angiotensin-aldosterone system, resulting in further kidney function decline and histopathological changes[73].

CLINICAL ASSESSMENT AND MANAGEMENT STRATEGIES IN COVID-19 INDUCED HRS

Key components of clinical assessment and management in COVID-19 induced HRS are summarized in Table 1. Clinical assessment of patients presenting with COVID-19 induced HRS should encompass a holistic understanding of an individual's medical history. This should be followed by physical examination to elicit specific signs, before urine, serum and imaging investigations are conducted, with these investigations forming the crux of the diagnostic criteria. Management strategies in this scenario should focus on achieving spontaneous recovery of liver function and resolution of HRS *via* medical management and only if this fails, then to consider the potential for liver transplantation.

Clinical assessment of COVID-19 induced HRS

Our current understanding of the typical signs and symptoms which appear with COVID-19 induced liver injury and HRS remains premature, and there are likely non-specific presentations in most instances. It is reasonable to suggest patients who develop acute liver injury following a positive COVID-19 diagnosis would likely have a COVID-19 infection severe enough to present as such[74]. From a thoroughly taken medical history, clinicians should aim to determine the likely course of COVID-19 infection and rule out other differentials more likely to explain the development of acute liver injury/fulminant liver failure[57]. There should be close observation for systemic (*i.e.*, septic symptoms, monitor hemodynamic stability as likely to have low mean arterial pressure) as well as respiratory-specific signs and symptoms[57]. It has been reported from prospective studies conducted in China that risks of severe liver injury is greater in patients who develop gastrointestinal symptoms such as diarrhea, nausea and vomiting, anorexia and abdominal pain (OR 2.71, 95%CI 1.52-4.83, $P < 0.05$) following acute COVID-19 infection[75]. Given patients with underlying CLD such as cirrhosis are more likely to develop HRS regardless of COVID-19 status, classical features of CLD/decompensated liver disease including jaundice, altered mental status, malnutrition, and ascites (ascites resistant to the use of diuretic medications is characteristic of type 2 HRS) should be meticulously monitored[76]. Whilst there is relative clarity regarding hepatic signs and symptoms in HRS, the same cannot be said for renal-specific signs and symptoms. Both oliguria and normal levels of urine output have been observed for patients diagnosed with HRS[62]. Due to inability in establishing definitive renal symptoms in HRS, there is wide opinion that HRS should be diagnosed mainly on the basis of laboratory results rather than symptomatic presentation.

The utilization of diagnostic tests should initially confirm COVID-19 status *via* a real-time reverse-transcriptase-polymerase chain reaction (rRT-PCR) test and determine the severity of disease through chest imaging (*i.e.*, chest X-ray or computed tomography)[77]. Serum tests evaluating the systemic inflammatory state (*i.e.*, full blood cell count, C-reactive protein, interleukin-6 Levels) should follow alongside tests to identify the presence of liver pathology, typically indicated by the rise in serum ALT, AST, total bilirubin, gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP) levels and reduction in serum albumin[78]. Recent observational data reported the pooled prevalence of the elevated liver enzymes ALT, AST, and total bilirubin in COVID-19 positive patients to be 18% (95%CI 13%-25%), 21% (95%CI 14%-29%), and 6%(95%CI 3%-11%), respectively[79]. These serum tests may prove to be a strong marker of poor prognosis, as a fatal outcome with COVID-19 induced liver disease is estimated to be between 58% and 78%[80]. The presence of hypoalbuminemia also signifies a more severe disease process with poorer prognosis[81,82]. Liver ultrasound could also be a useful front-line diagnostic imaging tool to detect the presence of any acutely developed liver lesions.

Investigations to ascertain kidney function in confirming a HRS diagnosis should encompass urinalysis, a serum urea & electrolytes screen and other renal panel testing to rule out differential diagnoses[83-86]. Important results to look out for in urinalysis is concentrated urine with low urine sodium (< 10 mmol/L) where there is usually no proteinuria or hematuria[85,86]. There would be absence or few granular (hyaline or muddy-brown) casts identified in urine microscopy, in contrast to acute tubular necrosis (ATN) which is a known renal complication of cirrhosis[84]. ATN in the context of liver injury most likely occurs as a result of exposure to toxic medications or the development of decreased blood pressure, and proximal tubular cells are unable to reabsorb sodium from urine[62,84,85]. Because of this, urinary sodium levels in ATN would be expected to be higher than that of HRS[84]. It is expected that there is marked reduction in eGFR with HRS, with no improvements in kidney function despite treatment with intravenous fluids (kidney function improvements are observed in most other causes of pre-renal kidney failure following intravenous fluid administration with reduction in serum creatinine and increased sodium excretion) due to the intra-renal vasoconstricted state[87]. Serum sodium concentration would be low due to retention of fluid together with sodium leading to dilutional hyponaetremia[88]. Plasma renin activity would be elevated considering the metabolic changes in HRS [89]. Kidney ultrasound would rule out obstruction of the kidney outflow tract.

Table 1 Key components of clinical assessment and management in COVID-19 induced hepatorenal syndrome

Clinical Assessment of HRS	Management of HRS
Medical history: (1) Identify likely course of disease progression; and (2) rule out other causes of acute liver injury/fulminant liver failure	Minimize potential drug-induced hepato- and nephron-toxicities: (1) Monitor response to immunosuppressive treatments; (2) monitor response to antivirals and other COVID-19 treatment regimes; and (3) aim to prescribe these medications through a dose-dependent approach
Clinical examination: (1) Identify signs of systemic and/or respiratory decompensation; (2) identify evidence of cirrhosis/decompensated liver disease; and (3) monitor for oliguria	Medical management strategies in COVID-19 induced HRS: (1) Extracorporeal membrane support therapy & dialysis; (2) potential utilization of MARS or other liver support devices; (3) TIPSS to reduce portal vein pressure (if renal function allows and known CLD); (4) adding intravenous albumin to other procedural/medical therapies to expand plasma volume; and (5) combined use of Midodrine (α-agonist) and Octreotide (somatostatin analogue) to regulate blood vessel tone in the gastrointestinal tract and act as systemic vasoconstrictors to inhibit splanchnic vasodilation. Terlipressin may be used as an alternative
Laboratory and imaging tests: (1) Confirm positive COVID-19 status; (2) assess systemic hemodynamic stability through basic observations; (3) chest imaging to assess degree of COVID-19 severity for the respiratory system; (4) serum tests to evaluate the degree of inflammation; (5) liver pathology could be evaluated *via* serum markers (*e.g.*, increased ALT, AST, total bilirubin, GGT and ALP, reduction in albumin) and liver ultrasound; (6) urinalysis to identify low urine sodium *i.e.*, < 10 mmol/L, proteinuria, hematuria and urinary casts seen in ATN; (7) serum eGFR reductions, low serum sodium (dilutional hyponatremia) and elevated plasma renin would be classically observed in HRS; and (8) kidney ultrasound should be performed to rule out obstruction of the kidney outflow tract	Consider liver transplantation if kidney function and hepatic recovery is unlikely with medical management

AKI: Acute kidney injury; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; ATN: Acute tubular necrosis; COVID-19: Coronavirus disease 2019; eGFR: Estimated glomerular filtration rate; GGT: Gamma-glutamyl transferase; HRS: Hepatorenal syndrome; MARS: Molecular adsorbents recirculation systems; TIPS: Transjugular intrahepatic portosystemic shunt.

Management strategies in COVID-19 induced HRS

The mainstay of treatment in HRS from either acute liver failure or cirrhosis, whether the cause is COVID-19 related or not, is usually medical management to aim for gradual liver recovery and resolution of HRS, with supportive dialysis or haemofiltration if required. Liver transplantation would not be indicated if recovery of liver function and resolution of HRS is achieved solely through medical management, with this usually resulting in the best outcome for patients. A key priority is to limit drug-induced hepato- and nephrotoxicity through a dose-dependent adjustment approach of managing immunosuppressive medications, antivirals and other COVID-19 treatment regimes if and when COVID-19 treatment is indicated[57].

The combination of extracorporeal membrane support and dialysis in HRS demonstrated significant effects in removing toxins from the circulation, including systemic inflammatory molecules generated from COVID-19 infection[83,90-92]. There has been greater use of molecular adsorbents recirculation systems in HRS as both a supportive treatment option or as a bridging therapy to liver transplantation if indicated, though wider work is needed to improve accessibility with this technology still being relatively novel[93]. Close haemodynamic monitoring during hemodialysis is recommended, given concerns that this may further deteriorate blood pressure stability in HRS, increasing the risk of mortality[94]. TIPS involves decompressing the high pressures in the portal circulation by placing a small stent between a portal and hepatic vein, *via* placement of a radiologically-guided catheter passed into the hepatic vein either through the internal jugular vein or the femoral vein[95]. This will theoretically reduce portal vein pressure, which as discussed in the previous section is a key factor in the hemodynamic process leading up to HRS[17,18,62,95]. Previous studies in patients with cirrhosis largely noted improvements in kidney function when TIPS is performed, particularly as a bridging treatment if liver transplantation might be indicated[96].

Intravenous albumin can expand plasma volume, and provide other benefits in the form of its immunological, antioxidant, endothelial protective functions. Combining intravenous albumin with other medical and/or procedural treatments displayed better outcomes compared to administering intravenous albumin alone[97]. Other pharmacological options which have demonstrated efficacy across all forms of HRS may include the combined use of Midodrine, an α agonist with somatostatin analogues such as Octreotide[98,99]. In Europe, Terlispressin and albumin are recommended in the best practice guidelines[100]. These drugs regulate blood vessel tone in the gastrointestinal tract, and also systemic vasoconstrictors which inhibit splanchnic vasodilation[97]. Interestingly, these drugs were only found to be effective when used in combination and not when independently prescribed[100]. There is preliminary data that other vasopressin analogues (*e.g.*, Ornipressin), Pentoxifylline, Acetylcysteine and Misoprostol amongst other treatments are potentially useful treatments in HRS, but this will require

further study[101-103].

Liver transplantation would be the ideal treatment in HRS, if renal function cannot be corrected with medical management and hepatic recovery is unlikely with conservative management alone. These situations mostly occur in patients with CLD, where the medical management options aforementioned serve as bridging therapies towards transplantation[17,18,20,62]. The optimal strategy would usually observe effects from treatment of the underlying cause of HRS first before planning for liver transplantation. During the COVID-19 pandemic, most hepatology societies advised the deferral of liver transplantation in stabilized patients[104]. There has been continuous debate throughout the pandemic on how to optimize the procedures of liver transplantation for patients with COVID-19 positive status, such as those with active COVID-19 infection inducing HRS[105]. Currently, there is only one reported case of liver transplantation in HRS with COVID-19 infection, performed 28 d after hospital admission [23]. Individuals with HRS who receive liver transplantation almost universally achieve recovery in kidney function[91]. Previous studies have demonstrated that survival rates at 3-year follow-up for liver transplant recipients in HRS are comparable to liver transplant recipients for other causes of liver disease[106]. Although differences in long-term outcomes are significant between patients who receive liver transplantation and those who do not, acute mortality rates after liver transplantation were found to be up to 25% in the first month[107]. Patients who present with further decline in liver and kidney function following liver transplantation are at higher risk[17]. Kidney function decline following liver transplantation in HRS is usually transient and most likely attributed to drug-induced nephrotoxicity, specifically the introduction of immunosuppressants such as Tacrolimus and Cyclosporine which are known to affect kidney function[17,105].

CONCLUSION

There is increased attention towards the extra-respiratory manifestations of COVID-19 as the pandemic continues to affect billions of lives. Hepatic consequences of COVID-19 infection are now recognized as an important complication of COVID-19. The development of HRS following COVID-19 induced liver injury suggests severe and perhaps life-threatening disease, particularly for individuals with multi-morbidities including pre-existing CLD. The prognosis of HRS is largely dependent on whether liver transplantation would be viable and accessible for the patient. Confounding effects of drug-induced hepato- and nephrotoxicity in exacerbating the systemic damage from COVID-19 induced HRS should always be considered and avoided if possible. A greater understanding of the multi-faceted pathophysiological mechanisms which result in HRS following acute COVID-19 infection is important to guide clinical decisions in a timely manner for the optimization of patient outcomes.

FOOTNOTES

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REVIEW

Receptor of advanced glycation end-products axis and gallbladder cancer: A forgotten connection that we should reconsider

Armando Rojas, Cristian Lindner, Iván Schneider, Ileana Gonzàlez, Miguel Angel Morales

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Abstract

Compelling evidence derived from clinical and experimental research has demonstrated the crucial contribution of chronic inflammation in the development of neoplasms, including gallbladder cancer. In this regard, data derived from clinical and experimental studies have demonstrated that the receptor of advanced glycation end-products (RAGE)/AGEs axis plays an important role in the onset of a crucial and long-lasting inflammatory milieu, thus supporting tumor growth and development. AGEs are formed in biological systems or foods, and food-derived AGEs, also known as dietary AGEs are known to contribute to the systemic pool of AGEs. Once they bind to RAGE, the activation of multiple and crucial signaling pathways are triggered, thus favoring the secretion of several proinflammatory cytokines also involved in the promotion of gallbladder cancer invasion and migration. In the present review, we aimed to highlight the relevance of the association between high dietary AGEs intakes and high risk for gallbladder cancer, and emerging data supporting that dietary intervention to reduce gallbladder cancer risk is a very attractive approach that deserves much more research efforts.

Key Words: Gallbladder cancer; Advanced glycation end-products; Receptor of advanced glycation end-products; Chronic inflammation; Nutrition

Core tip: A growing body of data has demonstrated a positive association between the risk of gallbladder cancer and high dietary intake of advanced glycation end-products (AGEs). These noxious compounds are important contributors to the onset of a chronic inflammatory response, through the activation of the receptor of AGEs (RAGE). We herein discuss how RAGE activation is crucial in the development of gallbladder cancer and the relevance of new incoming data supporting the role of dietary interventions to reduce the risk of gallbladder cancer.

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INTRODUCTION

Gallbladder cancer development is linked to both genetic and environmental factors, and where the onset of chronic inflammation is a crucial contributor to gallbladder carcinogenesis. This chronic inflammatory condition can be triggered by several factors including not only chronic infection by *Salmonella* spp., or *Helicobacter pylori*[1-4] but also some dietary habits or metabolic conditions[5-9], which are associated with an overactivation of the receptor of advanced glycation end-products (RAGE).

At present, the onset of many of both age- and diet-related noncommunicable diseases, including different cancer types, is widely associated with the chronicity of low-grade inflammation[10,11]. At this point, the diet is widely recognized as an important modulator of this chronic and systemic inflammation[12,13], particularly the western-type dietary patterns[14].

One common and important element in this unhealthy diet is the advanced glycation end-products (AGEs), which are a large and heterogeneous group of compounds that were initially recognized in the Maillard reaction, but they can also form by other reactions, including the oxidation of sugars, lipids, and amino acids[15,16].

Food-derived AGEs, also known as dietary AGEs, substantially contribute to the systemic pool of AGEs. Their intake has been linked in humans and mice to an increased level of oxidative stress and inflammation, thus playing an important role in the onset and development of several health disorders [17,18].

The pathogenic mechanisms of dietary AGEs are the same as those endogenously produced, either by activation of the RAGE or by covalent crosslinking of proteins, thus altering protein structure and function. The receptor-dependent and receptor-independent mechanisms are recognized as important contributors to tumor growth and development[19,20].

In the present review, we aim to highlight the burden of RAGE axis activation on gallbladder cancer, its therapeutic potential, as well as the significance of lowering dietary consumption of AGEs in subjects at risk.

THE RAGE/AGEs AXIS AND GALLBLADDER CANCER: NEW INCOMING PIECES OF EVIDENCE

There is growing evidence supporting the key role of dietary AGEs as major contributors to the systemic pool of AGEs[21], which notably increase oxidative stress and chronic/acute inflammation, contributing to the pathophysiology of many human inflammatory and malignant diseases[18,22,23].

Since the multicenter prospective European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study, which investigated the relationship of dietary and environmental factors with the incidence of cancer and other chronic diseases[24-27], a growing body of evidence has revealed strong findings to support that a proinflammatory diet with high levels of dietary AGEs intake increases the risk of several types of cancer[28], such as breast, skin and those originating from the digestive tract[29-31].

Recently, Mayén *et al*[32] conducted a multinational cohort study using the EPIC database to characterize the daily dietary intake (mg/d) of three AGEs including Nε-[carboxymethyl] lysine (CML), Nε-[1-carboxyethyl] lysine (CEL), and Nδ-[5-hydro-5-methyl-4-imidazolon-2-yl]-ornithine (MG-H1) for each study participant, to assess AGE consumption with hepatobiliary cancer risk. In this study, the authors found a positive association between the risk of gallbladder cancer and high dietary intake of CML [hazard ratio (HR) = 1.30, 95% confidence interval (CI): 1.07–1.57] and MG-H1 (HR = 1.26, 95%CI: 1.06–1.50), and thus suggesting that higher intakes of dietary AGEs may increase the risk of gallbladder

cancer.

Although the study of Mayen *et al*[32] has some limitations, particularly in estimating dietary AGEs exposure, other epidemiological studies have revealed an increased tumor progression and mortality of gallbladder cancer patients, with inflammatory comorbidities related to overactivation of the RAGE axis, such as high-fat diet consumption[33], metabolic syndrome[34], and diabetes mellitus [35-38], due to the increased endogenous formation of AGEs reported in these entities.

Some studies have shown increased expression of RAGE in gallbladder cancer cells, which were directly in concordance with the invasive ability of the neoplastic cell lines[39]. Additionally, compelling evidence has been reported of a strong increase in AGEs formation under hyperglycemic conditions[40, 41]. Noteworthy, the gallbladder accumulation of AGEs is significantly higher in the gallbladder of diabetic mice when compared to control animals. These findings support the role of the RAGE/AGEs axis activation in gallbladder carcinogenesis[42].

Furthermore, other *in vivo* analyses of adenocarcinoma cells treated under a hyperglycemic milieu, a condition favoring the increased accumulation of AGEs, have been revealed to promote tumor cell proliferation and migration[43].

Emerging *in vitro* and *in vivo* analyses have revealed overexpression of several RAGE ligands such as high mobility group B1 (HMGB1) and members of the S100P protein family in malignant gallbladder epithelial cells compared to benign tissue[44,45].

This increased expression of those RAGE ligands in gallbladder cancer cells has been closely correlated with malignant progression and therefore may then be considered an independent risk factor for poor prognosis and proliferation in gallbladder cancer[45-47].

A key consequence of RAGE binding with its ligands is the activation of multiple and crucial signaling pathways[48], that are involved in gallbladder carcinogenesis, such as reactive oxygen species (ROS)[49], Erk1/2 (p44/42) mitogen-activated protein kinases (MAPKs)[50], C-Jun n-terminal kinase and p38 MAPK[51], and phosphatidylinositol 3-kinase pathways[52].

These signals trigger important downstream inflammatory and procarcinogenic consequences such as activation of signal transducer and activator of transcription 3[53,54], activator protein-1[55], and nuclear factor (NF)-κB pathways[49,54,56-58], favoring the secretion of several proinflammatory cytokines also involved in the promotion of gallbladder cancer invasion and migration such as tumor necrosis factor-α[59,60]. Hence, this proinflammatory milieu continuously fuels chronic inflammation in gallbladder carcinogenesis in a RAGE-dependent manner[19,61].

THERAPEUTIC POTENTIAL OF THE RAGE/AGEs AXIS IN TUMOR BIOLOGY

RAGE is recognized as a pattern recognition receptor, and its activation plays a pivotal role in the propagation of immune responses and inflammatory reactions[62]. It is expressed at low levels in most differentiated adult cells in a regulated manner. However, upregulation of RAGE expression is associated with many inflammation-related pathological entities, including cancer[63].

RAGE engagement subsequently converts transient cellular stimulation into a sustained cellular dysfunctional state driven by long-term activation of NF-κB[64]. There is compelling evidence that RAGE activation promotes many crucial steps during tumorigenesis, from DNA damage and genetic instability to supporting many phenotypic changes in tumor cells favoring their growth and dissemination[65].

Since the work by Taguchi *et al*[66], which experimentally reported that *in vivo* blockade of the RAGE–amphotericin axis suppresses tumor growth and dissemination, intense research efforts have been focused towards the development of new therapeutic approaches to modulate both deleterious proinflammatory and procarcinogenesis effects of RAGE axis activation[67,68].

The use of novel RAGE-targeting antibodies and blocking peptides derived from RAGE ligands such as S100P and HMGB1 has demonstrated to block the ability of ligands to stimulate RAGE activation in cancer cells both *in vitro* and *in vivo* models, thus inhibiting tumor growth, metastasis, and inflammation [69], as well as significant reductions in tumor growth with acceptable toxicity levels in several *in vivo* mouse adenocarcinoma models[70,71]. Furthermore, the treatment of cancer cell lines with anti-RAGE antibodies demonstrates that RAGE blocking may even enhance the chemotherapeutic effects of antineoplastic drugs[72,73].

Recent evidence has also revealed that the antibody targeting of RAGE ligands such as HMGB1 and AGEs may effectively decrease tumor progression in solid malignancies[74]. This approach can even enhance the antitumoral response of cancer immunotherapies by remobilizing the antitumor immune response[75].

Another emerging therapeutic approach is based on the high binding affinity to RAGE of some members of the family of glycosaminoglycans such as chondroitin sulfate, heparan sulfate (HS), and low molecular weight and semisynthetic glycosaminoglycan[76]. These molecules have been reported to be involved in effectively inhibiting RAGE signaling pathways in *both in vitro* and *in vivo* models[71,77].

Strikingly, new evidence has revealed that HS acts as a crucial element for RAGE signaling, leading to the formation of stable RAGE–HS complexes, which drive the RAGE oligomerization and subsequent

downstream functional signaling[78,79].

These observations have revealed a new strategy for treating RAGE-associated diseases by hindering RAGE oligomerization.

The use of synthetic compounds with both anticarcinogenic and anti-inflammatory activities based on their capacities to interfere with the HMGB1–RAGE axis seems to be a promising strategy for several cancer types, including gallbladder cancer[80,81].

A novel molecule, recently discovered by Tanuma *et al*[82], 7-methoxy-3-hydroxy-styrylchromone (c6), is not only an effective suppressor of cell cycle/proliferation but also an initiator of apoptosis in cancer cells, and a promising potentiator of the anticancer effects of DNA-damaging antineoplastic agents.

RAGE gene silencing has been demonstrated to significantly downregulate AGE-induced inflammation and RAGE-dependent release of proinflammatory cytokines in normal human cells[83], while in malignant cells, *RAGE* gene silencing can decrease the colony-forming ability, proliferation, migration, and the invasive potential of cancer cells, through inhibiting RAGE-dependent mechanisms that sustain cancer cell progression and invasion[84].

The requirement of the cytoplasmic tail of RAGE to interact with its molecular effector DIAPH1 to mediate downstream signal transduction has been highlighted as a promising approach to inhibit RAGE signaling[85-87].

This novel screening strategy of searching for molecules able to block protein–protein interactions has been demonstrated to be successful to inhibit the RAGE-mediated expression of inflammatory genes in diabetes complications[88,89] and atherosclerosis[90].

A growing body of experimental data using the DNA-aptamer technology against RAGE has demonstrated that this novel approach can inhibit the inflammatory reactions triggered by activation of the RAGE axis in different *in vivo* models[91-93].

Experimental research has reported interesting results in different cancer types, as revealed in tumor-bearing mice treated with RAGE-aptamers, where marked inhibition of tumor growth was achieved [94]. The use of this technology on tumor-bearing mice is also able to inhibit macrophage infiltration and neoangiogenesis through the inhibition of RAGE/NF-κB/VEGF-A-dependent signaling pathways [94-96] (Figure 1).

In many clinical entities where the activation of the RAGE/AGEs axis is crucial in the underlying pathogenic mechanisms, restriction of dietary AGEs has been extensively studied in clinical trials[97-101]. Under the same rationale, and based on the active role of RAGE-mediated mechanisms in tumor biology, different interventional clinical studies already published[102-107] (Table 1), or in progress, have supported the use of restriction of AGEs intake in human cancers, as documented on the website ClinicalTrials.gov (ClinicalTrials.gov identifier: NCT03712371, NCT04716764, NCT02946996, NCT03092635, NCT01820299, NCT01363141, NCT03147339). However, it must be emphasized that therapeutic interventions, including dietary interventional actions on the RAGE axis, have been focused on achieving clinical improvements in disease course, including dietary interventional actions, and therefore the potential of modulating RAGE activation in terms of cancer prevention is still controversial.

REDUCING DIETARY AGEs INTAKE IN SUBJECTS AT RISK OF GALLBLADDER CANCER. A HOPEFUL APPROACH?

International consensus estimates that almost 40% of cancer cases are preventable through a healthy lifestyle[108]. Compelling evidence derived from epidemiological studies of different cancer types suggests that lifestyle changes, including dietary habits, may play a crucial role in determining the risk of various cancers[109-113].

Currently, the western diet is considered a major driver of chronic, low-grade, metabolic inflammation, which is a crosswise element in the pathogenesis of many human diseases, including cancer [114]. Data derived from preclinical investigations, and observational and interventional studies, has provided conclusive evidence that the western diet is associated with an increased incidence of many malignancies, such as colorectal, pancreatic, prostate and breast cancers[115-118].

In modern society, dietary AGE consumption – as a component of modern westernized diets – is markedly increased. Therefore, dietary AGE restriction is now recognized as a useful intervention, as demonstrated in several pathologies[119-123].

Western diet generally contains large amounts of fructose, thus promoting AGE formation[124]. This diet is also an important source of AGE precursors, such as methylglyoxal and glyoxal[125]. In light of these findings, dietary AGEs have gained particular importance due to their capacity to support the onset of many human diseases, including cancer, mainly due to their proinflammatory and pro-oxidant properties[17,18].

The role of RAGE/AGEs axis activation has emerged as a crucial element in the tumor microenvironment to promote cancer cell migration, invasion, survival, and even resistance to chemotherapy[19]. Additionally, the accumulation of AGEs in tissues can promote protein structural damage and

Table 1 Some clinical trials supporting the usefulness of restriction of advanced glycation end-products intake in human cancers

Ref.	Year	Condition	Outcome
Jiao *et al*[102]	2015	Pancreatic cancer	Increased risk of pancreatic cancer
Peterson *et al*[103]	2020	Breast cancer	Increased breast cancer risk in postmenopausal women
Omofuma *et al*[104]	2020	Breast cancer	Increased risk of breast cancer
Aglago *et al*[105]	2021	Colorectal cancer	Increased risk of CRC
Mao *et al*[106]	2021	Colorectal cancer	Increased CRC mortality in non-T2D patients
Omofuma *et al*[107]	2021	Breast cancer	Increased breast cancer mortality
Mayén *et al*[32]	2021	Hepatobiliary cancers	Increased risk of gallbladder cancer

CRC: Colorectal cancer; T2D: Type 2 diabetes.

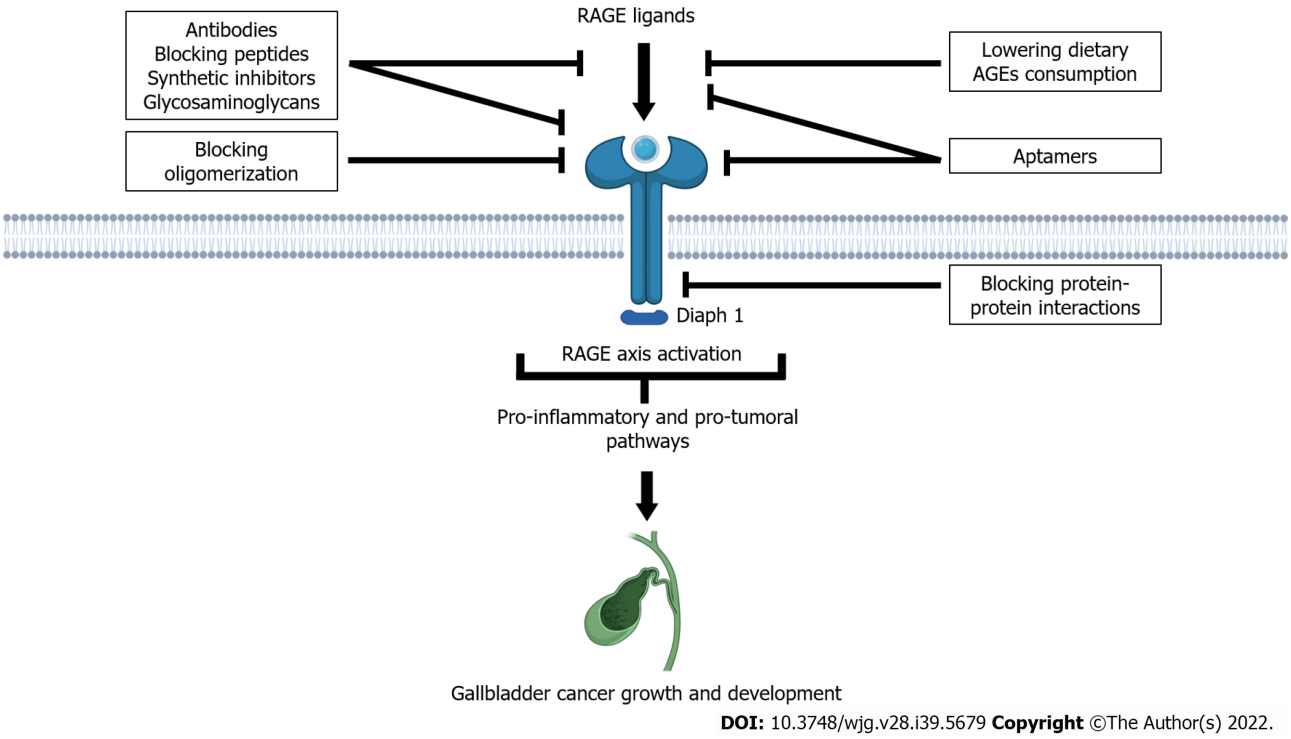

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Figure 1 Different therapeutic approaches used to inhibit the consequences of the receptor of advanced glycation end-products axis activation in cancer. RAGE: Receptor of advanced glycation end-products; AGEs: Advanced glycation end-products.

modification of the mechanical and physiological functions of the extracellular matrix, thus contributing to carcinogenesis and inflammation[20].

Therefore, the report recently published by Mayén *et al*[32] showed a positive association between dietary AGEs and the risk of gallbladder cancer in the EPIC cohort, which deserves special attention. We believe that actions such as dietary recommendations for the reduction of dietary AGEs intake to individuals at risk of gallbladder cancer will be beneficial. In this regard, it is important to highlight that some pre-existing clinical conditions such as diabetes mellitus and metabolic syndrome are risk factors for the development of gallbladder cancer[34,35-38]. Additionally, the demonstrated links between genetic ancestry and gallbladder cancer development may represent another risk factor for some populations[126,127]. Other recommendations that focus on reducing the RAGE/AGEs axis activation are attractive, particularly the consumption of polyphenol-rich foods due to the inhibitory activities of polyphenols on the RAGE/AGEs axis at different levels, such as by inhibition of ROS formation during glycation reactions, chelation of transition metal ions, trapping dicarbonyls, and activation of AGE detoxification pathways[128].

CONCLUSION

Gallbladder cancer is an aggressive and rare neoplasm with an unusual geographic distribution. Most patients are diagnosed in the advanced stages of the disease, and therefore the life expectancy is low. Compelling evidence supports the role of several risk factors, which are linked to the onset, and chronicity of an inflammatory reaction. The report of Taguchi *et al*[66] represented a critical point in understanding the role of the RAGE axis in tumor biology, and highlighting the potential of therapeutic interventions on a hyperactive cellular signaling pathway that causes disease, as the RAGE axis is[129].

The role of RAGE axis activation in gallbladder cancer is supported by its active contribution to the pathogenic framework of the main risk factors associated with this neoplasm, such as infectious agents [130,131], some metabolic conditions[132,133], and dietary habits[32].

Although much research is needed, lowering dietary AGEs intake as well as increasing the consumption of foods rich in polyphenols in subjects at risk of gallbladder cancer, either by pre-existing metabolic conditions or genetic ancestry, seems to be a plausible recommendation, to avoid the hyperactivation of the RAGE/AGEs axis.

FOOTNOTES

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REVIEW

Interplay between metabolic dysfunction-associated fatty liver disease and chronic kidney disease: Epidemiology, pathophysiologic mechanisms, and treatment considerations

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Abstract

The recently proposed nomenclature change from non-alcoholic fatty liver disease to metabolic dysfunction-associated fatty liver disease (MAFLD) has resulted in the reappraisal of epidemiological trends and associations with other chronic diseases. In this context, MAFLD appears to be tightly linked to incident chronic kidney disease (CKD). This association may be attributed to multiple shared risk factors including type 2 diabetes mellitus, arterial hypertension, obesity, dyslipidemia, and insulin resistance. Moreover, similarities in their molecular pathophysiologic mechanisms can be detected, since inflammation, oxidative stress, fibrosis, and gut dysbiosis are highly prevalent in these pathologic states. At the same time, lines of evidence suggest a genetic predisposition to MAFLD due to gene polymorphisms, such as the *PNPLA3* rs738409 G allele polymorphism, which may also propagate renal dysfunction. Concerning their management, available treatment considerations for obesity (bariatric surgery) and novel antidiabetic agents (glucagon-like peptide 1 receptor agonists, sodium-glucose co-transporter 2 inhibitors) appear beneficial in preclinical and clinical studies of MAFLD and CKD modeling. Moreover, alternative approaches such as melatonin supplementation, farnesoid X receptor agonists, and gut microbiota modulation may represent attractive options in the future. With a look to the future, additional adequately sized studies are required, focusing on preventing renal complications in patients with MAFLD and the appropriate management of individuals with concomitant MAFLD and CKD.

Key Words: Metabolic dysfunction-associated fatty liver disease; Chronic kidney disease; Hepatic steatosis; inflammation; Type 2 diabetes mellitus; Obesity

Core Tip: Metabolic dysfunction-associated fatty liver disease (MAFLD) is a recently defined pathological state aiming to identify individuals at increased risk of adverse prognosis. Numerous epidemiological studies propose that chronic kidney disease may be among its complications. Their shared risk factors, molecular mechanisms, and genetic predisposition represent the basis for this relationship. Accordingly, treatment approaches with combined efficacy in MAFLD and chronic renal impairment are expected to positively impact the natural history of this deleterious interaction, which remains to be confirmed in future studies.

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INTRODUCTION

Metabolic abnormalities, namely obesity and type 2 diabetes mellitus (T2DM) constitute contemporary pandemics with a high prevalence and rising incidence[1,2]. Although cardiovascular diseases remain the most prominent complication of metabolic derangement, hepatic insult is frequent, as documented in recently reported epidemiologic trends of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH)[3,4]. However, the existing NAFLD definition required the exclusion of other forms of liver disease instead of providing positive criteria for the diagnosis of metabolic dysfunction-associated fatty liver disease (MAFLD). A recent expert consensus tried to resolve this gap in evidence by providing a simple and comprehensive MAFLD definition and diagnostic criteria[5]. These included the presence of steatosis along with a main metabolic abnormality (overweight/obesity or T2DM) or at least two metabolic risk factors.

The establishment of MAFLD as an entity may promote the need for intense research in this field to define its epidemiology better, identify predisposing and prognostic factors, and evaluate effective therapeutic approaches. Moreover, investigating the association between MAFLD and other pathological states, primarily cardiac and renal diseases, will improve our understanding of this complex entity. Even though the link between MAFLD and cardiovascular disease has been the most extensively studied[6], ample evidence suggests the relationship between MAFLD and chronic kidney disease (CKD).

In this narrative review, we elaborate on this interaction by assessing its epidemiological features, the involved pathophysiologic pathways, and the potential therapeutic interventions.

MAFLD AND CKD; EPIDEMIOLOGICAL TRENDS

Due to the recent change in terminology and diagnostic criteria, we are now beginning to reevaluate the epidemiological characteristics of MAFLD. In a recently reported study that followed a meta-analytic approach, the prevalence of MAFLD in overweight or obese subjects was 50.7%[7]. The authors pointed to potential geographic variations in MAFLD prevalence, with South American populations exhibiting the highest prevalence rates (approximately 71%). Moreover, they detected a significantly higher prevalence in male subjects and in obese compared to overweight. No differences according to age or income were reported. Finally, T2DM and metabolic syndrome prevalence was 19.7% and 57.5%, respectively. A similar prevalence (47%) was detected in a cross-sectional study of the Mexican population, with male sex, older age, and increasing body mass index (BMI) being predictive factors[8]. Other than high prevalence rates, there is an association between MAFLD and all-cause mortality, which extends to cancer- and cardiovascular disease-related mortality[9]. Moreover, a higher risk of atherosclerotic disease, heart failure, obstructive sleep apnea, and malignancy has been reported[9].

According to the available evidence, MAFLD is tied to a higher incidence of CKD. To begin with, in an analysis of approximately 270000 individuals that underwent National Health Insurance Service health examinations, MAFLD was associated with an increased risk of incident CKD compared to non-metabolic NAFLD (adjusted hazard ratio 1.18, 95% confidence interval [CI]: 1.01-1.39; P = 0.04)[10]. In the study by Tanaka *et al*[11] in a sizeable Japanese population followed up for 10 years, MAFLD was a determinant of incident CKD irrespective of age, sex, smoking, coronary artery disease, estimated glomerular filtration rate (eGFR), and metabolic risk factors (diabetes mellitus [DM], hypertension, hyperlipidemia, obesity). Notably, such observations were not made for the presence of NAFLD or only fatty liver[11]. In a Chinese cohort of 6873 participants with a 4.6-year follow-up, the investigators noted a higher risk of CKD in MAFLD subjects (risk ratio 1.64, 95%CI: 1.39-1.94)[12]. Last but not least, the

authors of a recently published systematic review and meta-analysis found a potent association between a MAFLD diagnosis and new onset of CKD (hazard ratio 1.53, 95%CI: 1.38-1.68)[9]. Contradictory to the findings mentioned above, in an analysis of the National Health and Nutrition Examination Surveys of the United States 2017-2018, the relationship between MAFLD and CKD was not statistically significant after the propensity score matching[13]. Scientific interest is intense in this field due to the recently proposed change in the nomenclature of NAFLD into MAFLD. Future studies are eagerly awaited to assess the association between MAFLD and CKD and the prognosis of individuals with concomitant CKD and MAFLD.

Concerning the interplay between MAFLD and CKD, the use of transient elastography is of great importance. Ciardullo *et al*[14], in their meta-analysis of seven cross-sectional studies, detected an association of non-invasively assessed liver fibrosis with increased urinary albumin-to-creatinine ratio (UACR) (odds ratio [OR] 1.98, 95%CI: 1.29-3.05; $P = 0.002$) and incident CKD (OR 2.49, 95%CI: 1.89-3.29; $P < 0.001$). The study by Freitas *et al*[15] further stressed the role of transient elastography. Liver fibrosis, assessed by the liver stiffness measurements (LSM), was associated with early kidney dysfunction, characterized by the development of microalbuminuria (UACR 30-300 mg/g) or a drop in eGFR to < 60 mL/min/1.73 m^2 in MAFLD individuals[15]. LSM values of over 6.1 kPa were predictive of the endpoint, with a sensitivity and specificity of 85.7% and 67.6%, respectively[15]. It appears that Fibroscan-derived controlled attenuated parameter (CAP) may be a more crucial predictor of prevalent CKD in subjects with MAFLD than LSM. Specifically, CAP values of 353 dB/m were associated with CKD, even after multivariable adjustment (OR 1.07, 95%CI: 1.00-1.20; $P = 0.01$)[16].

PATHOPHYSIOLOGIC PATHWAYS LINKING MAFLD WITH CKD

According to those recently published reports, it is evident that MAFLD is a growing pandemic due to the constantly rising prevalence of its underlying risk factors. Moreover, the association between MAFLD and incident CKD is remarkable but unsurprising, due to the common pathophysiologic mechanisms surrounding those entities (Figure 1). To begin with, the main risk factors for CKD development, T2DM and arterial hypertension[17-19], are among the established diagnostic criteria for MAFLD. The same could be argued for obesity, prediabetes, dyslipidemia, and insulin resistance since studies have proposed an independent association between the risk factors mentioned above and incident CKD[17,19-21].

Inflammatory hypothesis in MAFLD and CKD

Regarding the involved molecular mechanisms, we should stress the role of inflammation. We know that inflammation is among the cardinal features of MAFLD, with elevations of high-sensitivity C reactive protein (hsCRP) being among the criteria of metabolic dysregulation. hsCRP elevation was correlated with the extent of liver steatosis and fibrosis in 393 obese individuals with MAFLD, even after adjustment for confounding factors[22]. Chronic, low-grade inflammation may propagate oxidative stress and endothelial dysfunction in MAFLD[23,24]. Ultimately, liver fibrosis ensues due to extracellular matrix formation and collagen deposition[25], potentially progressing to cirrhosis and hepatocellular carcinoma. Intriguingly, this pro-inflammatory state could facilitate the development of CKD, with nuclear factor kappa B (NF-κB) mediating the activation of endothelial cells, mesangial cells, podocytes, and tubular epithelial cells, resulting in increased permeability, the release of inflammatory mediators, and proteinuria[26]. In this deleterious setting, the additional extracellular matrix remodeling, epithelial-to-mesenchymal transition, and interstitial fibrosis contribute to the progression of CKD[27]. We should also stress that the contribution of MAFLD and CKD to the systemic inflammatory milieu could have deleterious cardiovascular implications[28-32].

Obesity and adipokines

Obesity, another shared risk factor for MAFLD and CKD, is also pivotal in their development. Adipose tissue is a known endocrine organ with critical regulatory functions on satiety, insulin sensitivity, inflammation, and the renin-angiotensin system through the secretion of adipokines[33,34]. The most well-characterized hazardous adipokine in FLD, leptin, by interacting with its primary receptor Ob-Rb, results in Janus kinase 2 phosphorylation, in turn leading to the upregulation of the Akt/mammalian target of rapamycin (mTOR), signal transducer and activator of transcription 5, and mitogen-activated protein kinase (MAPK) pathways[35]. This may aid in the development and progression of hepatic steatosis, steatohepatitis, and liver fibrosis. By contrast, low levels of the protective adipokine adiponectin are significantly associated with advanced fibrosis[36]. Moreover, an increased leptin-to-adiponectin ratio is positively correlated with the increasing severity of steatosis[37]. The imbalance in leptin and adiponectin may influence the development of CKD, as leptin could induce sympathetic nervous system activation and blood pressure increases[38], as well as transforming growth factor-β synthesis[39]. A recently reported longitudinal study of 2646 Koreans without CKD showed that higher plasma leptin was predictive of incident CKD after a 2.8-year mean follow-up[40]. On the other hand, adiponectin could have renoprotective effects by ameliorating renal inflammation, oxidative stress, and

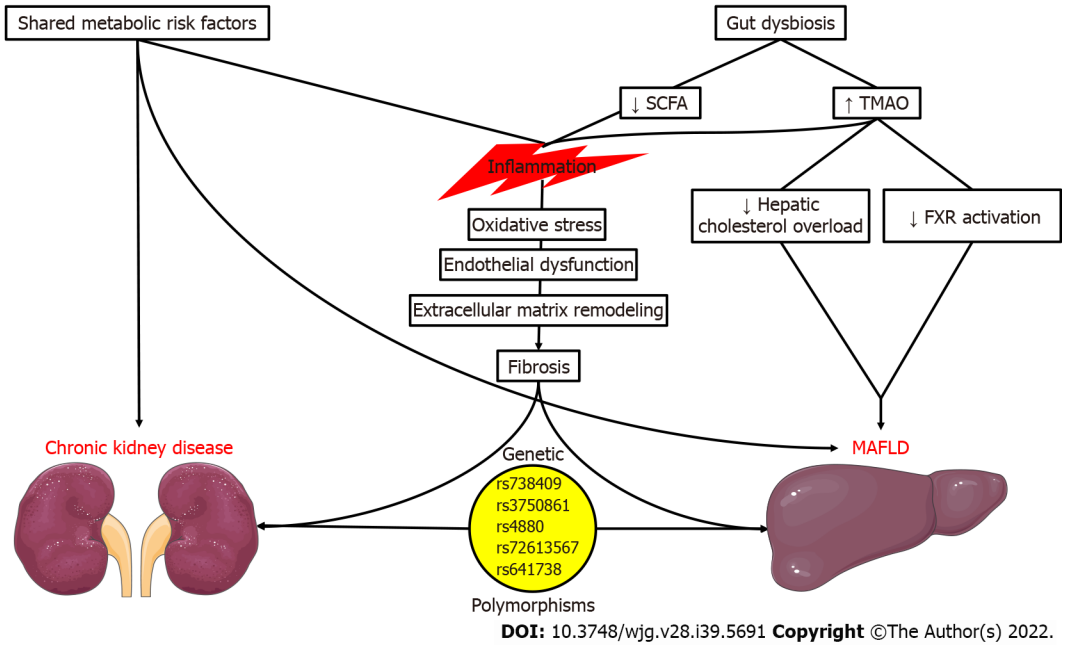

Figure 1 Common pathophysiologic mechanisms in metabolic dysfunction-associated fatty liver disease and chronic kidney disease.
FXR: Farnesoid X receptor; MAFLD: Metabolic dysfunction-associated fatty liver disease; SCFA: Short-chain fatty acid; TMAO: Trimethylamine N-oxide.

fibrosis[41]. However, multiple studies have shown that high adiponectin levels are inversely associated with eGFR in individuals with CKD[42] and were predictive of renal function deterioration in subjects without CKD[43].

Gut dysbiosis

The role of the gut microbiome in human health and disease is a highly relevant field of scientific interest. Therefore, potential associations of gut dysbiosis with MAFLD and CKD have been suggested in the past years, strengthening the importance of the gut-liver-kidney axis. In altered gut microbiome synthesis, hazardous metabolites such as trimethylamine N-oxide (TMAO), p-cresyl sulfate, and indoxyl sulfate may be formed.

TMAO is the most extensively studied metabolite regarding its health implications[44]. According to preclinical studies, it may aggravate hepatic steatosis and steatohepatitis by modulating bile acid metabolism, inhibiting farnesoid X receptor activation, and reducing hepatic cholesterol overload[45,46]. NAFLD presence and severity were correlated with circulating TMAO in a study of Chinese individuals[47]. The levels of TMAO were higher in individuals with obesity and NASH, only in the presence of T2DM[48]. TMAO was also associated with all-cause mortality only in NAFLD patients in the Prevention of Renal and Vascular End-stage Disease cohort study, even after adjustment for confounders[49]. Regarding the kidney, TMAO may exert deleterious effects, such as promoting inflammation and fibrosis[50-52]. In a meta-analysis of kidney function indices involving 32 clinical studies with 42062 participants, TMAO concentration was associated with advanced CKD, inversely correlated with eGFR, and positively correlated with UACR, serum creatinine, and serum cystatin C[53]. Circulating TMAO was predictive of all-cause and cardiovascular mortality in a recently reported systematic review and meta-analysis, with this finding being irrespective of kidney function and common risk factors (DM, hypertension, dyslipidemia, inflammation)[54].

Depletion of bacteria responsible for the production of beneficial short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, in the setting of a disrupted gut microbiome may lead to deleterious effects in the liver and kidney. These SCFAs could promote anti-inflammatory and anti-oxidative actions by limiting neutrophil recruitment, macrophage secretion of pro-inflammatory mediators, and histone deacetylase-induced NF-κB activation while promoting anti-inflammatory interleukin-10 formation by T regulatory cells[55]. Increased availability of SCFA-producing bacteria or SCFA treatment in clinical studies of patients on hemodialysis patients has resulted in lowering inflammatory markers and ameliorating renal function[56,57]. SCFAs are also helpful in the prevention of MAFLD due to the effects mentioned above, together with hepatic AMP-activated protein kinase (AMPK) activation and glucagon-like peptide 1 receptor (GLP1-R) activation, promotion of satiety, and abrogation of insulin resistance[58].

Gene polymorphisms

Polymorphisms in a few NAFLD-associated genes may also be associated with CKD. *PNPLA3* rs738409

G allele polymorphism is the most well-studied and correlated with NAFLD risk and severity[59]. Its potential association with renal outcomes has been investigated with conflicting evidence, as it may propagate podocyte activation and lipid nephrotoxicity. Initially, Sun *et al*[60] found a significant link between the G/G *PNPLA3* genotype with glomerular and tubular injury. In a United Kingdom Biobank analysis, the rs738409 single nucleotide polymorphism was associated with decreased eGFR, independently of metabolic risk factors[61]. Patients homozygous for the *PNPLA3* rs738409 had a higher prevalence of CKD and lower eGFR irrespective of liver stiffness and other risk factors in the study of Mantovani *et al*[62]. This study also found similar expression of *PNPLA3* in podocytes, hepatocytes, and hepatic stellate cells[62]. The same study group had previously proven the independent association of the G/G *PNPLA3* rs738409 polymorphism phenotype with eGFR and CKD in post-menopausal women with T2DM[63]. However, no associations between *PNPLA3* rs738409 gene polymorphism and kidney function were detected in other studies[64-67].

Other polymorphisms have also been investigated, albeit to a lesser degree. Risk alleles for *KLF6* rs3750861 and *SOD2* rs4880 polymorphisms correlate with kidney function in MAFLD[65]. Moreover, in a population of biopsy-proven NAFLD, the *HSD17B13* rs72613567 A alleles were protective against albuminuria but not eGFR decline[68]. Lastly, an association between the *MBOAT7-TMC4* rs641738 T/T genotype and lower eGFR was detected in a cohort of Asian individuals with biopsy-proven NAFLD [67]. While the genetic predisposition of kidney dysfunction in FLD represents an exciting hypothesis, further studies are required to improve our understanding of this link.

THERAPEUTIC APPROACHES

Treating patients with MAFLD and CKD requires therapeutic interventions to ameliorate their prognosis by targeting their shared risk factors and pathophysiology. Although studies have not explicitly assessed this subgroup of patients, we may assume that interventions with documented efficacy in MAFLD[69-81] and CKD[82-90] could lead to positive outcomes in this combination of diseases (Table 1). Moreover, due to the recent change in the nomenclature with the introduction of MAFLD, we should stress that most of the available clinical evidence discussed below is derived from studies of NAFLD patients. Therefore, future appropriately designed studies considering the novel MAFLD diagnostic criteria will shed additional light on managing this entity.

Bariatric surgery

Since obesity is among the main risk factors for the development of MAFLD, the role of bariatric surgery may be crucial in carefully selected eligible individuals. Initially, we should state that the prevalence of MAFLD may be exceptionally high in those morbidly obese patients that are eligible for bariatric surgery. Ciardullo *et al*[91] have demonstrated this association in a study of 434 potential candidates for bariatric surgery, with the prevalence of steatosis and fibrosis being 76.7% and 23.1%, respectively. In the only study assessing bariatric surgery in MAFLD patients, Meneses *et al*[69] prospectively enrolled 52 subjects whose MAFLD status was evaluated *via* liver biopsy. Those with a histological diagnosis of steatohepatitis were followed up with an additional biopsy 12 mo after the index procedure. Most subjects with steatohepatitis did not experience any disease progression, while a significant proportion (56.5%) exhibited complete resolution. Additionally, fibrosis and fibrotic scores were improved, highlighting a non-negligible benefit of bariatric surgery in this small-scale study.

Bearing in mind the increased prevalence (~80%) of steatosis in morbidly obese patients (BMI > 40 kg/m^2)[92], several clinical implications can be made regarding kidney outcomes. To begin with, compared with individuals who have undergone a bariatric surgery procedure, severely obese subjects had greater odds of having stage III CKD (OR 3.10, 95%CI: 3.05-3.14, $P < 0.001$) and end-stage renal disease (OR 1.13, 95%CI: 1.09-1.18, $P < 0.001$). This finding was consistent even after adjustment for CKD risk factors[93]. The performance of sleeve gastrectomy could have renoprotective effects, as shown in a retrospective analysis of 1330 individuals undergoing this procedure. The investigators noted a greater improvement of eGFR in subjects with impaired kidney function 12 mo after the procedure[82]. A rise in eGFR, together with albuminuria reduction, was observed by Wee *et al*[83] in their retrospective study of 557 Asian patients after metabolic bariatric surgery. Importantly, the CKD stage improved in 12.9% of the study participants, while the prevalence of albuminuria (UACR > 3.5 mg/mmol) decreased from 24.8% to 1.9% at the 1-year follow-up[83]. Fathy *et al*[84] also noted an astonishing albuminuria remission rate (83%) in 137 non-diabetic, non-hypertensive, severely obese subjects with albuminuria who underwent bariatric surgery. Moreover, in another study, subjects undergoing bariatric surgery had a lesser incidence of kidney disease than the control group (hazard ratio 0.46, 95%CI: 0.22-0.92)[85]. A systematic review and meta-analysis of 19 studies revealed that bariatric surgery led to ameliorated eGFR and lesser odds of incident albuminuria[94]. The observed benefits may be attributed to enhanced glomerular hyperfiltration, reduction in detrimental adipocyte-derived mediators such as leptin, and alterations in pro-inflammatory and pro-fibrotic molecule expression[95].

Table 1 Selected human studies assessing various treatment approaches in metabolic dysfunction-associated fatty liver disease and chronic kidney disease

Study	Treatment	Finding
MAFLD		
Meneses *et al*[69]	Bariatric surgery	Stabilization of fibrosis or complete resolution; ↓ NAFLD fibrosis score
Li *et al*[70]	GLP1-RA	↓ Liver fat
Morieri *et al*[71]	GLP1-RA	↓ MAFLD prevalence
Jianping *et al*[72]	GLP1-RA	Improvement in histological MAFLD features
Akuta *et al*[73]	Canagliflozin	↓ Histological steatosis, lobular inflammation, and fibrosis stage
Takahashi *et al*[74]	Ipragliflozin	↓ Hepatic fibrosis; Steatohepatitis resolution
Pakravan *et al*[75]	Melatonin	↓ Inflammation; Improvement of ultrasonographic fatty liver grade
Akhavan *et al*[76]	Melatonin	↓ Hepatic enzyme levels
Rinella *et al*[77]	Obeticholic acid	↓ Hepatic enzyme levels↓ Liver fibrosis
Neuschwander-Tetri *et al*[78]	Obeticholic acid	Improvement in histological features
Mohamad Nor *et al*[79]	Probiotics	↔ Elastography-derived hepatic steatosis and fibrosis
Derosa *et al*[80]	Probiotics	↓ Hepatic steatosis index; ↓ Ultrasonographic steatosis
Musazadeh *et al*[81]	Probiotics	↓ Hepatic enzyme levels
CKD		
Funes *et al*[82]	Bariatric surgery	↑ eGFR
Wee *et al*[83]	Bariatric surgery	↑ eGFR↓ Albuminuria
Fathy *et al*[84]	Bariatric surgery	↓ Albuminuria
Dash *et al*[85]	Bariatric surgery	↓ Kidney disease incidence
Shaman *et al*[86]	GLP1-RAs	↓ Albuminuria; Halted eGFR decline
Perkovic *et al*[87]	Canagliflozin	Reduction in the renal outcome (ESKD, doubling of serum creatinine, or renal death) by 34%
Heerspink *et al*[88]	Dapagliflozin	Reduction in the renal outcome (decline in eGFR of ≥ 50%, ESKD, or renal death) by 44%
Bhatt *et al*[89]	Sotagliflozin	Reduction in the renal outcome (decline in eGFR of ≥ 50%, ESKD, renal transplantation) by 29%
Wang *et al*[90]	Probiotic	Halted eGFR decline; ↓ Inflammation

↑: Increase; ↓: Decrease; ↔: No change. CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate; ESKD: End-stage kidney disease; GLP1-RA: Glucagon-like peptide 1 receptor agonist; MAFLD: Metabolic dysfunction-associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease.

GLP1-R agonists

GLP1-R agonists (GLP1-RAs) are novel potent antidiabetic agents with proven efficacy in reducing major adverse cardiovascular events. Besides their glucose-lowering action, their beneficial hepatic effects may be related to the influence on the AMPK/mTOR pathway, as shown by Reis-Barbosa *et al*[96] in obese C57BL/6 mice treated with subcutaneous semaglutide. Other inflammatory and oxidative pathways in the liver could be inhibited by GLP1-RAs, such as the receptor for advanced glycation end products/nicotinamide-adenine dinucleotide phosphate oxidase 2, limiting liver injury and fibrosis in mice on a high-fat diet[97]. Concerning human studies, patients with MAFLD treated with GLP1-RAs have exhibited a significant reduction in liver fat, which may be positively correlated to fibroblast growth factor 21[70]. The use of GLP1-RAs also resulted in a significant reduction of MAFLD prevalence (defined based on hepatic steatosis index > 36) during a 24-mo follow-up[71]. Interestingly, the effect was evident only in subjects on human-based GLP1-RAs[71]. Moreover, in a meta-analysis of 4 randomized clinical trials, semaglutide was associated with significant decreases in body weight, alanine aminotransferase, liver steatosis, and stiffness[98]. GLP1-RAs may also improve histologic features on MAFLD, such as liver fat deposition, steatohepatitis, and fibrosis, as shown by the systematic review and meta-analysis of Jianping *et al*[72].

GLP1-RAs have shown benefits in preventing the development or halting the progression of CKD. As demonstrated above, their effect in ameliorating steatosis and promoting anti-oxidative and anti-inflammatory actions may be among the determining factors in this renoprotective effect, together with weight loss, blood pressure, and glucose-lowering[99]. Other speculated mechanisms include glomerular hyperfiltration, the regulation of the renin-angiotensin system, sodium-hydrogen exchanger-3, and renal endothelial vasodilation[100]. Regarding clinical evidence, and as recently shown in a pooled analysis of the SUSTAIN 6 and LEADER trials of patients with T2DM, semaglutide and liraglutide diminished albuminuria and eGFR decline, especially in subjects with CKD (eGFR < 60 mL/min/1.73 m^2)[86]. Moreover, efpeglenatide, an exendin-4-based GLP1-RA, also led to favorable renal outcomes compared to placebo in the AMPLITUDE-O trial of individuals with T2DM, irrespective of eGFR and concurrent sodium-glucose co-transporter-2 (SGLT2) inhibitor use[101,102]. As far as head-to-head comparisons, the renoprotective effects of GLP1-RAs were of greater magnitude compared with dipeptidyl peptidase-4 inhibitors[103], whereas SGLT2 inhibitors may promote increased renal benefits[104,105].

Dual GLP1 and glucose-dependent insulinotropic peptide receptor agonists have recently emerged into the spotlight owing to the results of the SURMOUNT-1 clinical trial of tirzepatide for the treatment of obesity[106]. Regarding FLD, the administration of a hybrid agonist by the name of 19W in C57BL/6J on a high-fat diet decreased the area of liver fibrosis[107]. Moreover, dual GLP1/2 receptor agonists may also ameliorate NASH prognosis, as shown in C57BL/6J mice on a high-fat diet/high fructose and sucrose solution through an improvement in liver fibrosis[108]. However, these concepts need further validation in preclinical settings. Concerning clinical evidence, tirzepatide dose-dependently ameliorated biomarkers of NASH such as alanine transaminase, aspartate aminotransferase, keratin-18, and procollagen III compared to placebo. At the same time, it was associated with an increase in adiponectin[109]. Lastly, in a recently published substudy of the SURPASS-3 MRI clinical trial, administration of tirzepatide in patients with T2DM decreased the liver fat content along with the volume of visceral and abdominal subcutaneous adipose tissue, compared to insulin degludec[110].

SGLT2 inhibitors

SGLT2 inhibitors have been at the forefront of scientific research owing to the remarkable reduction in the rate of heart failure hospitalization and their ability to impact cardiac remodeling[111,112]. Their pleiotropic mechanisms of action have been a topic of continuous investigation[111,113], and their therapeutic indications are constantly expanding. In the field of MAFLD, specifically in obese, diabetic mice with FLD treated with empagliflozin, Kurtz *et al*[114] documented a reduction in hepatic steatosis, which was correlated with the whitening of the adipose tissue. Empagliflozin may also attenuate hepatocyte lipotoxicity through the calcium/calmodulin dependent protein kinase beta/AMPKα pathway[115]. Another SGLT2 inhibitor, ipragliflozin, ameliorated the progression of MAFLD in STAM mice with β cell depletion, evidenced by decreased histologic steatosis, hepatocyte ballooning, inflammation, and fibrosis[116]. This effect was accompanied by antioxidant and mitochondrial transport-related gene upregulation, and overexpression of miR-19b-3p[116]. Additionally, dapagliflozin reduced liver fat accumulation in male NIH mice on a high-fat diet by acting on the AMPK/mTOR pathway [117]. Moving to clinical evidence, empagliflozin may lessen liver fibrosis, insulin resistance, and hepatic enzyme concentrations, as shown by the systematic review and meta-analysis of Zhang *et al* [118]. An interesting study on the importance of SGLT2 inhibition in MAFLD was performed by Akuta *et al*[73], who retrospectively reviewed patients with T2DM and FLD initiated on canagliflozin with consequent biopsy results over a period of 5 years. Compared to pre-treatment biopsy, the investigators noted a histologic improvement in 50% of the participants and a decrease in steatosis, lobular inflammation, and fibrosis stage in 67%, 33%, and 33%, respectively, at the 5th year. In line with this study, ipragliflozin use in patients with T2DM and FLD led to significant improvements in hepatic fibrosis and greater rates of steatohepatitis resolution compared to the control group[74]. According to the available evidence, we can assume that SGLT2 inhibitors will become an essential tool in the prevention and treatment of MAFLD.

While the use of SGLT2 inhibitors in MAFLD is gaining ground, this drug class is an established treatment option for CKD. Among the putative nephroprotective mechanisms are the regulation of autophagy and the resulting inflammation, oxidative stress, endothelial dysfunction, fibrosis, and apoptosis, the reduction of intraglomerular and blood pressure, and the improvement of podocytopathy. Large-scale randomized clinical trials on CKD patients such as CREDENCE[87], DAPA-CKD [88], and SCORED[89] demonstrated the unequivocal benefit of SGLT2 inhibition in reducing the rate of adverse renal outcomes and eGFR decline. The upcoming EMPA-KIDNEY trial was stopped early due to clear efficacy detected in the interim analysis, and the detailed results are eagerly awaited. Subanalyses of the abovementioned trials stressed the effect of SGLT2 inhibitors on kidney outcomes independently of T2DM status, baseline hemoglobin A1c, CKD etiology, and stage[119-122]. The upcoming revision of existing CKD guidelines should incorporate this option in CKD treatment algorithms.

Melatonin

Melatonin, a crucial hormone produced in response to darkness, could be an additional approach to managing MAFLD and CKD due to its pleiotropic effects, as we have previously reviewed[123]. Starting

with its impact on MAFLD, fine particulate matter-induced hepatic steatosis was ameliorated with the administration of melatonin in apolipoprotein E knockout (ApoE$^{-/-}$) mice through anti-oxidative mechanisms involving protein tyrosine phosphatase 1B and nuclear factor erythroid 2-related factor 2 signaling pathways[124]. Furthermore, melatonin promoted anti-inflammatory actions by modulating NACHT, LRR, and PYD domain-containing protein 3 inflammasome activation and downregulating the toll-like receptor 4/NF-κB pathway in C57BL/6 mice models of high-fat diet-induced steatohepatitis [125]. This resulted in histopathological improvement of steatosis, ballooning, inflammation, fibrosis, and overall disease score[125]. In clinical studies, the administration of oral melatonin thrice daily for 3 mo ameliorated metabolic and inflammatory indices, as well as ultrasonography fatty liver grade, in patients with histologically proven NAFLD[75]. In a meta-analysis of studies with NAFLD patients, alanine transaminase, alkaline phosphatase, gamma-glutamyl transferase, triglycerides, and total cholesterol were significantly reduced after melatonin supplementation[76]. However, more clinical trials are needed to improve our understanding of the importance of melatonin treatment in MAFLD development, progression, and prognosis.

Melatonin supplementation has also been attempted in CKD, both preclinically and clinically. Based on experimental studies, several mechanisms of nephroprotection have been suggested, including anti-oxidative, anti-inflammatory, anti-fibrotic, and anti-apoptotic[123]. Although there is no reliable clinical evidence concerning CKD prognosis after melatonin therapy, human trials have proven an anti-oxidative and anti-inflammatory effect, paired with improved glycemia[126,127]. Moreover, ameliorating mitochondrial damage and promoting autophagy could represent other putative effects of melatonin treatment[123].

Farnesoid X receptor agonists

Farnesoid X receptor agonists have demonstrated efficacy both in fatty liver disease regression and kidney disease. The most commonly used agent of this drug class, obeticholic acid, at a dose of 25 mg, led to significant improvement in liver function tests, elastography-derived and histologically proven liver fibrosis in patients with steatohepatitis and liver fibrosis (F2-F3)[77]. Based on the results of the FLINT trial of patients with non-cirrhotic, non-alcoholic steatohepatitis, obeticholic acid 25 mg administration led to an improvement in liver histology in 45% of the participants compared to in the control group (relative risk 1.9, 95%CI: 1.3-2.8)[78]. Although the dosage of 25 mg may be more efficacious than 10 mg, it may be met with a more significant burden of side effects and possibly higher discontinuation rates[128]. Preclinical evidence has suggested the potential of farnesoid X receptor agonists in experimental kidney disease by abrogating inflammation, oxidative stress, fibrosis, and apoptosis[129-132]. Due to the lack of clinical data, the efficacy of farnesoid X receptor agonists in CKD remains speculative to date.

Gut microbiome modulation

Targeting the gut microbiome may represent an appealing approach to the holistic management of MAFLD and CKD. Probiotics such as *Bifidobacterium animalis, B. bifidum, B. adolescentis, Lactobacillus paracasei, L. plantarum, L. reuteri,* and *Weissella cibaria* have been assessed in preclinical FLD models and may alter gut permeability, ultimately affecting the processes of inflammation and oxidative stress among others[133-138]. Unfortunately, a probiotic supplement containing six different Lactobacillus and Bifidobacterium species for 6 mo did not improve hepatic steatosis and fibrosis evaluated by elastography in ultrasonography-diagnosed NAFLD subjects[79]. By contrast, a high-concentration probiotic combination of *Streptococcus thermophilus*, multiple *Bifidobacteria* and *Lactobacilli* led to a reduction of hepatic steatosis index as well as ultrasonographic steatosis in a double-blind, placebo-controlled, randomized clinical trial of NAFLD patients[80]. A recently reported umbrella systematic review and meta-analysis also suggested liver biochemical improvement through the administration of probiotics in NAFLD patients[81]. Although probiotics appear helpful in experimental FLD settings, more clinical trials are required to improve our understanding of their importance in human MAFLD.

Moving to CKD, *L. rhamnosus* administration for 14 wk in 5/6 nephrectomized mice diminished gut-derived uremic toxins and systemic inflammatory markers by restoring intestinal integrity and protecting against renal fibrosis[139]. Anti-inflammatory, anti-apoptotic, and anti-fibrotic effects with *L. rhamnosus* were demonstrated in cisplatin-induced CKD rat models by acting on the MAPK/NF-κB/cyclooxygenase-2, the p53/B-cell lymphoma 2-associated X protein/caspase-3, and the signal transducer and activator of 3 pathway[140]. Moreover, a lactobacillus mixture consisting of *L. paracasei* and *L. plantarum* led to attenuated kidney injury, inflammation, and fibrosis in adenine-induced CKD mouse models, while also restoring gut microbial composition[141]. Translating these findings in a clinical setting of patients with advanced CKD, 6 mo of treatment with a probiotic formulation containing *L. acidophilus*, *B. longum*, and *B. bifidum* significantly halted the eGFR decline, together with lowering of inflammatory markers[90].

Although still experimental, TMAO inhibitors may represent a possible approach to modulating gut microbiota. Using 3,3-dimethyl-1-butanol, a trimethylamine formation inhibitor, decreased plasma TMAO levels and attenuated renal inflammation, oxidative stress, and fibrosis in C57BL/6 mice on a high-fat diet[142]. Importantly, no changes in blood pressure and weight adiposity parameters were noted[142]. Iodomethylcholine (IMC), a selective gut microbial choline TMA-lyase inhibitor, was also

able to diminish TMAO production and revert the renal function decline and tubulointerstitial fibrosis in isoproterenol-induced CKD mouse models on a choline diet[143]. Similar observations were made in ApoE$^{-/-}$ mouse models with adenine-induced CKD treated with IMC, together with ameliorated microalbuminuria, cardiac hypertrophy, and vascular inflammation indices[144]. These molecules have not been assessed yet in FLD, and upcoming studies evaluating their efficacy are awaited.

CONCLUSION

In conclusion, it has become evident that the newly defined MAFLD is associated with high prevalence and mortality rates and is an independent predictor of CKD. The degree of hepatic steatosis and fibrosis in this group of patients correlates with kidney function indices such as urinary albumin-to-creatinine ratio and estimated glomerular filtration rate. This interaction is unsurprising, as these entities have shared risk factors and deleterious molecular mechanisms such as inflammation, oxidative stress, and gut dysbiosis. At the same time, gene polymorphisms associated with fatty liver disease predisposition may also propagate renal dysfunction. In the field of treatment, pharmacologic interventions have demonstrated considerable preclinical and clinical efficacy in ameliorating surrogate disease markers and clinical outcomes in these pathological states. Future studies should aim at the subpopulation of MAFLD patients with renal impairment to appropriately determine their prognosis and the impact of treatment approaches.

FOOTNOTES

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MINIREVIEWS

Pitfalls and promises of bile duct alternatives: A narrative review

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Abstract

Biliodigestive anastomosis between the extrahepatic bile duct and the intestine for bile duct disease is a gastrointestinal reconstruction that abolishes duodenal papilla function and frequently causes retrograde cholangitis. This chronic inflammation can cause liver dysfunction, liver abscess, and even bile duct cancer. Although research has been conducted for over 100 years to directly repair bile duct defects with alternatives, no bile duct substitute (BDS) has been developed. This narrative review confirms our understanding of why bile duct alternatives have not been developed and explains the clinical applicability of BDSs in the near future. We searched the PubMed electronic database to identify studies conducted to develop BDSs until December 2021 and identified studies in English. Two independent reviewers reviewed studies on large animals with 8 or more cases. Four types of BDSs prevail: Autologous tissue, non-bioabsorbable material, bioabsorbable material, and others (decellularized tissue, 3D-printed structures, *etc.*). In most studies, BDSs failed due to obstruction of the lumen or stenosis of the anastomosis with the native bile duct. BDS has not been developed primarily because control of bile duct wound healing and regeneration has not been elucidated. A BDS expected to be clinically applied in the near future incorporates a bioabsorbable material that allows for regeneration of the bile duct outside the BDS.

Key Words: Bile duct alternative; Bile duct substitute; Biliary regeneration; Bile duct reconstruction; Peribiliary gland; Bioabsorbable polymer

Core Tip: The bile duct-intestinal anastomosis eliminating the function of the papilla of Vater causes chronic inflammation due to the reflux of bile and is not an ideal reconstruction method. Bile duct alternatives for bile duct defects have not been developed for over 100 years. In the present situation where the wound healing of the bile duct defect cannot be controlled, only the use of a bioabsorbable material, such as a scaffold, and the regeneration of the bile duct outside the scaffold can be expected as a bile duct substitute.

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INTRODUCTION

The treatment of gastrointestinal diseases in the 21st century involves robotic and endoscopic surgeries, which aim to minimize potential risks and side effects[1-3]. Minimally invasive endoscopic treatments have been developed to facilitate functional preservation[4,5]. For diseases of the biliary system, the use of laparoscopic cholecystectomy as a treatment for cholelithiasis has become widespread, and minimally invasive approaches have been pursued[6]. In contrast, the incidence of severe iatrogenic extrahepatic bile duct injury due to laparoscopic cholecystectomy has increased significantly worldwide compared to that due to laparotomy surgery[7]. With regard to bile duct injuries, there is currently no bile duct substitute (BDS) for partially defective or damaged parts of the bile duct. Reconstruction by anastomosis of the hepatic bile duct and intestine is typically performed[8].

Bile duct-intestinal anastomosis is a biliary tract reconstruction procedure that was first performed by von Winiwater in 1880[9]. However, liver abscess, cirrhosis, and liver dysfunction were often observed in patients who underwent this anastomosis. In the early 20th century, retrograde cholangitis tended to cause chronic inflammation if duodenal papilla function was not preserved[10,11]. The suboptimal nature of this approach resulted in attempts to preserve papilla function and to develop alternatives for addressing bile duct defects and injury. Chronic inflammation caused by abnormal pancreatic-bile duct junctions, intrahepatic stones[12,13], and exposure to organic solvents from the printing industry is considered a high-risk factor for cholangiocarcinoma[14]. Therefore, the development of BDS has emerged as a critical, unmet need. To date, various alternatives, including autologous tissue[10,15-18], non-bioabsorbable[19-23], bioabsorbable materials[24-28], and decellularized tissue[29,30], have been investigated. Nevertheless, BDSs with widespread clinical applications have not yet been developed. In this review, we discuss potential factors underpinning the failure to develop clinically usable products despite efforts to develop BDSs for more than a century. Furthermore, we highlight the types of BDSs that may be clinically applied in the near future.

MATERIALS AND METHODS

We searched the PubMed electronic database to identify studies conducted to develop bile duct alternatives until December 2021 and identified studies in English. The following search items for the data relevant to "why has a product that can replace bile ducts not been developed?" were included: "bile duct alternative", "bile duct substitute", "bile duct regeneration", "biliary alternative", "extrahepatic bile duct", "biliary regeneration", and "bile duct reconstruction". Studies using large animals such as dogs, pigs, and goats were included, whereas studies using small animals such as rats and mice were excluded. To evaluate the efficacy of the BDS, the "type of substitute", "shape and length of substitute", "method of reconstruction of the bile duct by substitute", and "observation period" were included as search terms. The items to be examined were "presence or absence of regeneration process", "localization of regenerated bile duct to substitute", "number of large animals that could be sacrificed and killed intentionally", and "cause of narrowing of substitute". We also cited high-quality articles in *Reference Citation Analysis* (https://www.referencecitationanalysis.com).

For studies in which the items could be determined from the abstract or text, the number of experimental large animals used was eight or more. The full text was reviewed by two researchers (Miyazawa M, Takashima J). In each of these studies, the cases in which BDS transplantation was successful were as follows: These cases were sacrificed as planned before BDS transplantation, no stenosis was observed at the anastomotic site between the BDS transplantation site and the natural bile duct, and there was no

liver dysfunction after BDS transplantation.

Animal type

In small animals, such as rats, jaundice is unlikely to occur even if the extrahepatic bile duct is narrowed, and liver dysfunction may be difficult to evaluate. As such, BDS transplantation may be difficult[31]. Accordingly, only large-animal studies were considered.

Size of BDS

If the length of the BDS implantation segment was less than 1 cm, tissues such as the omentum may migrate around it after inserting the T-tube into the defective bile duct segment[32]. Therefore, BDSs ≤ 1 cm and > 1 cm in length were considered separately. For the same reason, patch-like or circular implantation of the BDS was examined separately. Regarding the implantation method, the site of BDS implantation (between the common bile duct or between the common bile duct and intestine) was examined separately because it affects the patency of the BDS lumen.

Observation period after BDS implantation

Early after BDS transplantation, stenosis of the BDS may not occur in the event of retrograde cholangitis or severe inflammation at the alternative transplant site. After chronic inflammation, the bile duct becomes narrowed due to the gradual hyperplasia of connective tissue around the substitute[33]. After stent insertion into the BDS, bile may flow through the stent and a bile plug may not be formed in the BDS in the early stage of BDS transplantation[34]. As anastomotic stenosis was considered less likely to occur when a stent was inserted, the observation period after BDS transplantation was included in the examination items.

Bile duct regeneration

After the formation of connective tissue in the shape of the bile duct, the BDS lumen does not become completely stenotic early after BDS transplantation, even if bile duct regeneration does not occur and bile remains in the lumen. However, stenosis tends to occur after a prolonged period[35]. Tissue regeneration in the defective bile duct area occurs due to wound healing[35]. The mature bile duct takes time (3 mo or more) to regenerate from bile duct stem cells[25,36,37] (Figure 1). Therefore, to examine the effectiveness of BDS for inducing bile duct regeneration, histological images of bile duct regeneration at the BDS transplantation site were included in the examination. If the study did not report neo-bile duct regeneration and the histology was similar to that of the native bile duct, the tissue was excluded from evaluation. The histology of the anastomotic site between the BDS and native bile duct was examined because the bile duct epithelium is continuous at the anastomotic site if stenosis of the anastomotic site does not occur[38].

Localization of bile duct regeneration with respect to BDS implants

We did not identify any reports of bile duct regeneration on the inner surface of the T-tube when it was placed in the injured part of the bile duct. This suggests that the bile duct does not regenerate on the inner surface of non-bioabsorbable BDSs. However, it is important from the viewpoint of wound healing whether the localization of the regenerated bile duct is the outer surface of the BDS, the part of the BDS itself, or the inner surface of the BDS (only the bile passage surface). For these reasons, the site where the regenerated bile duct regenerates was included in the examination items for the BDS transplantation site. When the localization of bile duct regeneration was not specified, it was judged from the BDS implantation site and bile passage position in the paper.

Causes of stenosis

Narrowing of the anastomotic site between the BDS and the native bile duct or the BDS lumen was evaluated as a separate cause of wound healing. We also examined the stenotic tissue type. For BDS lumen and anastomotic site stenosis, scar contraction was considered to occur over a prolonged period if granulation or connective tissue growth was reported[35,39,40].

BDS REPORTED TO DATE

The literature search enabled the classification of BDSs into four categories: Autologous tissues[10,15-18], non-bioabsorbable materials[19-23], bioabsorbable materials[24-28], and others (decellularized tissues[29,30], structures made with 3D printers[41,42], *etc.*).

BDS using autologous tissue

Tissues with a similar morphology to that of the extrahepatic bile duct and lumen have been investigated as BDSs. In practice, arteries[43], veins[15,17-19,44], ureters[45], skin[46], and jejunum[16] have been used as grafts. Due to the thickness of the extrahepatic bile duct and wall, the femoral vein was

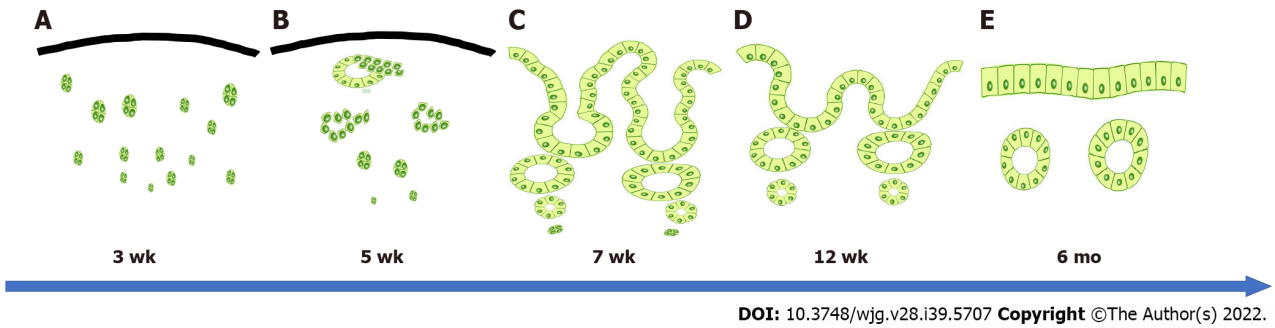

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Figure 1 Ideal bile duct regeneration process. A: Bile duct regeneration at approximately 3 wk. Numerous cell masses appear in the stroma that are thought to form peribiliary glands; B: Bile duct regeneration at approximately 5 wk. A ring-shaped peribiliary gland-like structure is observed, in which cell masses that are thought to form peribiliary glands are fused; C: Bile duct regeneration at approximately 7 wk. Numerous bile duct-attached glandular structures are observed on the bile passage surface, and the epithelial surface exhibits a high papillary morphology; D: Bile duct regeneration at approximately 12 wk. The number of peribiliary glands on the bile passage surface is decreased, the peribiliary glands become fused, and the epithelial surface becomes more even; E: Approximately 6 mo after bile duct regeneration. Similar to the native bile duct, the epithelial surface becomes a single layer of cubic columnar epithelium.

often used. In BDSs using these autologous tissues, necrosis of the substitute in the early stage of transplantation and obstruction due to attachment of the bile plug to the substitute in the middle stage of transplantation were common[10,17,18,44,47]. In the long term, scar contraction occurs due to the growth of connective tissue around the substitute and at the anastomotic site[10,17-19,44]. To address these issues, attempts have been made to wrap the omentum around the substitute to supply blood flow or to use stents and cuffs to prevent obstruction of the BDS lumen and anastomotic site[17,48,49]. As the BDS did not regenerate to the extent of the native bile duct, few BDSs were successful, and no clinically usable product was developed[48,49]. The localization of neo-bile ducts to the BDS, which attempted to promote bile duct regeneration, formed a part of the autologous tissue itself.

The most commonly used species in these studies was dog. Pearce *et al*[10] investigated autologous alternative veins as a BDS and concluded that 1 in 32 successful cases over 3 mo was insufficient for the use of autologous tissue as a BDS. A study by Dunphy and Stephens[19] using large animals (44 sheep and 8 pigs) evaluated autologous arteries, veins, and homozygous arteries. Only sheep that received autologous arteries as a BDS survived for 6 mo or more, but bile duct dilation on the liver side was observed. Myers *et al*[18] conducted a circular transplantation experiment using autologous bile ducts, arteries, veins, and genomic grafts to treat bile duct defects in 28 dogs. All homografts were rejected, and the dogs that received transplants died within 13 d.

Even in transplantations using autologous tissue, histological assessment revealed that the bile duct epithelium did not regenerate on the epithelial surface, fibrous connective tissue was increased on the epithelial surface, and the BDS transplantation site became stenotic. The authors concluded that self-organization is not possible in BDS. In 2009, Palmes *et al*[48] reported a high success rate of transplantation of the external jugular vein and a bioabsorbable stent as a BDS in pigs. However, histological changes in the bile ducts of autologous veins have not been reported (Table 1).

Due to the lack of blood supply in autologous tissue BDSs, the tissue becomes necrotic, and the anastomotic site is scar-contracted. A BDS capable of allowing bile to flow freely into the duodenum over a prolonged period has not yet been developed. It is unlikely that autologous tissue will resemble the native bile duct in the context of wound healing[40,50,51]. Stenting through the anastomotic site was effective in preventing narrowing[17,48,49]. High success rates have been reported when bioabsorbable stents are placed in the venous lumen; however, it is unclear how autologous venous tissue is induced for good bile duct regeneration. These findings suggested that autologous tissue cannot be used as a BDS.

BDS using non-bioabsorbable material

Since the 1930s, polyvinyl sponge[20], polytetrafluorethylene[22,23], Teflon[19,52], Dacron[53], and polyethylene[54,55] have been used as non-bioabsorbable BDS materials. These alternatives were used experimentally as patches or rings, but most studies reported high rejection rates early in transplantation and varying degrees of cholangitis, narrowing of the alternative lumen, and stenosis of the anastomotic site with the native bile duct[19,27,28]. Although partial success has been reported, the bile duct epithelium failed to regenerate on either the medial side (bile passage surface) or lateral side of these alternatives[29,30]. As a result, the perimeter of these BDSs was covered with fibrous connective tissue, and the luminal surface was clogged with bile plugs[19-22]. This resulted in stenosis of the anastomosis with the native bile duct, which prevented bile passage. As such, research on cyclic BDS made solely from non-bioabsorbable materials has ceased.

Sherman *et al*[21] transplanted a BDS made from acrylamide into dogs. In the stented group, 7 of the 33 cases were successful over 3 mo. However, bile duct regeneration has not been reported. Bergan *et al*

Table 1 Bile duct substitute using autologous tissue

Ref.	Journal	Substitute (*n*)	Stent (*n*)	Animal type (*n*)	Size of BDS (cm)	Method of reconstruction of bile duct by BDS (*n*)	Observation period after implantation	Localization of regenerated bile duct	Causes of stenosis	Note (planned sacrificial death and epithelialization)
Shea and Hubay[15], 1948	*Ann Surg*	Femoral vein (21)	Vitallium tube	Dog (21)	Ring (1.5)	CBC (21)	Maximum 208 d	BDS itself	Necrosis of BDS itself, narrowing of the BDS lumen, and narrowing of the anastomosis with the native bile duct	Although 14 out of 21 dogs were intentionally killed, the tissue of the regenerated bile duct was shown only as a result, and the process of bile duct regeneration was not demonstrated
Kirby and Fitts[16], 1950	*Arch Surg* (1920)	Jejunum (9)	T-tube	Dog (9)	Jejunum (2.5)	GBC (5); CBC (4)	Maximum 13 mo	BDS itself	BDS stenosis was not observed when the T-tube was inserted	Seven out of nine dogs were intentionally killed; however, no epithelial regeneration was observed at the anastomotic site. The procedure was too complicated
Pearce *et al* [10], 1951	*Ann Surg*	Femoral vein (32)	Lord and blakemore tube	Dog (32)	Ring (1.0)	CBC (10); CBJ (20); GBC (2)	Maximum 6 mo	BDS itself	Necrosis of autologous tissue, narrowing of the lumen of autologous tissue, narrowing of the anastomosis with the native bile duct, necrosis of BDS itself, narrowing of the BDS lumen, and narrowing of the anastomosis with the native bile duct	Only 1 of the 32 dogs survived for more than 6 mo. It was investigated in 32 dogs; however, in the end, fibrosis of the vein and stenosis of the anastomotic site with the native site occurred, and bile duct epithelial regeneration was not observed. It was concluded that the vein was not suitable for BDS
Ulin *et al* [17], 1955	*Ann Surg*	Vascularised jugular vein (10)	Polyethylene tube	Dog (10)	Ring (2.0-5.0)	CBC (10)	Maximum 10 mo	BDS itself	Necrosis of autologous tissue, narrowing of the lumen of autologous tissue, narrowing of the anastomosis with the native bile duct, necrosis of BDS itself, narrowing of the lumen of BDS, and narrowing of the anastomosis with the native bile duct	The omentum was used to maintain blood flow to the BDS, but in some cases, it functioned as a BDS only during the period when the stent was in place (6 out of 10 dogs). Bile duct regeneration process was not studied. No regeneration of the bile duct epithelium was observed
Myers *et al* [18], 1960	*Ann Surg*	Femoral vein and artery, bile duct (17), and homologous bile duct (6)	Polyethylene tube	Dog (28)	Ring (unknown)	CBC (23)	Maximum 449 d	BDS itself	Necrosis of autologous tissue, narrowing of the lumen of autologous tissue, narrowing of the anastomosis with the native bile duct, necrosis of BDS itself, narrowing of the BDS lumen, and narrowing of the anastomosis with the native bile duct	BDS using autologous veins, arteries, or allogeneic arteries also narrowed shortly after transplantation. No bile duct epithelial regeneration was observed
Dunphy and Stephens [19], 1962	*Ann Surg*	Autologous vein and artery (20), and homologous artery (32)	T-tube	Goat (44), dog (8)	Ring (1.0)	CBC (52)	Maximum 9 mo	BDS itself	Necrosis of autologous tissue, narrowing of the lumen of autologous tissue, narrowing of the anastomosis with the native bile duct, necrosis of BDS itself, narrowing of the BDS lumen, and narrowing of the anastomosis	In an experiment using autologous veins and T-tube as BDS, 2 dogs survived for more than 6 mo; however, both dogs demonstrated dilation of the bile duct on the liver side. No bile duct epithelial regeneration was observed

									with the native bile duct	
Belzer *et al* [44], 1965	*Ann Surg*	Femoral vein (20)	T-tube	Goat (20)	Patch (3.0-4.0)	Patch (20)	Maximum 11 mo	BDS itself	Necrosis of autologous tissue, narrowing of the lumen of autologous tissue, narrowing of the anastomosis with the native bile duct, necrosis of BDS itself, narrowing of the BDS lumen, and narrowing of the anastomosis with the native bile duct	Only 3 out of 20 dogs were intentionally killed, but no good bile duct epithelial regeneration was observed
Lindenauer and Child [47], 1966	*Ann Surg*	Vascularized jugular vein (14)	(-)	Dog (14)	Ring (unknown)	CBC (14)	Maximum 18 mo	BDS itself	The omentum increased blood flow to the BDS; however, it resulted in scar contraction. Necrosis of BDS itself, narrowing of the BDS lumen, and narrowing of the anastomosis with the native bile duct	No dog survived for more than 3.5 mo, although the omentum was used to maintain BDS blood flow
Palmes *et al* [48], 2009	*J Invest Surg*	External jugular vein (18)	PLA stent (12)	Pig (18)	Ring (2.0)	CBC (18)	Maximum 6 mo	BDS itself	When the stent was not inserted, the BDS was necrotic. When the stent was inserted, the BDS lumen was preserved, but eventually, it became necrotic and narrowed	Of the 18 dogs, all 12 stented dogs were deliberately killed. However, the process of regeneration of veins into the bile duct was not reported
Liang *et al* [49], 2012	*World J Gastroenterol*	Omentum (8)	Bioabsorbable stent	Pig (8)	Ring (0.5-1.0)	CBC (8)	Maximum 4 mo	BDS itself	The BDS lumen was preserved when the stent was inserted	The bile duct defect was repaired with an omentum, which was similar to inserting a T-tube into the defect. Bile duct regeneration was also poorly demonstrated

BDS: Bile duct substitute; CBC: Common bile duct to bile duct substitute to common bile duct; CBJ: Common bile duct to bile duct substitute to jejunum; GBC: Gallbladder to bile duct substitute to common bile duct; PLA: Polylactide acid.

[20] attached blood vessels to a BDS composed of polyvinyl sponge in dogs. Of the 12 dogs examined, 4 survived for more than 60 d, but the transplant was ultimately unsuccessful due to the formation of bile plugs inside the substitute. Dunphy and Stephens[19] examined Teflon as a BDS in four dogs and four sheep, but all failed because of severe rejection early in the transplantation. Recently, Gómez *et al*[23] investigated Gore-Tex as a BDS in 12 dogs and reported that 11 cases were successful. However, BDS was surrounded by strong fibrotic tissue and exhibited narrowing, indicating that it was not clinically usable in the long term (Table 2).

Currently, cyclic non-absorbable materials, such as polytetrafluoroethylene, are used clinically as artificial blood vessels. Anticoagulants are used to prevent blood from coagulating in artificial blood vessels when the blood vessel diameter is small[56]. Compared to blood, bile is more viscous and has a slower flow velocity. The bile duct diameter is similar to that of small blood vessels[57]. As such, this material may be unsuitable because the BDS lumen may be blocked by a bile plug[21,22]. In addition, the epithelium does not regenerate continuously at the anastomotic site between the BDS and native bile duct, scar contraction occurs in the long term after transplantation[38]. A bile plug may form if a tubular stent is inserted to secure bile passage, even when using non-bioabsorbable materials. This phenomenon

Table 2 Bile duct substitute using non-bioabsorbable material

Ref.	Journal	Substitute (*n*)	Stent	Animal type (*n*)	Size of BDS (cm)	Method of reconstruction of bile duct by BDS (*n*)	Observation period after implantation	Localization of regenerated bile duct	Causes of stenosis	Note (planned sacrificial death and epithelialization)
Dunphy and Stephens[19], 1962	*Ann Surg*	Teflon (8)	(-)	Dog (4), goat (4)	Ring (1.0)	CBC (8)	Maximum 7 wk	BDS outside	Narrowing of the anastomosis with the native bile duct; narrowing of the lumen of BDS	In all cases, the hepatobiliary enzyme levels increased, and no survivors were observed for more than 7 wk after transplantation
Bergan *et al*[20], 1962	*Arch Surg*	Vascularized polyvinyl sponge (21)	(-)	Dog (21)	Ring (0.5)	CBC (21)	Maximum 14 mo	BDS outside	Narrowing of the anastomosis with the native bile duct; narrowing of the lumen of BDS	Four out of 21 dogs survived for > 60 d after transplantation, and four of them had stenosis of BDS
Sherman *et al* [21], 1963	*Ann Surg*	Acrylamide with Dacron (33)	(-)	Dog (33)	Ring (1.5-3.5)	CBC (33)	Maximum 31 mo	BDS outside	Narrowing of the anastomosis with the native bile duct; narrowing of the lumen of BDS	Twenty-six out of 33 dogs died within 3 mo. In all cases, fibrotic thickening around the BDS and severe scar contraction had occurred at the site of anastomosis
Mendelowitz *et al*[22], 1982	*Am J Surg*	Gore-Tex (6), dacron (2)	(-)	Dog (8)	Ring (2.0-3.0), patch (2.0 cm × 1.0 cm)	Patch (Gore-Tex) (2), CBJ (Gore-Tex) (2), CBC (Gore-Tex) (1), CBC (Dacron) (2), GBJ (Gore-Tex) (1)	Maximum 40 d	BDS outside	Narrowing of the anastomosis with the native bile duct; narrowing of the lumen of BDS	In all cases, a bile plug was found in the lumen of the BDS, and a high degree of fibrotic thickening was found around the site of anastomosis
Gómez *et al*[23], 2002	*J Gastrointest Surg*	Gore-Tex (12)	(-)	Dog (12)	Ring (2.0-3.0)	CBC (12)	Maximum 3 mo	BDS outside	Narrowing of the lumen of BDS	Eleven out of 12 dogs were intentionally killed, but severe fibrotic thickening was observed around the BDS

BDS: Bile duct substitute; CBC: Common bile duct to bile duct substitute to common bile duct; CBJ: Common bile duct to bile duct substitute to jejunum; GBJ: Gallbladder to bile duct substitute to jejunum.

is similar to current endoscopic stenting for bile duct stenosis[58,59].

BDS using bioabsorbable material

Due to the failure to develop clinically applicable BDSs made from autologous tissue or non-bioabsorbable materials, increasing focus has been placed on bioabsorbable materials as BDSs. This concept is based on the technique of tissue engineering proposed by Langer and Vacanti[60] in 1993 for bile duct regeneration. The complex formed by the bioabsorbable material and cells attached to the material is absorbed in the body while the bioabsorbable material acts as a scaffold to maintain the environment and shape of the organ[60,61]. Concurrently, the cells attached to the scaffold regenerate the target organ. Natural polymers (particularly collagen)[24,27,28] and engineered synthetic polymers [25,26,62-64] have been investigated as BDSs based on bioabsorbable materials.

BDS using natural polymers

Alternative natural polymers to collagen have been investigated, such as the small intestinal submucosa (SIS) using porcine submucosa. Using this SIS, Rosen *et al*[24] reported that 9 cases of patch-like transplantation and 6 cases of circular transplantation were performed as BDS, and 13 of them were successful. However, bile duct regeneration was not observed as a pathological finding. On the other hand, in another study using SIS as a BDS, scar contraction was high[65]. As such, this material has not been clinically applied as a BDS (Table 3).

Nakashima *et al*[66] reported the successful use of collagen as a BDS in consideration of cell adhesion. However, collagen was attached to a non-bioabsorbable material (polypropylene) to maintain the hardness of this BDS; therefore, it may not be a strictly bioabsorbable BDS. The BDS scaffold was sutured to the native bile duct. As bile passes through the lumen, the scaffold must maintain an annular shape and therefore a degree of hardness. Collagen tends to lose its shape when immersed in water[66, 67]. Collagen-based BDSs require hardness in other substances to maintain the scaffold hardness, resulting in an absorption period longer than several months. For this reason, SIS uses collagen and submucosal tissue to maintain hardness[24].

To promote bile duct regeneration within the bioabsorbable material itself, the period of *in vivo* absorption of the bioabsorbable material is important for suppressing scar contraction. In the case of artificial skin using collagen for skin regeneration, it has been reported that the half-life of the bioabsorbable material, which most strongly suppresses scar contraction, is approximately 14 d[67]. If a BDS made of a bioabsorbable material has a half-life of 3-4 wk or more, scar contraction may be high. When bioabsorbable materials other than the skin are used as alternatives for bile duct regeneration, the absorption period of such materials must also be considered, but this is not the case for SIS[24].

In collagen-based BDSs, the density of the material ligand is important for cell adhesion to promote bile duct regeneration[68]. However, there is a paucity of research in this area. If attempts are made for bile duct regeneration within the bioabsorbable material itself, scar contraction of the BDS part cannot be suppressed after transplantation unless these points are taken into consideration.

BDS using synthetic polymers

BDSs based on synthetic polymers are predominantly composed of polyglycolic acid (PGA)[26] and polycaprolactone[24,62], BDSs produced using PGA fibers may fail to maintain their radial shape. In many studies, the major axis of the bile duct has been replaced by approximately 1 cm. The absorption period of PGA is approximately 3 mo; as such, chronic inflammation occurs, and bile duct regeneration occurs at the implanted site. As such, attempts to regenerate the bile duct epithelium on the inner surface (bile passage surface) of this BDS eventually cause scar contraction similar to that of non-bioabsorbable BDS, and previous efforts have been unsuccessful[24,61].

Studies employing polycaprolactone-based BDSs have reported good bile duct regeneration outside the BDS implant. The material in these studies comprised a 50:50 copolymer of lactic acid and caprolactone, which was reinforced with latticed PGA fibers to facilitate suturing[25]. Generally, the absorption period is 6-8 wk, and the material becomes vulnerable *in vivo* for approximately 3 wk. After the BDS becomes fragile and sheds into the duodenum, a bile duct thicker than the outer circumference of the BDS regenerates outside the BDS. Cells migrating to the BDS as a foreign body reaction may promote bile duct regeneration[25]. Previous studies have reported good results, including BDS experiments in infectious reservoirs[69]. The occurrence of bile duct regeneration on the outside of the BDS resembles bile duct regeneration after insertion of a T-tube into a defective bile duct followed by T-tube removal (Table 3).

As BDSs using synthetic polymers can be engineered, the period of absorption in the body can be adjusted[70]. However, if the BDS is hard to maintain the shape of the bile duct, the absorption period may be prolonged. As BDS results in longer chronic inflammation due to a foreign body reaction, the results are likely to be similar to those of non-bioabsorbable materials. In contrast, if the absorption period is too short, the BDS transplantation site becomes fragile and is destroyed after transplantation. Bile may flow out of the BDS transplantation site, making this material unsuitable for BDS. With regard to cell adhesion, the scaffold itself (for example, PGA only) has lower cell adhesion compared to collagen-based scaffolds owing to the lack of receptors for cell adhesion[38]. Therefore, to regenerate the scaffold itself within the bile duct, materials in which receptors are added to the scaffold have been investigated[38]. Failure of the bile duct to regenerate in the scaffold part while being absorbed prevents clinical application as a BDS; hence, these materials remain in the development stage.

BDS made of other materials

In recent years, attempts have been made to utilize decellularized tissues as BDSs because of the lack of an immune reaction when transplanted into a living organism[29,30]. The use of decellularized tissues as scaffolds for liver regeneration has been investigated. The bile was reported to drain into the duodenum as a tube for a prolonged period and functioned while the stent was inserted. However, reports of long-term bile duct regeneration and function after stent removal are lacking.

Scaffolds of the same shape as that of the extrahepatic bile duct have been produced using 3D printing[41,42]. The extrahepatic bile duct has been reported to regenerate in a ring shape, but no

Table 3 Bile duct substitute using bioabsorbable material

Ref.	Journal	Substitute	Stent	Animal type (*n*)	Size of BDS (cm)	Method of reconstruction of bile duct by BDS (*n*)	Observation period after implantation	Localization of regenerated bile duct	Causes of stenosis	Note (planned sacrificial death and epithelialization)
Rosen *et al* [24], 2002	*Surgery*	SIS	(-)	Dog (15)	Patch: (2.0 cm × 1.0 cm), ring: (2.0-3.0)	Patch (9), CBC (6)	Maximum 5 mo	BDS itself	Scar contraction at the site of anastomosis on the duodenal side	Thirteen out of 15 dogs were intentionally killed. The regenerated bile duct tissue was shown consequently, and the process of regenerating the bile duct was not shown
Miyazawa *et al*[25], 2005	*Am J Transplant*	P (CL/LLA) with PGA	(-)	Pig (18)	Ring (3.0)	CBJ (18)	Maximum 6 mo	BDS outside	No narrowed BDS	All pigs were intentionally killed. A good bile duct regeneration process was shown
Nau *et al* [26], 2011	*HPB (Oxford)*	PGA and TMC	5 Fr pancreatic stent	Dog (11)	Ring (1.0)	CBC (11)	Maximum 12 mo	BDS itself	Narrowing of the BDS lumen	Ten out of 11 dogs were intentionally killed. No good bile duct epithelial regeneration was observed
Li *et al*[27], 2012	*Biomaterials*	Collagen with bFGF	(-)	Pig (26)	Patch (2.0 cm × 1.0 cm)	Patch (26)	Maximum 6 mo	BDS itself	No narrowed BDS	All pigs were intentionally killed. The regenerated bile duct tissue was shown consequently, while the process of regenerating the bile duct was not shown
Tao *et al*[28], 2015	*Artif Organs*	Collagen	Plastic stent	Pig (20)	Patch (2.0 cm × 0.6 cm)	Patch (12)	Maximum 12 wk	BDS itself	No narrowed BDS	All pigs were intentionally killed. The regenerated bile duct tissue was observed consequently, while the process of regenerating the bile duct was not shown
Tanimoto *et al*[62], 2016	*Langenbecks Arch Surg*	P (CL/LLA)	T-tube	Pig (11)	Ring (2.0)	CBC (11)	Maximum 6 mo	BDS itself	No narrowed BDS	All pigs were intentionally killed. A high degree of fibrosis was observed in the regenerated bile duct tissue
de Abreu *et al*[63], 2020	*J Biomater Appl*	Bacterial cellulose film	T-tube	Pig (10)	Patch (2.0 cm × 1.0 cm)	CBC (20)	Maximum 330 d	BDS itself	No narrowed BDS	All pigs were intentionally killed. No process of bile duct regeneration was shown

BDS: Bile duct substitute; bFGF: Basic fibroblast growth factor; CBC: Common bile duct to bile duct substitute to common bile duct; CBJ: Common bile duct to bile duct substitute to jejunum; P(CL/LLA): Polyε-caprolactone/poly l-lactide; PGA: Polyglycolic acid; SIS: Small intestinal submucosa; TMC: Trimethylene carbonate.

studies to date have examined bile duct regeneration using these BDSs. Furthermore, there have been efforts to develop an actual bile duct as an organoid *in vitro* for transplantation[70]. However, it has not been possible to produce organoids of a certain length in the longitudinal direction for clinical use and with a length that can be sutured.

FACTORS TO CONSIDER FOR BDS DEVELOPMENT

Below, we discuss the factors that should be considered in the development of clinically applicable BDSs.

Bile duct wound healing

Bile duct regeneration must occur concurrently with wound healing[40,50,51]. Notably, the natural course of wound healing involves scar contraction. Therefore, if the BDS does not reduce scar contraction, the migrating cell mass results in scar contraction and lumen narrowing after removal of the BDS or decomposition and absorption[35,51]. For the BDS to promote bile duct regeneration, the BDS implant must be guided into the regeneration process while maintaining the shape of the bile duct rather than increasing scarring of the cell mass[40]. As such, which allows the cell mass that regenerates the bile duct to maintain the shape of the bile duct when the BDS is absorbed or removed.

A BDS made of a non-bioabsorbable material that has been present in the body for a prolonged period retains its shape until the BDS is removed, but the lumen is prone to clogging by bile plugs[20-22]. Further, the anastomotic site with the native bile duct will be narrowed due to connective tissue growth[50,51]. Current bile duct regeneration methods using BDSs suggest that after the cell mass gathers around the foreign body and the BDS forms the shape of the bile duct, the cell mass regenerates as a bile duct after BDS removal[25]. If the BDS cannot maintain the shape of the bile duct, a stent may be used instead.

Studies have demonstrated that bone marrow-derived and adipose-derived cells are effective in suppressing scar contraction[72,73]. In addition, it has been reported that fibrosis is suppressed by controlling the function of macrophages that have migrated to the injured region during the remodeling period of wound healing[40,51]. However, methods for reliably suppressing scar contraction have yet to be developed[72,73]. For bile duct regeneration and retention of normal tissue structure, it is necessary for the dynamics and function of multiple types of cells that have migrated around the BDS to be tightly regulated and to reduce scar contraction.

Bile properties

Compared to blood, bile is more viscous and has a slower velocity[57]. If a BDS is not absorbed in the body, a bile plug may adhere to the BDS at the anastomosis site with the lumen or native bile duct. This results in impaired bile flow and narrowing of the lumen. In studies using Teflon (a non-bioabsorbable material) as a BDS, anticoagulants are often used even for blood. Therefore, further measures are required to prevent bile plug formation. This resembles the insertion of metal or tube stents for the treatment of bile duct stenosis, which often clogs the stent with a bile plug[58,59].

Bile duct regeneration

The extrahepatic bile duct is a component of the digestive tract, and the mature bile duct is regenerated *via* a regeneration process similar to that of the stomach and intestine[36,74,75]. In pigs, it takes approximately 6 mo for bile duct stem/progenitor cells to undergo regeneration to form a mature bile duct similar to the native bile duct. Furthermore, the bile duct epithelium is covered with a layer of cubic columnar epithelial cells[25] (Figures 1 and 2). Therefore, it is controversial in studies that do not show the process of bile duct regeneration, even if BDS transplantation is reported to be successful. For example, in the subsequent regeneration of the T-tube insertion site, the portion of the hole with the T-tube inserted reproduced well without any narrowing. This suggests that small partial bile duct regeneration often occurs without stenosis even if a patch of omentum or blood vessel is applied externally[76]. However, if bile duct defects are extensive and the BDS is placed between the native bile ducts, the cell mass must undergo substantial regeneration from the early stage of bile duct regeneration to regenerate the bile duct without stenosis. Studies have demonstrated that cells attached to the bioabsorbable material regenerate the bile ducts, but the mechanisms by which these cells undergo bile duct regeneration in the presence of the bioabsorbable material remain unclear[24].

Immature cells attached to the scaffold migrate using the scaffold of the BDS as a foreign substance [40,50]. These cell masses first form an assembly of the peribiliary gland[25,74,75]. When good bile duct regeneration is achieved approximately 2 mo after the initial stage of regeneration, a tall papillary shape is formed on the inner surface through which bile passes. In pigs, after approximately 3 mo of bile duct regeneration, these peribiliary glands fuse and the epithelial cells become shorter; within approximately 6 mo, these structures mature into bile ducts that are similar to native bile ducts (Figures 1 and 2)[25].

With regard to the mechanisms of bile duct regeneration in the injured part of the bile duct, activated bile duct cells mobilize immune cells, vascular cells, and mesenchymal cells to the inflamed region as a ductular reaction during bile duct ligation and inflammation[77]. This process is involved in regeneration of the inflamed area. Inflammation-activated bile duct cells secrete chemokines, cytokines, and angiogenic factors, which are involved in wound healing[35,50,51]. Furthermore, reactive ductal cells generated *via* complex mechanisms depend on the nature and intensity of bile duct injury[35,50,51]. However, further investigations of bile duct regeneration mechanisms in various bile duct injuries, such as circular transplantation of BDS, are required to develop effective BDSs.

Regeneration of anastomotic site

When the anastomosis between a BDS and the native bile duct is narrowed, the flow of bile into the duodenum is obstructed, which limits the clinical application of the BDS. In a normal gastrointestinal anastomosis, a large anastomotic hole and fixed shape that does not experience deformation are

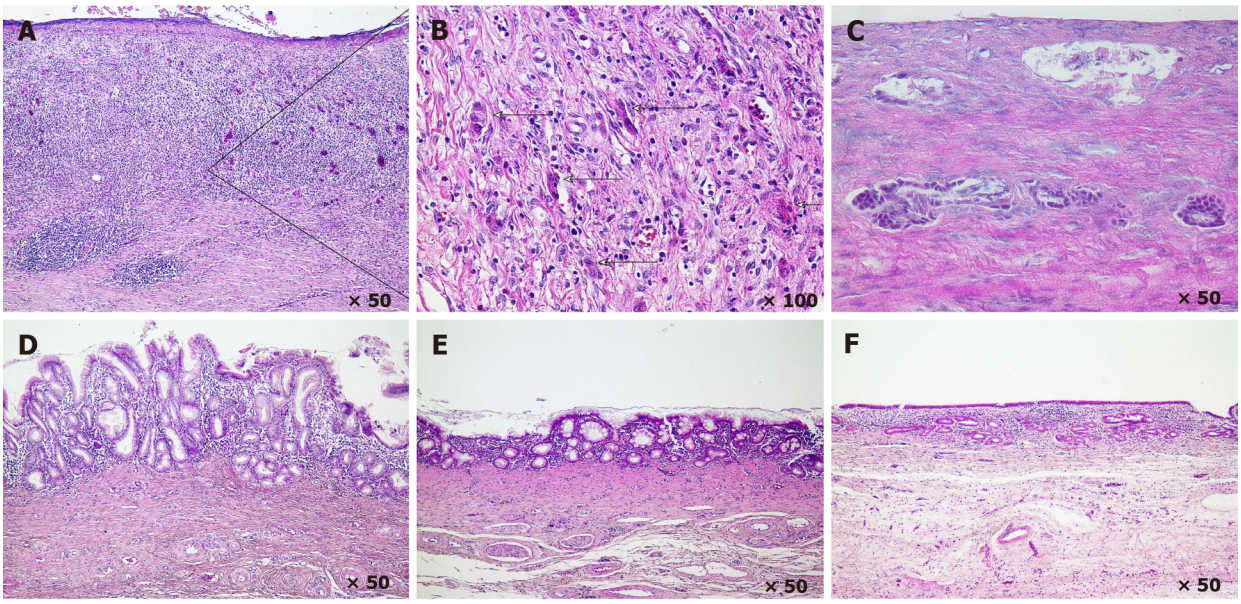

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Figure 2 Histology of bile duct regeneration on the outside of the short-term absorption type bile duct substitute. A and B: At 3 wk after bile duct substitute (BDS) implantation, a cell population in the stroma that may comprise the origin of the peribiliary gland is observed; C: At 5 wk after BDS implantation, a ring-shaped biliary gland-like structure is observed in which cell masses that are thought to form peribiliary glands are fused; D: At 7 wk after BDS implantation, many bile duct appendages are observed on the bile passage surface, and the epithelial surface exhibits a high papillary morphology; E: At 12 wk after BDS implantation, the number of peribiliary gland on the bile passage surface is decreased, the appendages begin to fuse, and the epithelial surface becomes more even; F: At 6 mo after BDS implantation, the epithelial surface becomes a single layer of cubic columnar epithelium, similar to the native bile duct.

prerequisites to prevent narrowing of the anastomosis[78]. Therefore, in surgical practice, a stent is inserted when the anastomotic hole is small[78]. For BDSs made from non-bioabsorbable materials, the extrahepatic bile duct is thin; hence, the anastomotic site with the native bile duct is likely to be narrowed by connective tissue in the absence of stent insertion. To prevent anastomotic stenosis between the BDS and the native bile duct, it may be necessary to insert a stent through the anastomotic site to maintain its shape. Even for BDSs made from bioabsorbable materials, it may be difficult to maintain the shape of the anastomotic site and stent insertion is recommended to prevent stenosis.

Localization of bile duct regeneration to BDS

Three regenerative localizations of the neo-bile duct with respect to the BDS have been identified: Outside the BDS, within part of the BDS itself, and inside the BDS (bile passage surface). If the BDS is present for a prolonged period, chronic inflammation will persist[50,51]. As such, it is unlikely that the cell mass that has migrated due to detection of the BDS as a foreign substance will regenerate into a structure similar to the native bile duct at the site of the BDS in the context of wound healing[20-22,51].

If the BDS is present for a prolonged period, the bile duct does not regenerate outside the BDS or as part of the BDS itself. Bile duct stem cells do not appear to adhere to the luminal surface of non-living non-absorbable materials, such as T-tubes. Given that the bile duct does not tend to narrow after T-tube removal, a cell mass that has migrated outside the BDS is formed. In the absence of BDS, the cell mass is exposed to fresh bile and regenerates as a bile duct, which may promote good bile duct regeneration at the BDS transplant site[25] (Figures 3 and 4).

The use of decellularized tissue as a BDS is thought to promote bile duct regeneration by cell adhesion to the luminal surface or inside the scaffold[29,30]. For BDSs made of bioabsorbable materials with a short absorption period, cell clusters contributing to bile duct regeneration migrate to the outside of the BDS and regenerate the bile duct. This bioabsorbable material may become fragile in approximately 3 wk and shed to the duodenal side, after which the cells surrounding the BDS regenerate the bile duct[25] (Figures 3 and 4). Although further research is needed, the extant literature suggests that the cell mass forms a ring-shaped structure more rapidly than during chronic inflammation and subsequently disappears from the site. Collectively, these findings suggest that bioabsorbable materials that induce good bile duct regeneration may be harnessed as effective BDSs.

RESEARCH LIMITATIONS

One study reported that when a stent was placed in the BDS, bile flowed through the stent for a certain period, resulting in a successful BDS. However, the study did not demonstrate bile duct regeneration.

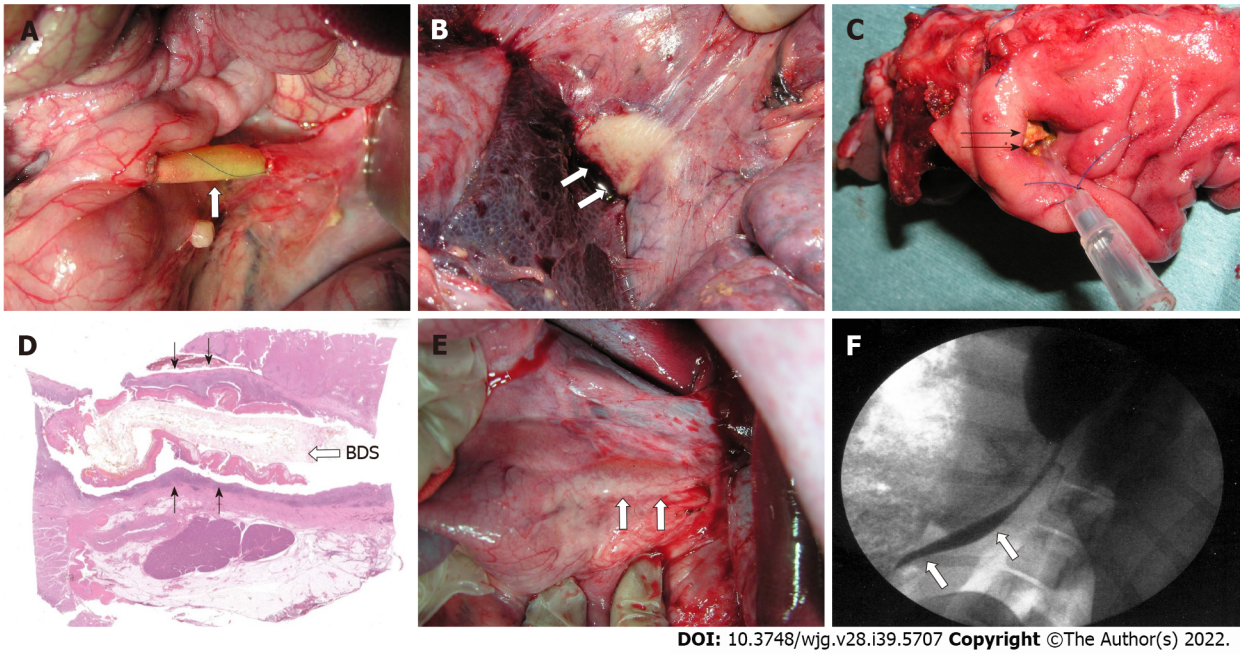

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Figure 3 Bile duct substitutes using bioabsorbable material (synthetic polymer with short absorption period). A: A bile duct substitute (BDS) using bioabsorbable material is implanted to bypass the extrahepatic bile duct (3 cm in size). Three weeks post-BDS implantation; B: White cell clusters are observed on the outside of the BDS; C: A vulnerable BDS is observed from the duodenal side; D: Dark purple connective tissue is noted on the outside of the BDS; E: At 6 mo after BDS implantation, the neo-bile duct is macroscopically similar to the natural common bile duct have been regenerated (arrow); F: Cholangiography (6 mo after BDS implantation). The BDS implant and anastomotic site become unknown, and the contrast medium flows smoothly into the duodenum (white arrows).

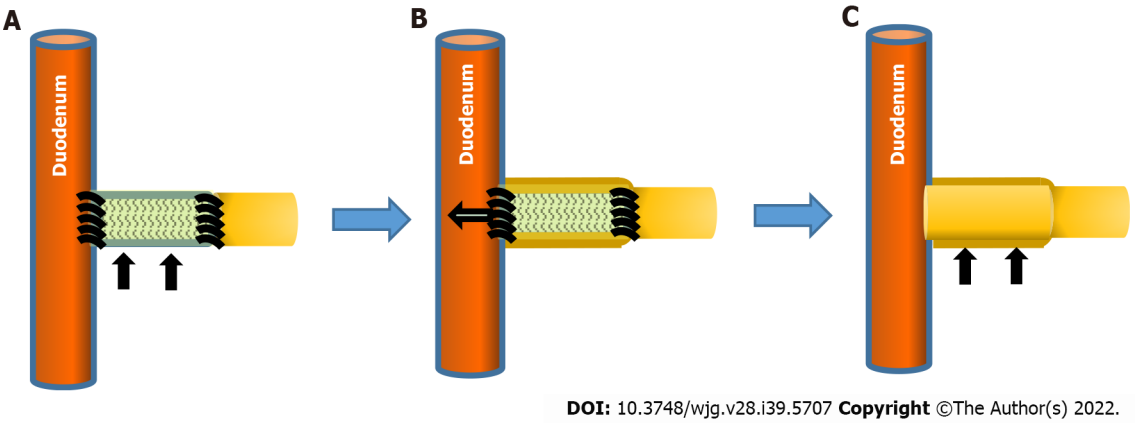

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Figure 4 Regeneration of the neo-bile duct outside the short-term absorption type bile duct substitute. A: Bile duct substitute (BDS) (black arrows) is anastomosed to the native extrahepatic bile duct; B: Immature cells attach around the BDS, forming a cylindrical cell mass outside the BDS. The bioabsorbable polymer that comprise BDS becomes fragile in the living body from approximately 3 wk and sheds to the duodenal side; C: After BDS is no longer present at the transplant site, immature cell clusters mature as bile duct cells and the bile ducts are regenerated as tissue (black arrows).

This process is similar to that of stent insertion during the treatment of benign bile duct stenosis. Nevertheless, the alternative portion eventually becomes stenotic and clinically unusable over a prolonged period. Our analysis was unable to identify these issues accurately. Although numerous studies using various BDSs have been conducted, the lack of success highlights the limitations of the field. Moreover, we did not analyze successful cases in small animals or studies using a small number of large animals.

FUTURE PERSPECTIVES

Extant research has provided novel insights into the mechanisms of repair after tissue damage[75]. If the injured area does not undergo normal repair, a high degree of fibrosis will occur in the injured area, resulting in scar formation at the site. For bile duct injuries, the injured area or the BDS implant may

become stenotic. For the regeneration of a bile duct similar to the native bile duct at the injury site, cells that have migrated to the bile duct injury site must suppress scar contraction, similar to the regeneration of other organs. In this regard, it is necessary to create a niche for bile duct regeneration similar to that of the native bile duct[73,75]. In addition, chronic inflammation is associated with prolonged and severe fibrosis, resulting in scar formation. Therefore, a BDS should not remain at the transplantation site for a prolonged period.

Based on these caveats, two methods can be considered for regenerating a bile duct similar to the native bile duct at the BDS transplantation site. The first involves the use of a short-term absorption type of bioabsorbable BDS, in which cells that have migrated to repair bile duct injury (BDS transplantation site) form the shape of the bile duct *via* a foreign body reaction. Ultimately, the BDS will no longer be present at that site and chronic inflammation does not occur (*i.e.*, the bile duct regenerates outside the BDS)[25] (Figure 4). The second method involves bile duct stem/progenitor cells adhering to part of the BDS itself, and wound healing is regulated such that bile duct regeneration progresses well while the BDS is being absorbed. Therefore, it is necessary to develop a method to control wound healing so that the scaffold portion of the decellularized tissue and adhering cells do not cause scar contraction. In summary, bioabsorbable BDSs for bile duct regeneration outside the bile duct constitute a promising development that will be clinically useful in the future.

CONCLUSION

To date, successful BDSs have not been developed. This is predominantly due to poor mechanistic understanding and lack of methods for regulating bile duct wound healing and bile duct regeneration. As an alternative to the extrahepatic bile duct, bioabsorbable materials can be used to form the shape of the bile duct, and the cell mass forming the shape of the bile duct can migrate to the external surface of this structure. Once the cell mass was able to maintain the shape of the bile duct, the BDS acting as a scaffold was removed. The development of BDSs that enable this process will permit the treatment of a wide range of bile duct defects.

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FOOTNOTES

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MINIREVIEWS

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Abstract

The novel coronavirus disease 2019 is an infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and was declared a global pandemic with more than 500 million reported cases and more than 6 million deaths worldwide to date. Although it has transitioned into the endemic phase in many countries, the mortality rate and overall prognosis of the disease are still abysmal and need further improvement. There has been evidence that shows the significance of SARS-CoV-2-related liver injury. Here, we review the literature on the various spectrum of SARS-CoV-2 infection-induced liver injury and the possible mechanisms of damage to the hepatobiliary system. This review aimed to illustrate the latest understanding regarding SARS-CoV-2-induced liver injury including the high-risk populations, the characteristic clinical manifestations, the possible pathogenic mechanism, the pathological changes, the current suggestions for clinical treatment for various spectrum of populations, and the prognosis of the condition. In conclusion, SARS-CoV-2 patients with a liver injury warrant close monitoring as it is associated with the more severe and poorer outcome of the infection.

Key Words: COVID-19; SARS-CoV-2; Pandemic; Liver injury; Pandemics; Prognosis

Core Tip: There are several reviews in the literature that discuss the pathophysiology, management, and outcomes of liver injury in coronavirus disease 2019 (COVID-19). Here, we reviewed the current understanding on various aspects of COVID-19-related liver injury, including the high-risk populations, the characteristic clinical manifestations, the possible pathogenic mechanism, the pathological changes, the current suggestions for clinical treatment for the spectrum of populations, and the prognosis of the condition.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the name given to the newly emerged zoonotic virus that causes coronavirus disease 2019 (COVID-19)[1]. It was first reported in Wuhan, China on December 29, 2019 and was declared a global pandemic on March 11, 2020[2]. SARS-CoV-2 is an enveloped, single-stranded positive-sense RNA genome virus that harbors the largest genome among currently known RNA viruses, with a genome length of around 26 to 32 kb. It has an oval shape and an average size of 100 nm in diameter. Electron microscopy revealed large club-shaped spikes of glycoprotein membrane on the viral surface making the viral particles appear like a typical crown-like shape[3].

COVID-19 is a syndrome with various systemic and respiratory symptoms such as fever, fatigue, dry cough, and breathing difficulties. It can be critical, causing severe pneumonia and cardiorespiratory failure that requires specialized management in intensive care units[4]. SARS-CoV-2 can also affect other systems, namely the nervous system causing headache, anosmia, paresthesia, and altered consciousness[5]. Abnormal liver function parameters are commonly found in patients with SARS-CoV-2 infection, indicating that SARS-COV-2 infection is associated with liver injury and even failure. Apart from that, several studies suggested that liver injury has a significant role in determining the severity and mortality rate of the disease. Considering the ongoing global threat of SARS-CoV-2 infection and the necessity to improve the prognosis of the disease, the treating physicians need to be aware of the association and significance of SARS-CoV-2 infection-related liver injury not only for the severity of the disease but also for the mortality rate and prognosis as a whole. Therefore, this review aimed to elucidate the importance of hepatobiliary involvement in SARS-CoV-2 infections and provide helpful information for managing the condition and improving the overall prognosis of the disease.

HIGH RISK POPULATIONS OF SARS-COV-2-INDUCED LIVER INJURY

Since the beginning of the pandemic, it was reported that patients with severe SARS-CoV-2 infection tended to develop liver injury compared to mild infection. Cai *et al*[6] reported that male patients of older age and higher body mass index have a higher tendency to develop liver injury during the course of the disease. A similar finding was seen in a study on 79 non-hospitalized SARS-CoV-2 patients by Xie *et al*[7], who reported that liver injury was more common among male patients. The authors also said that patients with an underlying severe chronic lung disease have a higher rate of liver injury, which was also reported by Zhang *et al*[8]. Cai *et al*[6] and Singh and Khan[9] both found that liver injury was more common among patients with underlying liver disease. According to Da *et al*[10], the common etiology of chronic liver disease that is prone to developing worsening liver injury during the infection is alcohol-related liver disease. Patients with nonalcoholic fatty liver disease and nonalcoholic steatohepatitis are usually associated with additional metabolic risk factors, such as obesity, that can increase the susceptibility to the infection and is commonly associated with a more severe presentation[11].

There has been significant concern about the increased susceptibility to SARS-CoV-2 infection among solid organ transplant recipients. In a systematic review by Piedade and Pereira[12], patients with liver transplant were not associated with an increased risk of SARS-CoV-2 infection. The risk is highly dependent on the sex, age, body mass index, history of hepatocellular carcinoma, and the immunosuppression drug dose of the patient. However, the prevalence of severe infection was higher among liver transplanted patients. A study by Becchetti *et al*[13] found that alterations in liver enzymes among liver transplanted patients with SARS-CoV-2 occurs more commonly among hospitalized patients. In addition, Ali Malekhosseini *et al*[14] showed that the admission rate of liver transplanted patients to the intensive care unit was as high as 33.3%.

No evidence shows that pregnancy increases susceptibility to SARS-CoV-2-induced liver injury. Nevertheless, a retrospective cohort study involving 122 pregnant patients with confirmed SARS-CoV-2 infection by Can *et al*[15] found that 13.9% developed an abnormal liver function that was generally mild, where most of them were critically ill and had a longer stay in the hospital compared to the normal liver function group.

THE CHARACTERISTIC MANIFESTATIONS OF SARS-COV-2-INDUCED LIVER INJURY

The most common manifestations of SARS-CoV-2 induced liver injury was the elevation of liver enzymes, such as alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase, and alkaline phosphatase. In a meta-analysis completed in the first few months of the pandemic by Cai *et al*[6], about 25% of SARS-CoV-2 patients had increased liver enzyme levels, which showed a direct association with the disease activity. The prevalence of increased AST was higher than ALT levels and was positively correlated with the severity of cases, where the level was higher in patients with severe cases[7,8,15]. Lei *et al*[16] reported a significant association between inpatient mortality in SARS-CoV-2 infected patients and liver injury based on liver enzymes, specifically AST elevation.

In a study on 417 SARS-CoV-2 infected patients by Cai *et al*[6], 41.0% of patients had abnormal liver tests, and 5.0% had liver injury upon presentation to the hospital. Throughout hospitalization, 76.3% developed some form of abnormal liver function, and it was high enough to be considered liver injury in 21.5% of patients. A similar finding was reported by Fan *et al*[17], who conducted a retrospective single center study on 148 patients with SARS-CoV-2 infection, where 37.2% had an abnormal liver function at hospital admission. Patients with the abnormal liver function were also found to have an extended hospital stays. A retrospective study of 79 patients with SARS-CoV-2 by Xie *et al*[7] found that patients with an abnormal liver test had an extended stay in the hospital.

Phipps *et al*[18] reported that 21.0% of 2273 patients with SARS-CoV-2 infection had a moderate liver injury, which was defined as elevated liver enzymes two to five times above the upper limit of normal, and 6.4% had severe liver injury, which was defined as liver enzymes more than five times the upper limit of normal. In this study, 69% of the patients with liver injury required intensive care unit care. The reports also mentioned that severe liver injury was associated with elevated inflammation markers, including ferritin and interleukin 6 (IL-6).

PROPOSED PATHOPHYSIOLOGICAL MECHANISM OF SARS-COV-2-INDUCED LIVER INJURY

The exact pathophysiological mechanism of SARS-CoV-2-induced liver injury is still poorly understood, but evidence has shown it to be multifactorial (as shown in Figure 1). One of the factors is direct invasion of SARS-CoV-2, which has been suggested in several studies. The primary receptor for SARS-CoV-2 cellular entry is the angiotensin-converting enzyme 2 (ACE2) receptors, which are found not only in the lung parenchyma but also in other parts of the body[19], such as the brain[5], gastrointestinal tract, biliary tree, and liver epithelia[20]. Zhou *et al*[21] stated that SARS-CoV-2 patients with gastrointestinal symptoms had higher AST and ALT levels, which reflected that ACE2 receptor was expressed within the gastrointestinal tract and the biliary tree. However, even though the ACE2 receptor is expressed more within the biliary tree than the liver parenchyma, most studies showed a predominant pattern of parenchymal liver injury based on the elevated levels of AST and ALT rather than the damage to the bile ducts, which was reflected by increased gamma-glutamyl transferase and alkaline phosphatase[22].

Wu *et al*[23] found that almost 50% of SARS-CoV-2 infected patients who recovered from the disease had persistent virus shedding in their fecal specimens for more than 10 d after viral detection in respiratory tract samples became negative. This may further support the possibility of viral replication in the hepatobiliary system. Similarly, the previous SARS-CoV strains that caused an outbreak from 2002 to 2004 have also been shown to directly injure the liver parenchyma causing lobular inflammation and apoptosis of hepatocytes[24].

Apart from the direct viral-induced hepatocytopathic hypothesis, autoinflammatory mediated injury to the liver is another plausible explanation. Immune dysregulation can occur in severe SARS-CoV-2 infection, which the overactivation of the immune system will lead to systemic hyperinflammation in extreme conditions. This condition is called 'cytokine storm syndrome', which is a phenomenon that will not only cause pulmonary inflammation but also multiorgan involvement, including the nervous system causing encephalitis[25] and peripheral neuritis[26] and the liver causing acute hepatitis and even failure[27,28].

Drug-induced liver injury is also common in SARS-CoV-2 patients, as the medications used to treat the infection can be hepatotoxic. These include lopinavir/ritonavir, remdesivir, tocilizumab, and others

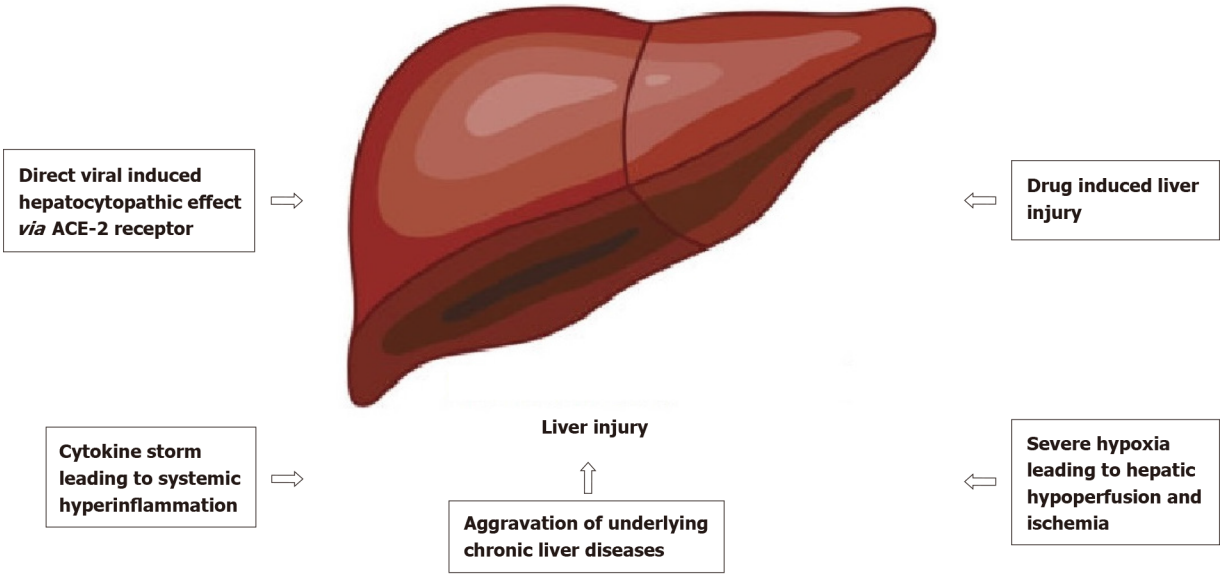

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Figure 1 Possible pathophysiological mechanisms of liver injury induced by severe acute respiratory syndrome coronavirus 2 infection.
ACE 2: Angiotensin-converting enzyme 2.

[29]. A study of 148 cases of SARS-CoV-2 infected patients in Shanghai by Li *et al*[30] found that the utilization rate of lopinavir/ritonavir among patients with abnormal liver function was higher than the patients with normal liver function. There was no significant difference in the pre-hospital medication between the two groups of patients. The exact mechanism of how lopinavir/ritonavir induces liver injury is still uncertain, but there is evidence that it activates the endoplasmic reticulum stress pathway in the liver and induces hepatocytes apoptosis[31].

Ritonavir is also widely metabolized by the liver through the cytochrome P450 system, where the production of toxic intermediates of any drugs that are metabolized by the system will have the potential of causing liver injury[32]. Tocilizumab, which is an IL-6 inhibitor that is used to reduce overactive inflammation, has been reported to cause drug-induced liver injury and liver failure, which in some cases requires a liver transplant[33]. The exact mechanism is still unknown but may be due to its inhibitory effect on the IL-6 pathway, which is essential for liver regeneration.

SARS-CoV-2 patients with underlying chronic liver diseases are more likely to suffer from liver injury. This may suggest that SARS-CoV-2 infection can aggravate underlying liver diseases. In addition, there is a possibility that the liver damage may be caused by the viral reactivation of existing liver diseases in SARS-CoV-2 infection. Some biological medications such as tocilizumab and baricitinib may cause reactivation of viral hepatitis B, which causes deterioration of liver function[34].

Another simpler hypothesis is that prolonged hypoxia and tissue ischemia in critically ill SARS-CoV-2 patients who suffer from severe pneumonia and acute respiratory distress syndrome can also be one of the mechanisms of liver injury and even failure[35]. This occurs due to prolonged tissue hypoperfusion leading to ischemia, including in the liver. The anaerobic metabolism and lactic acidosis will further depress the cardiorespiratory effort, which will cause the continuation of the vicious circle[36].

PATHOLOGICAL CHANGES IN SARS-COV-2-INDUCED LIVER INJURY

The first post-mortem autopsy on a patient who succumbed to SARS-CoV-2 infection was reported by Xu *et al*[37]. The liver histology showed a moderate degree of microvesicular steatosis with mild lobular and portal vein activity in the study. Ji *et al*[38] reported overactivation of T cells, suggesting viral-induced cytotoxic T cell liver damage. Liu *et al*[39] described various hepatic lesions, including focal lobular necrosis, lobular lymphocytic and monocytic infiltration, ballooning degeneration of liver cells, and sinusoidal congestion with microthrombosis. A study on 48 liver autopsies by Sonzogni *et al*[40] reported focal portal and lobular lymphocytic infiltrates with multiple vascular changes, which are suggestive of hepatic vascular involvement.

Tian *et al*[41] also reported a similar autopsy finding of mild lobular lymphocytic infiltration, with sinusoidal expansion of the central lobule and patchy necrosis in the periportal and centrilobular areas. There was no significant inflammatory cell infiltration around the portal tracts, which is consistent with the mode of acute liver injury. Autopsy reports on 7 SARS-CoV-2 infected patients who died noted multiple platelet-fibrin microthrombi in the hepatic sinusoids[42]. Wang *et al*[43] and Wang *et al*[44]

reported massive hepatic apoptosis, microvesicular steatosis, and inflammatory cell infiltration over the portal systems. In addition, there was a large amount of viral SARS-CoV-2 RNA titers detected in the liver *via* reverse transcriptase-polymerase chain reaction[41,45].

CURRENT MANAGEMENT OF SARS-COV-2-INDUCED LIVER INJURY IN VARIOUS POPULATIONS

Liver injury is a severe complication of SARS-CoV-2 infection and can significantly affect the outcome of the patient. Multiple studies have suggested regular monitoring of liver function parameters in SARS-CoV-2-infected patients. Based on the consensus statement of the American Association for the Study of Liver Diseases[46], it is recommended to consider etiologies outside SARS-CoV-2, such as other viral hepatitis. This has been proven in a case reported by Hambali *et al*[47], where a patient with SARS-CoV-2 infection presented with abnormal liver function and high IL-6, which was due to hepatocellular carcinoma. It is also essential to consider other indirect causes of liver injury such as myositis, cardiac injury, ischemia, and cytokine release syndrome. Patients with liver enzymes more than five times the upper limit of normal may be excluded but not contraindicated from using medications such as remdesivir, tocilizumab, and hydroxychloroquine. Every patient receiving the medications, especially remdesivir and tocilizumab should be regularly monitored for liver biochemical indicators regardless of baseline values. It should not be assumed that patients with autoimmune hepatitis and liver transplantation have a sudden onset of disease or acute cellular rejection without biopsy confirmation. Patients who are immunocompromised or are treated with immunosuppressive drugs should be considered at increased risk for SARS-CoV-2 infection and should be prioritized for testing[46].

SARS-CoV-2-infected patients with ongoing antiviral treatment for hepatitis B or C should be continued, but the initiation of antiviral treatment for hepatitis C may need to be delayed. Patients with an underlying liver disease requiring immunosuppressants should be continued in cases of mild infection, but in moderate to severe infection, the treatment dosage of calcineurin inhibitors should be reduced. The position statement from the European Association for the Study of the Liver-European Society of Clinical Microbiology and Infectious Diseases recommended that the dose of immunosuppressant drugs can be adjusted according to antiviral treatment regimens because the drugs in both regimens will likely interact with each other[48].

THE OUTCOME OF SARS-COV-2-INDUCED LIVER INJURY AND PREDICTORS OF INFECTION SEVERITY

The biomarkers of liver injury were significantly higher in severe cases of SARS-CoV-2 infection. In a meta-analysis by Henry *et al*[49], the severity and mortality rate of SARS-CoV-2 infection was related to the biomarkers of liver functions, which suggests that liver injury has a strong correlation with the severity of SARS-CoV-2 infection. A retrospective study that compared the clinical spectrum between patients with and without liver injury by Xie *et al*[7] found the hospitalization time was significantly longer in patients with liver injury. Lei *et al*[16] reported that abnormal AST in SARS-CoV-2 infection was associated with a higher risk of death during hospitalization than other indicators of liver injury.

Kulkarni *et al*[50] stated that the severity of elevated liver enzyme markers determined the outcome of SARS-CoV-2 infection, with the incidence of liver injury as high as 58%-78% among the death cases. A multicenter study involving 2780 SARS-CoV-2 infected patients by Singh and Khan[9] found that patients with underlying liver disease had higher mortality and hospitalization. In addition to the abnormal liver biochemistry, hypoalbuminemia during the illness is an important indicator of the severity of the SARS-CoV-2 infection. Both studies by Gong *et al*[51] and Huang *et al*[52] showed that hypoalbuminemia was associated with severe infection and an independent risk factor for death.

CONCLUSION

In conclusion, this review illuminated the significance of liver injury in SARS-CoV-2 infection based on the descriptions from the scientific literature. Although it is common and mild in the majority of cases, it is a strong predictor of the severity and a significant risk factor for the mortality rate of the disease, especially if it is associated with male sex, older age, the presence of other comorbidities or underlying chronic liver disease, and in severe respiratory symptoms. Therefore, it is prudent to monitor SARS-CoV-2-infected patients with liver injury and to individualize treatment for patients with an underlying disease who developed liver injury to improve the prognosis by delivering the appropriate management.

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FOOTNOTES

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ACADEMIC WRITING

All journals should include a correspondence section

Nikolaos Papanas, Dimitri P Mikhailidis, Debabrata Mukherjee

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Abstract

Letters to the editor can provide useful scientific information and evaluation of published work as well as acting as an additional level of peer review. Furthermore, letters are good reading material, especially if they involve a debate between authors. Finally, letters are relatively short. Therefore, inexperienced career researchers can use such an opportunity to practice putting together a cogent argument. However, it is far from an ideal situation if letters are the only (or main) type of article on which to base an academic career.

Key Words: Correspondence; Journals; Letters to the editor; Medical writing; Peer review; Debate

Core tip: Letters provide another level of worldwide peer review. Three editors express their opinions regarding the scientific value and structure of correspondence sections in journals.

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INTRODUCTION

In this brief overview, three editors express their opinions regarding the scientific value and structure of correspondence sections in journals. Interpretations and suggestions are based on experience and the literature.

THE NEED FOR A CORRESPONDENCE SECTION

We propose that a correspondence section is an essential part of all journals. The reasons are summarised as follows[1-3]: Letters provide an additional level in the peer review process. Essentially, anyone worldwide can comment on a publication. Letters often promote good reading, especially when they involve a debate between authors. This is especially true for journals that have letters openly available. Given that letters are short, they are relatively easy to write. Therefore, they provide a training opportunity for inexperienced authors. Letters do not count as items when the Clarivate journal impact factor is calculated but if they are cited, these citations count. Thus, any citations of a letter may prove helpful for journals. However, we also need to consider that most letters are probably not highly cited.

TIPS ON WRITING A LETTER

A general rule would be a short text (the shorter, the better); brevity is important[1,2]. Therefore, letters need to focus on a restricted number of topics. Most journals impose limits on the word count and number of references. Some journals allow inclusion of a figure or table in a letter[1,2]. However, some editors provide substantial flexibility. Most letters are usually related to publications in the same journal [1-3]. Indeed, some editors do not consider letters unless they relate to material published in their journal. There are broadly two types of letters[1].

Correspondence

This is the commonest type. Such letters aim at one of the following goals[1-3]: (1) To contradict a published finding, for example by citing omitted studies or presenting unpublished results. Letter authors may also wish to highlight methodological or statistical flaws in a published study; (2) To reinterpret a published finding; for example based on additional findings; and (3) To support a published finding; for example on the basis of additional findings, possibly unpublished. This may include indirect evidence (*e.g.*, involving a different gender, ethnicity, species, methodology or related disease).

Early unpublished findings or a case report/series

More rarely, letters present early (unpublished) findings or a case report/series[1,2]. Such letters are miniatures of full studies or case reports. Their main advantages for the authors include rapid publication and the ability to present data on smaller patient series[1,2]. Full papers take longer to be published and processed. This may even take several months and it is possible that during that time more recent and relevant findings become available. For the journals, a potential advantage of full papers (and reviews) is that they are likely to have a higher citation rate than letters.

One final tip for academics and clinicians: avoid exclusively writing letters to the editor without also authoring original or review articles[4]. Indeed, it has already been noted that some authors try to build their career solely on letters published in high-ranking journals[4]. This will be noticed by others and will not be to the authors' benefit.

SUGGESTIONS FOR JOURNAL EDITORS REGARDING MANAGING A CORRESPONDENCE SECTION

We suggest that all journals could benefit from a correspondence section as a peer review "safety net". One of us has resigned as Associate Editor from two journals, because they would not introduce a correspondence section on the grounds that it would require too much editorial work.

A dedicated editor for the correspondence section would be ideal. However, this may be impractical for some journals. One of us has recently experienced a 5-mo delay regarding a decision on a 300-word letter. In our opinion, this represents completely unacceptable standards by the editorial staff of this journal. However, this is probably and hopefully, a rare event.

Letters provide an opportunity for a rapid response by journal editors[1,2]. Based on our experience both as editors and authors, this may be, at least ideally, a matter of a few days. When letters refer to a specific publication, the authors of the latter usually provide a response, pointing out every possible

error.

What to do if authors decline to respond to a letter commenting on their work? There is no simple answer. Possibly, if a letter is highly critical of a study, it may be published together with an editorial message, stating that the authors of the original work declined to respond. It would be unfortunate if some authors avoid criticism just by refusing to respond to valid points raised in a letter. Again, this has happened to us, although the definition of valid comments is based on our knowledge/views. Nevertheless, in our opinion they were obvious. That is why, in similar circumstances, we prefer to underline that the letter containing criticisms will be published, whether the authors of the original work respond or not. Editors must not suppress valid criticism of a publication thinking that it may suggest an oversight of errors by the peer reviewers and editors involved. This is an example of how correspondence provides another valuable level of peer review. One of us is currently involved in resolving such a problem. Obviously, any improvements in peer reviewing are welcome, and are still being sought[5-7].

In defence of authors who refuse to respond to comments in a letter, we need to consider that responding may require considerable additional work, which they do not wish to carry out or would like to reserve for their next publication. In such circumstances, honesty is the best policy. The authors can just state why they cannot provide a detailed response at this time, but they will do so in their forthcoming work. However, the comments will remain in the literature. If they are not covered by future work, this deficiency may be pointed out. Citing an older letter to show that the queries raised were answered is not only professional behaviour, but will also suit the journal where the letter was published by delivering a citation.

Other editorial issues include whether to allow more than one round of exchanges regarding the same publication. The time allowed between publication of an item and the submission of related letters needs to be clearly stated in the instructions for authors.

Finally, in the event of an interesting but too long letter, an option may be to convert it to a commentary or brief communication.

CONCLUSION

Letters to the editor are useful for authors, readers and journals. They provide training for younger researchers and are another valuable level of peer review. For all these reasons, in our opinion as editors, a correspondence section is likely to be a useful part of all scientific journals.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Effects of COVID-19 on the liver: The experience of a single center

Valentina Liakina, Ieva Stundiene, Gabriele Milaknyte, Ramune Bytautiene, Rosita Reivytyte, Roma Puronaite, Gintare Urbanoviciute, Edita Kazenaite

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Abstract

BACKGROUND

The coronavirus disease 2019 (COVID-19) was perhaps the most severe global health crisis in living memory. Alongside respiratory symptoms, elevated liver enzymes, abnormal liver function, and even acute liver failure were reported in patients suffering from severe acute respiratory disease coronavirus 2 pneumonia. However, the precise triggers of these forms of liver damage and how they affect the course and outcomes of COVID-19 itself remain unclear.

AIM

To analyze the impact of liver enzyme abnormalities on the severity and outcomes of COVID-19 in hospitalized patients.

METHODS

In this study, 684 depersonalized medical records from patients hospitalized with COVID-19 during the 2020-2021 period were analyzed. COVID-19 was diagnosed according to the guidelines of the National Institutes of Health (2021). Patients were assigned to two groups: those with elevated liver enzymes (Group 1: 603 patients), where at least one out of four liver enzymes were elevated (following the norm of hospital laboratory tests: alanine aminotransferase (ALT) ≥ 40, aspartate aminotransferase (AST) ≥ 40, gamma-glutamyl transferase ≥ 36, or alkaline phosphatase ≥ 150) at any point of hospitalization, from admission to discharge; and the control group (Group 2: 81 patients), with normal liver enzymes during hospitalization. COVID-19 severity was assessed according to the interim World Health Organization guidance (2022). Data on viral pneumonia complications, laboratory tests, and underlying diseases were also collected and analyzed.

RESULTS

In total, 603 (88.2%) patients produced abnormal liver test results. ALT and AST levels were elevated by a factor of less than 3 in 54.9% and 74.8% of cases with increased enzyme levels, respectively. Patients in Group 1 had almost double the chance of bacterial viral pneumonia complications [odds ratio (OR) = 1.73, P = 0.0217], required oxygen supply more often, and displayed higher biochemical inflammation indices than those in Group 2. No differences in other COVID-19 complications or underlying diseases were observed between groups. Preexisting hepatitis of a different etiology was rarely documented (in only 3.5% of patients), and had no impact on the severity of COVID-19. Only 5 (0.73%) patients experienced acute liver failure, 4 of whom died. Overall, the majority of the deceased patients (17 out of 20) had elevated liver enzymes, and most were male. All deceased patients had at least one underlying disease or combination thereof, and the deceased suffered significantly more often from heart diseases, hypertension, and urinary tract infections than those who made recoveries. Alongside male gender (OR = 1.72, P = 0.0161) and older age (OR = 1.02, P = 0.0234), diabetes (OR = 3.22, P = 0.0016) and hyperlipidemia (OR = 2.67, P = 0.0238), but not obesity, were confirmed as independent factors associated with more a severe COVID-19 infection in our cohort.

CONCLUSION

In our study, the presence of liver impairment allows us to predict a more severe inflammation with a higher risk of bacterial complication and worse outcomes of COVID-19. Therefore, patients with severe disease forms should have their liver tests monitored regularly and their results should be considered when selecting treatment to avoid further liver damage or even insufficiency.

Key Words: COVID-19; SARS-CoV-2; Liver enzymes; Complications; Underlying disease; Disease severity

Core Tip: In our study, elevated liver enzymes were detected in 88.2% of patients hospitalized with coronavirus disease 2019 (COVID-19). Alanine aminotransferase and aspartate aminotransferase were elevated by a factor of less than 3 in 54.9% and 74.8% of cases, respectively. Regardless of underlying diseases, including hepatitis, these patients had higher biochemical indices of inflammation, required an O_2 supply, and exhibited bacterial pneumonia complications more often than those with normal liver tests. Male gender, older age, diabetes, and hyperlipidemia were confirmed as independent factors associated with a more severe course of COVID-19. All deceased patients (2.9%) had underlying diseases - most often heart disease, hypertension, and urinary tract infections.

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INTRODUCTION

In addition to the most common symptoms of coronavirus disease 2019 (COVID-19) - such as fever, dyspnea, sore throat, dry cough, headache, fatigue, restlessness, myalgia, anosmia, dysgeusia, and chest pain with ground-glass opacities seen on radiological investigations[1] - approximately half of patients

suffer from gastrointestinal symptoms such as a lack of appetite, nausea, and vomiting[2]. In some cases, gastrointestinal symptoms may precede respiratory symptoms or even occur as the sole symptom of COVID-19[3]. A wealth of evidence suggesting that elevated liver enzymes are also a common finding in COVID-19 pneumonia has already been published[4]. Depending on the population studied, elevated levels of liver enzymes– alanine aminotransferase (ALT) and aspartate aminotransferase (AST) - in the blood have been detected in the range of 14%-76%[5,6]. In patients with severe COVID-19, gamma-glutamyl transferase (GGT) and hypoalbuminemia have also been documented[7]. Although liver injury is often transient and is usually normalized without special treatment in mild cases of disease[8], in severe and critical cases it can be the first sign of life-threatening upcoming events such as acute liver failure[9].

However, the exact triggers of liver damage, how it affects patients, and whether it could predict the course and outcomes of COVID-19 itself remain unclear. To address this issue, this study examines liver enzyme abnormalities in patients hospitalized with COVID-19.

MATERIALS AND METHODS

Study design and participants

This is a retrospective cohort study of patients hospitalized at Vilnius University Hospital's Santaros Clinics during the 2020-2021 severe acute respiratory disease coronavirus 2 (SARS-CoV-2) viral pneumonia pandemic. The inclusion criteria for patients were as follows: documented SARS-CoV-2 infection, diagnosed according to NIH guidelines[10] based on manifestations of clinical pneumonia; positive real-time reverse transcription SARS-CoV-2 polymerase chain reaction (RT-PCR) test from nasopharynx swab specimens (MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit and TaqPath COVID-19 CE-IVD kit, Applied Biosystems); radiologically confirmed viral pneumonia; and age over 18 years.

Thus, exclusion criteria were: Age ≤ 18 years; patients with incomplete medical records, and negative SARS-CoV-2 test from nasopharyngeal swab specimen.

The depersonalized data of 684 patients were analyzed. Patients were assigned to two groups according to the results of liver tests: those with elevated liver enzymes (603 patients), where at least one of four liver enzymes were elevated (ALT ≥ 40, AST ≥ 40, GGT ≥ 36, or alkaline phosphatase (ALP) ≥ 150; following the norm of hospital laboratory tests) at any point of hospitalization from admission to discharge; or the control group (81 patients), with all four liver enzymes within normal range during hospitalization (Table 1). Depending on the severity of SARS-CoV-2 pneumonia - which was evaluated by radiological observation of lung damage (lung infiltration, pleura infiltration, ground glass opacities), level of respiratory failure (SpO_2, respiratory rate), and the overall clinical picture of the case – patients were assigned to the groups of moderate, severe, or critical COVID-19 pneumonia following the NIH COVID-19 disease guide (2022)[11].

Data collection

Depersonalized electronic medical records - including symptoms and clinical characteristics of COVID-19, laboratory and instrumental tests, therapeutic interventions, and outcome data - were collected for each patient. Demographic data included only age and gender. Underlying diseases were also recorded for all patients.

To confirm the diagnosis and evaluate the severity of COVID-19, the following tests were performed for all patients upon admission, and were repeated during treatment as required: throat roentgenogram; SARS-CoV-2 RT-PCR of nasopharyngeal swab; routine hematologic (full blood cell formula) and biochemical blood tests (troponin I, glucose, creatinine, blood urea nitrogen test, ferritin, procalcitonin, lactate, eGFR (CKD-EPI), ALT, AST, ALP, GGT, lactate dehydrogenase (LDH), bilirubin, C-reactive protein (CRP), interleukine-6 (IL-6), blood electrolyte tests (K, Na, Mg, Ca, Cl), coagulation tests (ADTL, Stago prothrombin assay, international normalized ratio, fibrinogen, D-dimers); and urea tests. In particular cases, an arterial blood analysis (pH, pCO_2, pO_2, HCO_3, SBC, ABE, SBE) was also performed, as well as additional instrumental, biochemical, and microbiological tests of blood and urea.

Hepatitis B and C markers, together with human immunodeficiency virus, cytomegalovirus, and Epstein-Barr virus markers as required, were also performed on admitted patients.

Statistical analysis

Quantitative data were presented as mean ± SD and range. Qualitative data were presented as numbers and percentages. The characteristics of the data distribution were evaluated using the Shapiro-Wilk test. Depending on data distribution normality, the difference in continuous variables between the groups of patients with elevated and normal liver enzymes was assessed using the Welch two independent sample *t*-test or the nonparametric Mann-Witney-Wilcoxon test. Pearson's chi-squared test was used to compare categorical variables between groups.

Table 1 Characteristics of the studied patients

Variables	Group 1 (with elevated liver enzymes)		Group 2 (with normal liver enzymes)		*P* value
		Number of tested		Number of tested	
n/%	603/88.2	603	81/11.8	81	
Male, *n*/%	356/59.0	356	28/34.6	28	< 0.0001
Female, *n*/%	247/41.0	247	53/65.4	53	
Age, yr ± SD	50.7 ± 9.5	603	51.9 ± 12.4	81	0.5075
Hospitalization, d ± SD	9.7 ± 5.9	603	8.7 ± 6.5	81	0.2039
ALT, U/L, range	149 ± 115, 40-728	603	22 ± 9, 7-39	81	< 0.0001
AST, U/L, range	90 ± 77, 3-818	552	22 ± 7, 11-39	70	< 0.0001
GGT, U/L, range	114 ± 125, 8-820	550	22 ± 8, 7-35	72	< 0.0001
ALP, U/L, range	101 ± 128, 29-1183	93	77 ± 25, 45-131	10	0.128
Bilirubin, μmol/L, range	9.1 ± 5.5, 3-67.1	460	7.6 ± 3.2, 3.2-18.5	61	0.0028
SPA, %, range	96.1 ± 19.5, 5-176	500	98.6 ± 21.9, 39-154	64	0.3868
INR, range	1.04 ± 0.18, 0.83-3.87	506	1.03 ± 0.11, 0.86-1.6	68	0.4728

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; INR: International normalized ratio; SPA: Stago prothrombin assay.

Univariate and multivariate logistic regression analysis was performed to assess the likelihood of the cohesion of the variables.

Data were considered statistically significant when the *P* value was < 0.05 for the confidence interval set at 95%. All statistical analysis was performed with the R software, version 4.1.2 (The R Project for Statistical Computing, r-project.org).

RESULTS

Elevated liver enzymes, especially ALT, were detected in the majority of patients hospitalized with SARS-CoV-2 pneumonia (603 out of 684). ALT and AST were elevated by a factor of less than 3 in 54.9% and 74.8% of cases with increased enzyme levels, respectively. Only 9.3% of the cases of elevated ALT, 2.7% of AST, and 11.2% of GGT were in concentrations higher than 300 U/L (Table 1).

In most patients (432, 71.6%), elevated ALT in the range of 41-728 U/L was detected on the first day of hospitalization. In almost half (209) of these patients, ALT increased to 80 U/L; in 91 patients, up to 120 U/L; and in only 17 patients did the level of ALT increase to more than 300 U/L (Table 1). ALT tended to rise during hospitalization and pneumonia treatment, and in some cases its level did not recede to the normal range even after SARS-CoV-2 infection recovery and discharge.

AST levels were elevated in 449 (72.1%) patients overall: for 241 (53.7%) of these patients, AST was found to be elevated in the range of 41-351 U/L on the first day of hospitalization; 159 patients had an up to two-fold elevation of AST; 50 patients up to 120 U/L; and only two patients had AST levels over 300 U/L (Table 1). Similarly to ALT, AST levels were prone to increase during the treatment of SARS-CoV-2 pneumonia, but for most patients, these levels returned to their normal range by the time of discharge.

GGT levels in the range of 40-820 U/L were detected in 438 (70.4%) of the patients tested, and in 353 (80.6%) patients, elevated GGT was detected on the first day of hospitalization. Up to two-fold elevated GGT was detected in 198 patients; 71 patients displayed CGT levels up to 120 U/L; and 25 patients displayed CGT levels over 300 U/L (Table 1). Like ALT, GGT tends to increase during hospitalization and slowly normalizes after patients recover from viral pneumonia.

ALP level was tested for only 103 patients, and most cases 89 (86.4%) were in the normal range (Table 1). Only 7 (6.8%) patients had up to three-fold elevated ALP, and in one patient with a critical course of COVID-19 ALP increased dramatically to 1183 U/L, along with ALT 162 U/L and AST 223 U/L. This patient suffered from acute liver and kidney failure, electrolyte and alkaline-acid imbalance, and sepsis aggravated by resistance to beta-lactam antibiotics, from which they did not recover.

Only 24 patients (3.5%) of the studied cohort had preexisting hepatitis of different etiologies (including two patients with chronic hepatitis C and two patients with chronic hepatitis B), and only two of these patients (8.3%), with hepatitis B in long-term remission, had normal levels of liver enzymes

at admission and during hospitalization. The other 22 (91.7%) patients had elevated liver enzymes, including two patients (8.3%) with chronic hepatitis C. Most patients with preexisting liver diseases had a moderate course of COVID-19 pneumonia (20, 83.3%), three (12.5%) had severe cases, and one patient (4.7%) had a critical course of COVID-19. Only one female patient in this group died of COVID-19. No differences in other comorbidities or COVID-19 complications were observed in the remainder of the patients with preexisting liver disease.

Comparison of patient groups with elevated liver enzymes versus normal liver enzymes

When comparing patients who showed signs of liver impairment with those who did not, the main difference was the severity of inflammation. Patients with elevated liver enzymes (Group 1) more often demanded oxygen, and all biochemical inflammation indices were higher than in those with normal enzymes (Group 2). Of the 684 patients studied, 209 (30.5%) required O_2 supply due to respiratory failure, mostly belonging to Group 1 (Table 2). In addition, these patients required a longer duration of supportive O_2 due to low blood O_2 saturation because of severe lung infiltration.

For most patients, Il-6 blood concentration was in the range of 2-626 ng/L. Only one 62-year-old male with a critical course of pneumonia and elevated liver enzymes had an Il-6 as high as 2499 ng/L. This patient died after the manifestation of a cytokine storm (Table 2).

The level of LDH in the blood was in the range of 134 U/L–979 U/L for most patients. Only one patient (a 72-year-old male), who had normal liver enzyme levels during all stages of the disease, had LDH 1304 U/L and died of critical COVID-19.

Patients with elevated liver enzymes had almost double the chance of bacterial complications of viral pneumonia (univariate logistic regression: odds ratio (OR) = 1.73 (1.087–2.789), P = 0.0217). The incidences of other complications were largely similar in both groups (Table 3).

Underlying diseases - including hypertension, the most common - did not substantially prevail in patients with elevated liver enzymes (Table 4).

Analysis of the association of variables with the seriousness of SARS-CoV-2 pneumonia

A moderate course of SARS-CoV-2 pneumonia was diagnosed in 500 (73.1%) patients; a severe course in 148 (21.6%); and a critical course of SARS-CoV-2 pneumonia was diagnosed in 36 (5.3%) patients. There were no significant differences in the distribution of severity between the two groups of patients studied (Table 2).

To clarify which factors predispose a patient toward a more severe course of COVID-19, univariate (Tables 5 and 6) and multivariate (Table 7) analyses were performed.

The age of patients had almost no influence on the course of the disease, while male patients had forms of COVID-19 that were almost 1.5 times more severe and critical (Table 5). Multivariate logistic regression analysis also confirmed that the male gender was independently associated with more severe COVID-19 (Table 7). Acute kidney failure, but not acute liver failure, was also found to be associated with a more severe course of the disease (Table 5).

Neither the underlying disease that was most frequently presented - primary hypertension - nor less frequent lung diseases, cancers, or obesity were confirmed to be associated with a more severe course of COVID-19. Only heart disease of various etiologies, type 2 diabetes, and hyperlipidemia were prone to aggravate COVID-19 (Table 6). Diabetes and hyperlipidemia, but not heart disease, were also independently confirmed to be associated with more severe COVID-19 (Table 7).

Multivariate logistic regression analysis included age, gender, underlying diseases, and complications of pneumonia. Among the pneumonia complications analyzed, only sepsis, increased respiratory rate, and respiratory failure were independently associated with the severity of COVID-19 (Table 7).

Analysis of patients with liver failure

In our study, five patients, all of whom were male, experienced acute liver failure (0.73%) (Table 8). Septic shock developed in four patients, who did not recover despite all efforts to stabilize their condition. Two patients experienced rapid progression of the disease, and died after three days (a 50-year-old male with angioblastic T lymphoma) and eight days (an 84-year-old male with intracranial abscess, epilepsy, and chronic heart disease) of hospitalization.

Disease outcome analysis

Twenty patients died from COVID-19, 85% of whom (17 patients) had elevated liver enzymes. When comparing between patients with elevated and normal liver enzymes, no significant differences were found in mortality rate - 2.8% for those with abnormal liver test results, and 3.7% for those with normal liver test results.

Most of the deceased patients were older males who experienced much more severe SARS-CoV-2 pneumonia, with more life-threatening complications than recovered patients (Table 9). All deceased patients had at least one underlying disease or a combination thereof, and suffered significantly more often from heart disease, hypertension, and urinary tract infections than patients who made recoveries (Table 9). It should be noted that despite the fact that deceased patients were characterized by developing resistance to antibiotics more often than recovered patients, bacterial complications of viral

Table 2 Coronavirus disease 2019 pneumonia severity indices of patients with elevated liver enzymes (group 1) versus normal liver enzymes (group 2)

	Group 1 (n = 603)		Group 2 (n = 81)		*P* value
		Number of tested		Number of tested	
Moderate COVID, n/%	436/72.2	603	64/79.0	81	0.4341
Severe COVID, n/%	134/22.4		14/17.3		
Critical COVID, n/%	33/5.4		3/3.7		
O_2 demand, n/%	188/31.2	603	21/25.9	81	0.3354
SpO_2, %, range	94.4 ± 3.5, 68-100	594	95.3 ± 2.9, 84-99	76	0.0169
Respiratory rate, n/min, range	18.4 ± 3.2, 14-40	543	17.7 ± 2.4, 14-28	72	0.0291
Mortality, total, n/%	17/2.8	603	3/3.7	81	0.6573
CRP, mg/L, range	72.3 ± 68.9, 0.4-459.0	571	54.2 ± 56.1, 0.6-327.0	70	0.0151
IL-6, ng/L, range	50.3 ± 120.0, 3-2499	546	35.7 ± 38.9, 2-188	65	0.0402
LDH, U/L, range	357.9 ± 134.0, 167-979	435	299.0 ± 173.4, 134-1304	49	0.0254

COVID: Coronavirus disease; CRP: C-reactive protein; LDH: Lactate dehydrogenase; IL-6: Interleukin-6.

Table 3 Comparison of coronavirus disease 2019 pneumonia complications in patients with (group 1) and without (group 2) elevated liver enzymes

	Group 1	Group 2	*P* value
Bacterial complication, n/%	335/55.6	34/42.0	0.0213
Sepsis, n/%	14/2.3	4/4.9	0.1672
Respiratory failure, n/%	63/10.4	7/8.6	0.6146
Acid-alkaline imbalance, n/%	11/1.8	0	0.2204
Electrolyte imbalance, n/%	67/11.1	8/9.9	0.7385
Hyperkalemia, n/%	10/1.7	0	0.2426
Hypokalemia, n/%	40/6.6	7/8.6	0.5023
Hypernatremia, n/%	1/0.2	2/2.5	0.0032
Hyponatremia, n/%	13/2.2	0	0.1821
Blood volume decrease, n/%	15/2.5	3/3.7	0.5209
Blood clotting disorder, n/%	6/1.0	3/3.7	0.0446
Acute kidney failure, n/%	15/2.5	1/1.2	0.4836
Acute liver failure, n/%	5/0.8	0	0.3672
Antibiotic resistance, n/%	18/3.0	1/1.2	0.3681

pneumonia were less frequently documented in this group. Only two deceased patients were obese (2, or 10%, *vs* 15, or 2.26%, of recovered patients).

DISCUSSION

In our cohort, 88.2% of hospitalized COVID-19 patients had elevated liver enzymes - most often ALT (88.2%) followed by AST (71.6%) and GGT (70.4%). Similar results were found in the Cai *et al*[7], where 76.3% of hospitalized patients displayed abnormal liver test results. On the contrary, the Hao study reported that 79.2% of hospitalized patients displayed normal liver tests[5].

An increase in LDH level was observed in patients with severe forms of COVID-19, and was associated with a poor prognosis. In the acute liver failure group, four patients had elevated LDH in the

Table 4 Prevalence of underlying diseases in patients with (group 1) and without (group 2) elevated liver enzymes

	Group 1	Group 2	*P* value
Hospitalization, d, *n* ± SD	9.7 ± 5.9	8.7 ± 6.5	0.2039
Primary hypertension, *n*/%	193/32.0	21/25.9	0.2638
Heart disease, *n*/%	32/5.3	6/7.4	0.4430
Lung disease, *n*/%	19/3.2	5/6.2	0.1652
Diabetes, *n*/%	50/8.3	9/11.1	0.4015
Obesity, *n*/%	14/2.3	3/3.7	0.4532
Hyperlipidemia, *n*/%	31/5.1	5/6.2	0.6962
Podagra, *n*/%	12/2.0	0	0.2002
Kidney disease, *n*/%	16/2.7	4/4.9	0.2518
Prostate disease, *n*/%	9/1.5	1/1.2	0.8546
Urinary tract disease, *n*/%	39/6.5	6/7.4	0.7487
Thyroiditis and goiter, *n*/%	21/3.5	4/4.9	0.5121
Gastrointestinal disease, *n*/%	32/5.3	3/3.7	0.5387
Liver disease, *n*/%	22/3.7	2/2.5	0.5865
Nervous and mental diseases, *n*/%	27/4.5	1/1.2	0.1666
Cancers, *n*/%	17/2.8	5/6.2	0.1098

Table 5 The association of the severity of coronavirus pneumonia with the demographic characteristics of patients and critical disease outcomes (data from univariate logistic regression analysis)

	OR	95% confidence interval	*P* value
Gender: male *vs* female	1.5989137	1.1317308-2.2732760	0.0083
Age	1.0260275	1.0116249-1.0409474	0.0004
Respiratory rate	1.1588882	1.0942282-1.2311821	< 0.0001
Acute respiratory failure	3.3114884	2.0001907-5.4967193	< 0.0001
O_2 demand	3.9476028	2.7654860-5.6573094	< 0.0001
Sepsis	7.4825581	2.7778683-23.601933	0.0002
Acute kidney failure	6.2586207	2.2427771-20.097089	0.0008
Antibiotic resistance	3.8879310	1.5485304-10.193077	0.0041
Mortality	26.8383223	7.6407747-169.97850	< 0.0001

OR: Odds ratio.

range of 448-900 U/L. One patient with normal liver enzymes had an LDH of 1304 U/L. These patients died during treatment. Our findings are consistent with data from an Indonesian meta-analysis in which an association between increased LDH levels and mortality was observed (OR = 4.22, $P < 0.001$)[12].

It should be noted that the proportion of patients with abnormal liver test results varies between published COVID-19 studies for several reasons. As in our case, some studies are restricted only to hospitalized patients[5,7,13,14], whereas other studies include all positive cases of COVID-19[9]. Moreover, the liver test norms that apply in particular hospitals vary, making it somewhat difficult to compare data. In our study, ALT and AST below 40 U/L, GGT below 36 U/L, and ALP below 150 U/L were considered normal for both male and female patients, as in the Cai *et al*[7], while in other studies the normal limits of liver tests were set lower. In the Hao study, the ALT norm for males was 35 U/L, while for females it was 25 U/L[5]; in the Wishniewska study, ALT and AST norms for males were 41 U/L, and for females they were 32 U/L[15].

In general, some reviews provide elevated liver enzymes for COVID-19 patients in the range of 14.8%-53%[16], while others consider 50%-78% to be elevated[6].

Table 6 The association of the severity of coronavirus pneumonia with underlying diseases (data from univariate logistic regression analysis)

	OR	95% confidence interval	*P* value
Diabetes	2.3041738	1.3274753-3.9642971	0.0027
Heart disease	2.5760479	1.3198252-4.9960747	0.0050
Hyperlipidemia	2.2857140	1.1426635-4.5048434	0.0173

OR: Odds ratio.

Table 7 Independent factors associated with the severity of coronavirus disease 2019 (data from multivariate logistic regression analysis)

	OR	95% confidence interval	*P* value
Age	1.0227924	1.0032076-1.0431564	0.0234
Gender: male *vs* female	1.7233575	1.1114172-2.7017507	0.0161
Respiratory rate	1.1444804	1.0724480-1.2254639	< 0.0001
Respiration failure	2.1878906	1.1163265-4.2384266	0.0209
Sepsis	14.923604	1.6112025-359.53433	0.0352
Diabetes	3.2206335	1.5539799-6.6834759	0.0016
Hyperlipidemia	2.6652639	1.1327787-6.2794095	0.0238

OR: Odds ratio.

Table 8 Data from patients with acute liver failure

Gender	Age	Outcome	COVID severity	Respiratory failure, SpO_2, respiratory rate	O_2	Laboratory tests	COVID complications	Comorbidities
Male	84	Deceased	Critical	Yes, 83%, 18/min	No	ALT 107, ALP 117, GGT 107, LDH 743, Alb 29.3	Sepsis; Electrolytes imbalance; Hypokalemia; Acid-alkaline imbalance	Heart disease; Epilepsy
Male	55	Deceased	Critical	Yes, 91%, 34/min	Yes	CRP 111, IL-6 87, ALT 115, AST 344, ALP 40, LDH 448, Alb 28.1	Sepsis; Hypokalemia; Hyponatremia; Blood coagulation disorder	Primary hypertension; Obesity; Antibiotic resistance
Male	44	Recovered	Moderate	No, 94%, 16/min	No	CRP 98, IL-6 54, ALT 113, AST 193, ALP 73, GGT 355, LDH 399	Hypokalemia	Primary hypertension; Heart disease; Kidney disease
Male	42	Deceased	Critical	Yes, 80%, 30/min	Yes	CRP 459, IL-6 99, ALT 162, AST 183, ALP 1183, GGT 67, LDH 900, Alb 16.6	Acute kidney failure; Sepsis; Bacterial pneumonia complication; Hypokalemia; Blood coagulation disorder	Urinary tract infection; Anemia; Antibiotic resistance
Male	50	Deceased	Moderate	No, 98%, 22/min	No	CRP 221, IL-6 626, ALT 521, AST 53, ALP 296, GGT 559, LDH 546, Alb 16.5	Acute kidney failure; Sepsis; Blood coagulation disorder; Hypokalemia	Heart disease; Cancer (lymphoma)

COVID: Coronavirus disease; CRP: C-reactive protein; LDH: Lactate dehydrogenase; IL-6: Interleukin-6; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; Alb: Albumin.

Despite the aforementioned discrepancies, abnormalities in liver tests deserve the attention of clinicians due to the wealth of evidence suggesting that patients with elevated liver enzymes, especially ALT and AST, generally have more severe SARS-CoV-2 pneumonia[6,7,17-20]. In different studies, elevated transaminases are associated with a 2–9-fold increased probability of poor outcomes of COVID-19[19,21].

Table 9 Analysis of patients hospitalized with coronavirus disease 2019 depending on the outcome of the disease

	Deceased	Recovered	*P* value
Total number, *n*/%	20/2.9	664/97.1	
Male/female ratio	17/3	367/297	0.0081
Age, yr ± SD	64.3±11.9	50.45	0.0004
With normal enzymes, *n*/%	3/15.0	78/11.7	0.6554
With elevated enzymes, *n*/%	17/85.0	586/88.3	
Moderate COVID, *n*/%	2/10.0	498/75.0	< 0.0001
Severe COVID, *n*/%	5/25.0	143/21.5	
Critical COVID, *n*/%	13/65.0	23/3.5	
Respiratory failure, *n*/%	17/85.0	53/8.0	< 0.0001
Respiratory rate, *n*/min ± SD	22.3 ± 5.6	18.2 ± 2.9	0.0099
SpO_2, % ± SD	88.7 ± 6.3	94.6 ± 3.2	0.0010
O_2 demand, *n*/%	11/55.0	198/29.8	0.0158
Bacterial pneumonia complication, *n*/%	5/25.0	364/54.8	0.0084
Sepsis, *n*/%	13/65.0	5/0.8	< 0.0001
Acute liver failure, *n*/%	4/20.0	1/0.15	< 0.0001
Acute kidney failure, *n*/%	10/50.0	6/0.9	< 0.0001
Electrolytes imbalance, *n*/%	11/55.0	64/9.6	< 0.0001
Antibiotic resistance, *n*/%	7/35.0	12/1.8	< 0.0001
Urinary tract infections, *n*/%	5/25.0	40/6.0	0.0007
Primary hypertension, *n*/%	10/50.0	204/30.7	0.0669
Heart disease, *n*/%	10/50.0	28/4.2	< 0.0001

COVID: Coronavirus disease.

In our study, moderate pneumonia was more frequent in patients with normal liver enzymes, while critical pneumonia prevailed in patients with elevated liver test results. Furthermore, the majority of the deceased patients had elevated liver enzymes. Despite this, it should be noted that we have not proved that abnormal liver test results are directly related with the likelihood of more severe COVID-19.

The broader field of research has no consensus on the pathological mechanism by which SARS-CoV-2 infection damages the liver because the histological view of liver injury rarely presents. In the Cai *et al* [7], postmortem histological liver analysis showed neither lesions of the lobular architecture nor portal tract infiltration - only slight vesicular steatosis, watery degeneration of hepatocytes with minimal plasma cells, and neutrophil infiltration of hepatic sinuses[7]. Such alterations prompted the formulation of a hypothesis of ischemic/hypoxic hepatitis due to altered O_2 blood saturation, and cardiac failure in critically affected cases[22].

However, the pattern of increase in transaminase in patients with COVID-19 was different from hypoxic hepatitis, suggesting that this was not the case. ALT and AST were elevated by a factor of less than 3 in 54.9% and 74.8%, respectively, of cases of abnormal liver test results in this study. Only 9.3% of the cases of elevated ALT, 2.7% of AST, and 11.2% of GGT were in concentrations greater than 300 U/L. Other studies also emphasize that transaminases rarely increase by a factor of more than 2-3 in COVID-19 patients[19,23].

It should also be borne in mind that elevated transaminases do not always originate exclusively from the liver; therefore, other causes such as myositis, ischemia, and cytokine release syndrome must be excluded to draw a definitive conclusion regarding liver injury in COVID-19 patients[24]. ACE-2, responsible for the virus entry receptor, is expressed not only in respiratory tract epithelium cells but also in vascular endothelium, cardiovascular tissue, renal tissue, and intestinal epithelia, which is why the possibility for the entry and replication of SARS-CoV-2 theoretically exists in practically all vasculated tissues of the human body[25]. Histopathological analysis of autopsies confirmed inflammatory infiltration of the lamina propria; epithelium of the digestive tract, skin, and kidney blood vessels; features of viral myocarditis; and hypoxic brain injury[26]. Furthermore, other research has established that SARS-CoV-2 can persist in the intestines of infected subjects even longer than in the

respiratory tract[27-29].

Abnormal liver test results are more likely to reflect the severity of COVID-19 in general than a particular liver injury[21]. A comprehensive meta-analysis of 35 studies with more than 10000 total participants concluded that COVID-19, despite its severity, has a minor impact on the liver[4]. We agree with this conclusion.

Patients with elevated liver enzymes also displayed increased indices of inflammation, such as CRP and IL-6 levels. Elevated serum levels of CRP, IL-1, IL-6, and the tumor necrosis factor were reported in several other studies, and this was associated with a non-favorable course of liver injury[30]. Additionally, the bacterial complication of SARS-CoV-2 pneumonia, which contributes to the systemic inflammatory response, was diagnosed more frequently in patients with increased liver enzymes (OR = 1.73, P = 0.0217).

In our study, resistance to antibiotics, most often beta-lactams, was found to be associated with more severe COVID-19 (OR = 3.89, P = 0.004) and was documented more frequently in deceased patients. This issue is rarely discussed in COVID-19-related studies and needs more careful evaluation, as only very few studies with a small number of patients have been published[31,32]. Although concerns that the pandemic has led to an increase in antibiotic resistance due to self-medication of this viral infection have already been raised[33,34], it is necessary to elucidate how resistance to antibiotics modulates COVID-19 itself as very little comprehensive analysis has been published so far.

It also appeared that male patients in our cohort were more likely to have elevated liver enzymes than female patients. Furthermore, the male gender was confirmed as an independent factor associated with more severe COVID-19. This finding is consistent with previous data which highlights the male gender as one of the indicators of liver affliction[7,35]. With some disagreements, the protective effect of estrogen is often mentioned in relation to liver diseases[36], but the specific reasons that males suffer from more severe COVID-19 should be elucidated in more studies involving not only clinical but also detailed epidemiological data.

Weber and co-authors reported that only rare cases of acute liver failure were diagnosed in infected patients[37]. Our findings are in line with this: only five (0.72%) patients experienced acute liver failure, all of whom were male. In four of those patients, sepsis developed, leading to death. Males also prevailed among the total number of deceased patients (20, or 2.9%), most of whom (17, or 85%) displayed abnormal liver test results. Other similar results have been posted elsewhere which suggest that the majority (58%-78%) of deceased COVID-19 patients had liver injuries[38,39]. However, a comprehensive meta-analysis of 158 studies involving 78798 patients drew the conclusion that elevated liver enzymes, despite being a common finding in COVID-19 patients, had no effect on mortality or the critical course of the disease[20].

It should be emphasized that none of the patients in our study who experienced liver failure during the course of COVID-19 had an underlying liver disease. Furthermore, there was no association of elevated liver enzymes with preexisting liver disease.

Overall, 3.5% of patients had underlying liver disease, the majority of whom (91.7%) exhibited abnormal liver test results during the course of COVID-19. Only 12.5% of these patients had severe COVID-19, and one female died of critical COVID-19.

Although chronic comorbid liver diseases are reported in 2.6%-11% of patients[19], it seems that in most cases this does not influence COVID-19-associated liver injury, severe COVID-19 infection, or poor patient outcomes. This was confirmed in our study, was also shown in a nationwide matched cohort study[40], and has been approved in several reviews[19,41]. However, in one publication with 99 patients, preexisting hepatitis B infection was reported as a condition for more severe COVID-19 infection compared to patients without hepatitis B[42]. It is assumed that the immunosuppressive effect of the SARS-CoV-2 virus may lead to the reactivation of the hepatitis B virus[43]. We could neither confirm nor deny this, as our cohort included only two patients with chronic hepatitis B in remission and two patients with chronic hepatitis C.

There is an opinion that the SARS-CoV-2 virus itself could be responsible for liver injury during COVID-19, but the histopathological mechanism remains uncertain[41]. In the liver, the majority of abandonment of ACE-2 expression is determined in cholangiocytes, but in patients with COVID-19, the cytopathic, not cholestatic, profile of elevated enzymes prevails. On the other hand, the level of expression of ACE-2 receptors in hepatocytes is believed to be regulated by the virus. There may also be additional ACE receptors or co-receptors[44]. Thus, we agree with the conclusion that COVID-19-associated liver injury usually occurs as a result of the progression of COVID-19 itself[45].

We did not find any differences between the groups of patients with and without elevated liver enzymes concerning the prevalence of underlying diseases. Therefore, we cannot predict nor draw any conclusions regarding how underlying diseases contribute to liver damage during COVID-19. However, patients with diabetes, heart diseases of various etiologies, and hyperlipidemia, but not obesity, have an increased likelihood of suffering from more severe SARS-CoV-2 pneumonia. Furthermore, diabetes and hyperlipidemia were independently confirmed to be associated with more severe COVID-19 (OR = 3.2, P = 0.0016, and OR = 2.7, P = 0.0238, respectively). Thus, concomitant pathology appeared to be more likely to affect the severity of COVID-19 than the probability of liver damage in our study. Many studies also mention cardiovascular and renal diseases, diabetes, obesity, and hypertension as factors that worsen the course of COVID-19[20].

In summary, elevated liver enzymes are often found in patients with COVID-19. This may be due to a variety of factors, including the effects of medications used to treat the disease, concomitant liver pathology, and the influence of the virus itself. In the cases that we examined, there were significantly more patients with hepatic impairment than with normal liver function, and hepatic-impaired patients had both a higher risk of bacterial complications and a more severe course of viral pneumonia, with increased oxygen demand. This is most likely due to the effects of the SARS-CoV-2 virus, which causes more damage not only to the respiratory system but also to other organs, including the liver, due to more pathogenicity and increased cytopathic aggression which causes a greater storm of cytokines.

Because we only examined inpatients with moderate to severe and critical COVID-19, we cannot compare the frequency of hepatic impairment in mild cases of COVID-19.

Limitations of the study

We must consider several limitations of our study. Because we studied only patients with COVID-19 who needed hospitalization, and outpatients were omitted, the groups of patients differed in size by a factor of almost 8: the group with elevated liver enzymes consisted of 603 patients, and the group with normal liver enzymes consisted of 81 patients. This circumstance perhaps prevented us from statistically proving an association between liver impairment and severity of COVID-19 course - although direct damage of the liver tissue by the SARS-CoV-2 virus itself should be proved histologically, since elevated transaminases do not entirely arise from damage to the liver tissue.

Our study featured no groups with asymptomatic or mild COVID-19; their liver enzymes would likely have been in the normal range. Thus, the group with normal enzymes could be larger. This circumstance is also necessary to consider when evaluating data on the impact of preexisting diseases on COVID-19 severity. We also have not followed up with patients after discharge, which is why we do not know how quickly signs of liver impairment are resolved after the recovery of patients from COVID-19 pneumonia.

CONCLUSION

Despite a wealth of published data analyzing liver tests in COVID-19 patients, it is still difficult to draw inferences not only about the cause of such an effect of the SARS-CoV-2 virus infection on the liver, but also about the prevalence of elevated liver enzymes in such patients.

In summarizing our results, we can conclude that liver impairment allows a more severe inflammation to be predicted, with a higher risk of bacterial complications and worse outcomes in patients with SARS-CoV-2 pneumonia. Because several drugs with potentially hepatotoxic effects are used in severe cases, patients with more aggressive forms of COVID-19 should have their liver enzymes monitored regularly; their results should be considered when selecting a treatment to avoid further hepatic impairment or even insufficiency.

ARTICLE HIGHLIGHTS

Research background

Alongside respiratory symptoms, elevated liver enzymes, abnormal liver function, and even acute liver failure were reported in patients suffering from severe acute respiratory disease coronavirus 2 (SARS-CoV-2) pneumonia. However, the exact triggers of liver damage, how it affects patients, and whether it could predict the course and outcomes of coronavirus disease 2019 (COVID-19) itself remain unclear.

Research motivation

Although liver injury in patients with COVID-19 is often transient and is usually normalized without special treatment in mild cases of the disease, it can be the first sign of life-threatening events such as acute liver failure in severe and critical cases. Therefore, it is essential for everyday clinical practice to have a more precise view of how the liver impairment affects the course and outcomes of SARS-CoV-2 infection itself. Our study contributes to this goal.

Research objectives

This study aims to analyze the impact of liver enzyme abnormalities on the severity and outcomes of COVID-19 in hospitalized patients to have a clearer view of how to evaluate the risk of severe liver impairment from elevated enzyme tests.

Research methods

In this study, 684 depersonalized medical records from patients hospitalized with COVID-19 during the 2020-2021 period were analyzed. Patients were assigned to two groups: those with elevated liver

enzymes, where at least one out of four liver enzymes were elevated at any point of hospitalization, from admission to discharge; and the control group, with normal liver enzymes during hospitalization. COVID-19 severity was assessed according to the interim World Health Organization guidance (2022). Data on viral pneumonia complications, laboratory tests, and underlying diseases were also collected and analyzed.

Research results

In total, 88.2% of patients with SARS-CoV-2 infection produced abnormal liver test results. Alanine aminotransferase and aspartate aminotransferase levels were elevated by a factor of less than 3 in 54.9% and 74.8% of cases with increased enzyme levels, respectively. Patients in Group 1 had almost double the chance of bacterial viral pneumonia complications, required oxygen supply more often, and displayed higher biochemical inflammation indices than those in Group 2. Like in other research, our patients rarely experienced acute liver failure. The majority of the deceased patients had at least one underlying disease or a combination thereof, and most were male. Alongside male gender and older age, diabetes and hyperlipidemia, but not obesity, were confirmed as independent factors associated with more a severe COVID-19 infection in our cohort.

Research conclusions

In our study, the presence of liver impairment allows us to predict a more severe inflammation with a higher risk of bacterial complication and worse outcomes of COVID-19. Therefore, monitoring liver enzyme levels should be a part of the qualitative care of patients with SARS-CoV-2 pneumonia.

Research perspectives

To find out more precisely the sources of increased liver enzymes in patients with COVID-19, it would be beneficial to elucidate whether the SARS-CoV-2 virus can enter and replicate in hepatocytes. For this purpose, an experimental study on the cell line of the liver origin or virus detection in hepatocytes during a histological analysis of autopsies could be promising.

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FOOTNOTES

Author contributions: Puronaite R, Milaknyte G, Reivytyte R and Urbanoviciute G performed raw data collection; Liakina V, Stundiene I, Bytautiene R and Kazenaite E revised collected data for relevance and sufficiency; Liakina V, Stundiene I, Milaknyte G and Bytautiene R wrote the manuscript draft; Liakina V edited draft and prepared the final version of the manuscript; Kazenaite E revised manuscript for important intellectual content.

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ORIGINAL ARTICLE

Retrospective Study

Immune checkpoint inhibitor-mediated colitis is associated with cancer overall survival

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Abstract

BACKGROUND

Immune checkpoint inhibitor-mediated colitis (IMC) is a common adverse event following immune checkpoint inhibitor (ICI) therapy for cancer. IMC has been associated with improved overall survival (OS) and progression-free survival (PFS), but data are limited to a single site and predominantly for melanoma patients.

AIM

To determine the association of IMC with OS and PFS and identify clinical predictors of IMC.

METHODS

We performed a retrospective case-control study including 64 ICI users who developed IMC matched according to age, sex, ICI class, and malignancy to a cohort of ICI users without IMC, from May 2011 to May 2020. Using univariate and multivariate logistic regression, we determined association of presence of IMC on OS, PFS, and clinical predictors of IMC. Kaplan-Meier curves were gen-erated to compare OS and PFS between ICI users with and without IMC.

RESULTS

IMC was significantly associated with a higher OS (mean 24.3 mo *vs* 17.7 mo, P = 0.05) but not PFS (mean 13.7 mo *vs* 11.9 mo, P = 0.524). IMC was significantly associated with OS greater than 12 mo [Odds ratio (OR) 2.81, 95% confidence interval (CI) 1.17-6.77]. Vitamin D supplementation was significantly associated with increased risk of IMC (OR 2.48, 95%CI 1.01-6.07).

CONCLUSION

IMC was significantly associated with OS greater than 12 mo. In contrast to prior work, we found that vitamin D use may be a risk factor for IMC.

Key Words: Immune checkpoint inhibitors; Immune checkpoint inhibitor-mediated colitis; Immune-related adverse events

Core Tip: Immune checkpoint inhibitor-mediated colitis (IMC) is a common adverse event following immune checkpoint inhibitor (ICI) therapy for cancer. We sought to determine the association of IMC with overall survival (OS) and progression-free survival (PFS) among cancer patients treated with ICI and identify clinical predictors of IMC. We performed a retrospective case-control study including 64 ICI users who developed IMC. In multivariate logistic regression analysis, IMC was significantly associated with a higher OS but not PFS. IMC was significantly associated with OS greater than 12 mo. Vitamin D supplementation was associated with increased risk of IMC.

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INTRODUCTION

Immune checkpoint inhibitors (ICI) have dramatically changed the landscape of cancer therapy. Early studies showed significantly prolonged survival in patients with metastatic melanoma compared to standard chemotherapy[1], and evidence now exists for improved outcomes in a variety of tumors ranging from lung cancers to urothelial carcinoma to breast cancer[2-5]. Although these are powerful treatments in our armamentarium against malignancy, ICI can cause immune-related adverse events (irAE) characterized by autoimmune-like inflammation in a variety of non-tumor organs, leading to increased morbidity for patients[6].

One of the most common irAE is immune checkpoint inhibitor-mediated colitis (IMC). IMC may occur in up to 40% of patients treated with ipilimumab, an antibody targeting CTLA-4, 11%-17% of patients treated with antibodies against anti-PD-1 or anti-PD-L1, such as nivolumab, pembrolizumab, or atezolizumab, and around 32% of patients treated with a combination of anti-CTLA-4 and anti-PD-1[7]. Prior retrospective analyses of patients with IMC have attempted to identify characteristics associated with development of IMC, including type of malignancy, ICI class, dose of ICI, cancer stage, and vitamin D use[8-11]. Intriguingly, two prior studies have suggested that development of IMC may positively correlate with improved progression-free survival (PFS) and overall survival (OS)[9,10]. One of these studies controlled for confounding effects of ICI class *via* frequency matching, but was limited to patients with melanoma, hindering wider applicability of their findings[10]. These findings also conflict with data suggesting that use of steroids and the anti-TNF antibody infliximab in patients treated with ICI are associated with worse cancer outcomes[12,13]. These discrepancies represent a significant knowledge gap that impedes our ability to evaluate and manage IMC and ICI use.

Here we present data from a retrospective study of patients treated with ICI at our institution who developed IMC across malignancy types. We compare this cohort to a matched control cohort to determine whether IMC was associated with improved progression-free survival and overall survival. We also evaluate which clinical characteristics increase the risk of developing IMC, including severe IMC.

MATERIALS AND METHODS

Study design and population

We conducted a retrospective case-control single-center study after obtaining approval from the Institutional Review Board at Stanford University (IRB 57125, approved 6/30/2020). Our primary aim was to determine the association of presence and severity of IMC on OS and PFS in ICI users. Our secondary aim was to identify clinical variables which predicted development of IMC in ICI users. We evaluated all patients over the age of 18 who had been treated with immune checkpoint inhibitors (ICI) for

malignancy at Stanford Health Care from May 2011 to May 2020, including anti-CTLA-4 (ipilimumab), anti-PD-1 (nivolumab, pembrolizumab), and anti-PD-L1 (atezolizumab, avelumab, durvalumab), with follow up through October 2020. Using the Stanford Research Repository tool, we screened patients treated with ICI who were assigned International Classification of Diseases (ICD) 9 and ICD 10 codes associated with non-infectious colitis and diarrhea (Supplementary Table 1). Each chart which passed the initial screen was further screened by review of clinic notes to confirm diagnosis of immune checkpoint inhibitor-related colitis by oncology providers. Any patient found to have other explanations for their clinical presentation was excluded from the study.

Control patients were matched one to one with each IMC patient for sex, age, malignancy, type of ICI used, prior ICI exposure, and duration of ICI exposure (matched to number of doses from initiation of ICI to development of colitis in study cohort). Control patients were initially screened by those lacking the above ICD codes and were confirmed *via* direct evaluation of each chart to lack diarrhea and/or colitis ascribable to ICI per their treating oncologist.

We extracted clinical data on IMC and control patient charts including demographics (age at time of ICI initiation, sex, body mass index, race per patient report), medical history (presence of prior non-liver and non-upper gastrointestinal disease, personal history of autoimmune disease, family history of autoimmune disease), and cancer history (type of malignancy, tumor stage at ICI initiation, prior chemotherapy, prior radiation therapy, type of ICI used, duration of ICI use, OS and PFS) (Supplementary Table 2). OS was determined as time from initiation of ICI to death, while PFS was determined as time from initiation of ICI to death or progression of disease as determined by oncology providers, based on radiographic evidence of progression. IMC severity was graded using commonly accepted determinants of IMC and irAE grading[14]. We specifically noted prior use of therapies designed to increase immune responses [interleukin (IL)-2, interferon (IFN)-γ, toll-like receptor (TLR)-9 agonist, tebentafusp, or anti-CD47 antibody]. Vitamin D and non-steroidal anti-inflammatory (NSAID) use were defined as vitamin D supplement or NSAID medication, respectively, noted in the history of present illness or on the patient's medication list at the clinic visit closest to their date of ICI initiation.

We collected data on IMC diagnosis including number of patients who received endoscopy (flexible sigmoidoscopy or colonoscopy), findings on endoscopy, and fecal calprotectin (Supplementary Table 3). Data on management of IMC included treatment with anti-diarrheal medications, mesalamine, steroids (prednisone, budesonide, dexamethasone), infliximab, and vedolizumab.

Statistical analysis

The rate of the primary outcomes (OS > 12 mo and PFS > 6 mo among all ICI users, OS > 12 mo and PFS > 6 mo in patients with IMC) and secondary outcomes (risks of IMC among patients with malignancy using ICI, IMC severity), predictive value of clinical variables on primary and secondary outcomes, odds ratio (OR) with its 95% confidence interval (CI), and *P* values were calculated using Statistics/Data Analysis (Stata/IC 15.1 for Windows, College Station, TX, United States). Dichotomous variables were analyzed for outcomes using the chi-squared test or the Fisher's exact test where appropriate, and continuous variables were analyzed using Student's *t*-tests if normally distributed, or the Wilcoxon signed-rank test for non-normal data. For our multivariate analyses, model building was based on forward stepwise logistic regression, with a *P* value of 0.05 required for entry, and known predictors were also included. We constructed Kaplan Meier curves for the outcomes of OS and PFS between patients with and without IMC and patients with mild *vs* severe IMC using GraphPad Prism (version 8.3; GraphPad Software, Inc., La Jolla, CA, United States). All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Clinical characteristics associated with IMC

We identified a total of 314 patients treated with ICI at Stanford Health Care from May 2011 to May 2020 who had ICD codes matching our query (Supplementary Table 1). Of these, 64 had a diagnosis of IMC per review of Oncology providers' notes, after excluding patients with alternative diagnoses for their symptoms. 24 (37.5%) of these IMC patients underwent an endoscopy (colonoscopy or flexible sigmoidoscopy) during workup, of which seven (29.2%) had a normal endoscopic appearance, consistent with prior reports demonstrating that approximately one third of patients with IMC related to anti-PD-1 therapy have microscopic colitis[15] (Supplementary Table 3). An additional 14 patients (21.9%) had imaging findings suggestive of IMC while 3 patients (4.69%) without imaging or endoscopy had an elevated calprotectin or fecal lactoferrin.

These 64 patients were manually matched 1:1 with control patients based on age, sex, malignancy, type of ICI, whether or not the patient had prior ICI exposure, and duration of ICI use. We compared clinical characteristics of patients from the IMC cohort and the control cohort (Table 1). None of the matched characteristics were significantly different between the two cohorts. The mean age across the combined cohorts was 66.6 years, with an average age of 67.4 in the cohort with IMC compared with 65.8 in the control cohort ($P = 0.42$). 57.81% of patients in each group were male ($P = 1.00$). Patients were

Table 1 Baseline characteristics of patients with immune checkpoint inhibitor use

Clinical variables	All patients (*n* = 128)		Patients with IMC (*n* = 64)		Patients without IMC (*n* = 64)		*P* value
Age, yr (mean ± SD)[1]	66.6 (± 11.5)		67.4 (± 11.7)		65.8 (± 11.3)		0.420
Sex[1]							
Male, *n* (%)	74	57.81%	37	57.81%	37	57.81%	1.000
Female, *n* (%)	54	42.19%	27	42.19%	27	42.19%	
Race							
White, *n* (%)	102	79.69%	52	81.25%	50	78.13%	0.660
Black, *n* (%)	4	3.13%	2	3.13%	2	3.13%	1.000
Asian, *n* (%)	9	7.03%	4	6.25%	5	7.81%	0.730
Type of malignancy[1]							
Melanoma, *n* (%)	66	51.56%	33	51.56%	33	51.56%	1.000
RCC, *n* (%)	15	11.72%	8	12.50%	7	10.94%	0.783
NSCLC, *n* (%)	12	9.38%	6	9.38%	6	9.38%	1.000
Sarcoma, *n* (%)	11	8.59%	5	7.81%	6	9.38%	0.752
Head and neck SCC, *n* (%)	7	5.47%	3	4.69%	4	6.25%	0.697
Other, *n* (%)	17	13.28%	9	14.06%	8	12.50%	0.795
Stage IV malignancy, *n* (%)	114	89.07%	56	87.50%	58	90.63%	0.778
Type of immune checkpoint inhibitor[1]							
Ipilimumab plus nivolumab, *n* (%)	48	37.50%	24	37.50%	24	37.50%	1.000
Ipilimumab, *n* (%)	22	17.19%	11	17.19%	11	17.19%	1.000
Nivolumab, *n* (%)	12	9.38%	6	9.38%	6	9.38%	1.000
Pembrolizumab, *n* (%)	38	29.69%	19	29.69%	19	29.69%	1.000
Atezolizumab, *n* (%)	8	6.25%	4	6.25%	4	6.25%	1.000
Number of Infusions[a] (mean ± SD)[1]	6.91 (± 8.40)		6.09 (± 7.20)		7.73 (± 9.40)		0.268
Dose of ICI (mg/kg) (mean ± SD)	2.47 (± 1.30)		2.63 (± 1.60)		2.31 (± 1.00)		0.318
Prior ICI use[1]	19	14.84%	10	15.63%	9	14.06%	0.500
Medical history, *n* (%)							
Non-liver, non-upper GI disease[b], *n* (%)	28	21.88%	18	28.13%	10	15.63%	0.087
Personal history of autoimmune disease[b], *n* (%)	30	23.44%	20	31.25%	10	15.63%	0.037
Prior irAE[b], *n* (%)	8	12.50%	7	10.90%	1	1.56%	0.062
Family history of autoimmune disease[b], *n* (%)	10	7.81%	8	12.50%	2	3.13%	0.048
Prior immune-enhancing therapy[b], *n* (%)	11	8.59%	2	3.13%	9	14.06%	0.027
Prior interferon-γ therapy, *n* (%)	7	5.47%	1	1.56%	6	9.38%	0.115
Vitamin D use, *n* (%)	38	29.69%	25	39.06%	13	20.31%	0.020
Smoking (current or prior), *n* (%)	61	47.66%	33	51.56%	28	43.75%	0.376
NSAID use, *n* (%)	21	16.41%	10	15.63%	11	17.19%	0.811
Any vaccine, *n* (%)	25	19.53%	9	14.06%	16	25.00%	0.119
Flu vaccine, *n* (%)	19	14.84%	7	10.94%	12	18.75%	0.214

Pneumonia vaccine, *n* (%)	11	8.59%	4	6.25%	7	10.94%	0.344
Other vaccine, *n* (%)	2	1.56%	1	1.56%	1	1.56%	1.000
Weight at start of ICI (kg) (mean ± SD)	78.1 (± 17.4)		79.4 (± 16.9)		76.8 (± 17.9)		0.396
Medications							
Steroid at start of ICI, *n* (%)	20	15.63%	11	17.19%	9	14.06%	0.626
Steroid duration (d)	N/A		107.7 (± 164.2)		N/A		
Infliximab use, *n* (%)	N/A		10	15.63%	N/A		
Vedolizumab use, *n* (%)	N/A		1	1.56%	N/A		
Malignancy outcomes							
Mean PFS (mo)	12.8 (± 15.3)		13.7 (± 14.9)		11.9 (± 15.8)		0.524
PFS > 6 mo, *n* (%)	63	49.22%	35	54.69%	28	43.75%	0.216
OS (mo)	21.0 (± 18.9)		24.3 (± 19.4)		17.7 (± 18.0)		0.050
OS > 12 mo, *n* (%)	72.0	56.25%	42	65.63%	30	46.88%	0.025
Death, *n* (%)	20	15.63%	6	9.38%	14	21.88%	0.051

[1]Variable matched between cases and controls.
[a]Number of infusions of immune checkpoint inhibitor prior to immune checkpoint inhibitor-mediated colitis diagnosis (cases) or total (controls).
[b]See Supplementary Table 2.
IMC: Immune checkpoint inhibitor-mediated colitis; ICI: Immune checkpoint inhibitor; SD: Standard deviation; RCC: Renal cell carcinoma; NSCLC: Non-small cell lung cancer; SCC: Squamous cell carcinoma; PFS: Progression-Free Survival; irAE: Immune related adverse event; OS: Overall survival.

predominantly white in both groups, with 52 (81.25%) white individuals in the IMC cohort compared to 50 (78.13%) in the control group (P = 0.66). The most common malignancy in each group was melanoma [33 (51.56%) in both cohorts], followed by renal cell carcinoma [8 (12.5%) in the IMC cohort and 7 (10.94%) in the control cohort] and non-small cell lung cancer [6 (9.38%) in both cohorts]. Both groups had similar numbers of patients with stage IV malignancy [56 (87.5%) in the IMC cohort and 58 (90.63%) in the control cohort, P = 0.778]. Combination ipilimumab and nivolumab was the most commonly used checkpoint therapy [24 (37.5%) of patients in each cohort], followed by nivolumab monotherapy [19 (29.69%) of each cohort] and ipilimumab monotherapy [11 (17.19%) of each cohort].

Among the remainder of the clinical characteristics evaluated, personal history of autoimmune disease (including prior irAE) and family history of autoimmune disease were significantly more common in patients with IMC (P = 0.037 and 0.048, respectively). Intriguingly, prior use of a therapy designed to increase immune responses was more common in the control cohort without IMC (P = 0.027). In contrast to prior data[11], use of vitamin D supplementation at the time of first dose of ICI was significantly more prevalent in patients with IMC (P = 0.020). Neither smoking status, NSAID use at time of ICI initiation, steroid use at the time of ICI initiation, nor recent vaccination were significantly more common in IMC patients compared to controls.

IMC significantly increases overall survival

As IMC has previously been associated with increased OS and PFS in cancer patients[9,10], we evaluated whether this association was seen in our study. We found that OS was significantly longer in patients who developed IMC compared to those who did not, with a mean OS of 24.3 mo in patients with IMC and 17.7 mo in control (P = 0.05, Table 1). OS at 12 mo following ICI initiation was significantly higher in patients who developed IMC compared to those who did not (P = 0.02, Figure 1). However, in contrast to prior findings, our study did not find a significant difference in PFS between IMC patients and controls, with a mean PFS 13.7 mo in IMC patients and 11.9 mo in controls (P = 0.524) (Table 1). PFS also did not differ between patients who developed mild *vs* severe IMC (P = 0.690, Supplementary Table 5).

Across both cohorts, we identified clinical characteristics significantly associated with OS greater than 12 mo and PFS greater than 6 mo, which are correlated with cancer outcomes in patients treated with ICI[16] (Tables 2 and 3) (Supplementary Tables 4 and 5). IMC was significantly and independently associated with OS > 12 mo in the multivariate model (OR 2.81, 95%CI 1.17-6.77, P = 0.021) (Table 2). Number of ICI infusions was also positively associated with OS > 12 mo (OR 1.23, 95%CI 1.09-1.40), while sarcoma as underlying malignancy was significantly associated with OS < 12 mo (OR 0.17, 95%CI 0.029-0.947). Within the IMC cohort, nivolumab use was associated with OS < 12 mo in the univariate analysis (OR 0.09, 95%CI 0.01-0.83), while only age was associated with OS < 12 mo in multivariate analysis (OR 0.93, 95%CI 0.88-0.99) (Table 3). No individual malignancy was significantly associated

Table 2 Univariate and multivariate predictors of overall survival > 12 mo among patients with malignancy using immune checkpoint inhibitor (*n* = 128)

Clinical variables	Univariate predictors			Multivariate predictors		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Demographics						
Age (yr)	1.00	0.97-1.03	0.970			
Male	0.92	0.45-1.87	0.822			
Female	1.08	0.53-2.20	0.822			
Race						
White	1.37	0.58-3.25	0.473			
Black	2.39	0.24-23.6	0.456			
Asian	0.97	0.25-3.79	0.965			
Other	0.45	0.14-1.45	0.181			
Type of malignancy						
Melanoma	0.87	0.43-1.74	0.688			
RCC	1.65	0.53-5.12	0.390			
NSCLC	2.52	0.65-9.80	0.181			
Sarcoma	0.15	0.03-0.72	0.018	0.17	0.03-0.95	0.043
Head and neck SCC	1.04	0.22-4.84	0.961			
Other	1.50	0.52-4.35	0.453			
Presence of IMC	2.16	1.06-4.41	0.034	2.81	1.17-6.77	0.021
Presence of high grade IMC	0.47	0.16-1.38	0.167			
Stage IV malignancy	0.48	0.14-1.61	0.233			
Type of Immune Checkpoint Inhibitor						
Ipilimumab plus nivolumab	1.32	0.30-5.77	0.714			
Ipilimumab	0.74	0.29-1.85	0.517			
Nivolumab	1.63	0.46-5.70	0.448			
Pembrolizumab	2.93	1.27-6.73	0.011	1.06	0.38-2.98	0.911
Atezolizumab	1.32	0.30-5.77	0.714			
Number of ICI infusions[a]	1.19	1.08-1.32	0.001	1.23	1.09-1.40	0.001
Dose of ICI (mg/kg)	1.33	0.86-2.05	0.198			
Prior ICI use	0.51	0.19-1.37	0.183			
Medical history						
Non-liver, non-upper GI disease[b]	0.87	0.38-2.02	0.747			
Personal history of autoimmune disease[b]	1.47	0.63-3.40	0.373			
Family history of autoimmune disease[b]	1.03	0.32-4.41	0.804			
Prior irAE	2.84	0.31 - 25.9	0.356			
Prior immune-enhancing therapy[b]	0.62	0.18-2.15	0.454			
Vitamin D use	0.60	0.28-1.29	0.190			
Smoking (current or prior)	0.74	0.37-1.50	0.410			
NSAID use	1.04	0.41-2.69	0.928			
Any vaccine	0.36	0.14-0.89	0.026	1.03	0.16-6.70	0.972

Flu vaccine	0.22	0.08-0.67	0.007	0.30	0.04-2.31	0.248
Pneumonia vaccine	0.41	0.11-1.48	0.175			
Other vaccine	0.77	0.05-12.66	0.858			
Weight at start of ICI (kg)	0.99	0.97-1.01	0.207			
Medications						
Steroid at start of ICI	0.74	0.29-1.93	0.541			
Steroid duration (d)	1.00	0.997-1.01	0.368			
Infliximab use	0.76	0.21-2.77	0.226			
Vedolizumab use	1.00	0.99-1.01	1.000			

[a]Number of infusions of immune checkpoint inhibitor prior to immune checkpoint inhibitor-mediated colitis diagnosis (cases) or total (controls).
[b]See Supplementary Table 2.
ICI: Immune checkpoint inhibitor; IMC: Immune checkpoint inhibitor-mediated colitis; OR: Odds ratios; CI: Confidence interval; SD: Standard deviation; RCC: Renal cell carcinoma; NSCLC: Non-small cell lung cancer; SCC: Squamous cell carcinoma; irAE: Immune related adverse event.

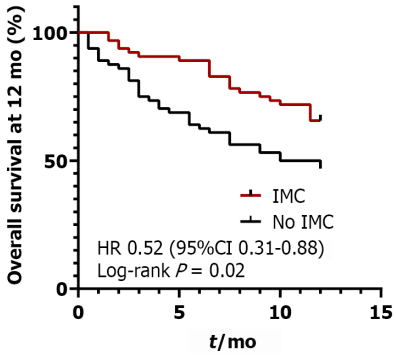

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Figure 1 Overall survival at 12 mo in patients with and without immune checkpoint inhibitor-mediated colitis. Kaplan-Meier curve of overall survival at 12 mo in patients with immune checkpoint inhibitor-mediated colitis (IMC, red) and without IMC (black). IMC: Immune checkpoint inhibitor-mediated colitis; HR: Hazard ratio.

with OS > 12 mo within the IMC cohort (Table 3).

Significant risk factors for developing IMC and severe IMC

As certain clinical characteristics were significantly more common in patients with IMC compared to controls, we evaluated whether any of these clinical characteristics were associated with risk of developing IMC (Table 4). In univariate analysis, history of autoimmune disease and vitamin D use were both significantly associated with increased risk of IMC (OR 2.45, 95%CI 1.04-5.78, P = 0.040 for autoimmune disease; OR 2.51, 95%CI 1.14-5.54, P = 0.022 for vitamin D use). Interestingly, the use of vitamin D supplementation has previously been associated with a decreased risk of IMC, in contrast to our findings here[11]. Prior use of an immune-enhancing therapy (Supplementary Table 2) was associated with a significantly decreased risk of IMC (OR 0.20, 95%CI 0.04-0.95, P = 0.043). In the multivariate model which incorporated these characteristics, only the use of immune-enhancing therapy remained significantly associated with decreased risk of IMC, with an OR of 0.20 (95%CI 0.04-1.00, P = 0.050).

We next determined if any variables were associated with an increased risk of severe IMC. Consistent with prior studies of irAE in ICI[17-19], we defined grade 1-2 IMC as mild and grade 3 or higher IMC as severe. In our study, 38 of the 64 patients (59.4%) had severe IMC (Supplementary Table 3). In the univariate model, ipilimumab and vitamin D supplementation were significantly associated with development of severe IMC (OR 8.93, 95%CI 1.07-74.8, P = 0.043 for ipilimumab; OR 3.33, 95%CI 1.10-10.14, P = 0.034 for vitamin D) (Supplementary Table 6). Combination therapy (ipilimumab plus nivolumab) trended towards an increased risk of severe IMC but did not reach significance (P = 0.053). In contrast, pembrolizumab was significantly associated with a decreased risk of severe IMC (OR 0.26, 95%CI 0.09-0.81, P = 0.020). In the multivariate model no characteristic reached significance for association with severe IMC, although both combination therapy and ipilimumab monotherapy approached significance for increased risk of severe IMC (P = 0.058 and 0.060, respectively).

Table 3 Univariate and multivariate predictors of overall survival > 12 mo among patients with immune checkpoint inhibitor colitis (*n* = 64)

Clinical variables	Univariate predictors			Multivariate predictors		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Demographics						
Age (yr)	0.96	0.92-1.01	0.103	0.93	0.88-0.99	0.023
Male	0.82	0.29-2.32	0.711			
Female	1.22	0.43-3.44	0.711			
Race						
White	0.87	0.23-3.27	0.835			
Black	1.00	0.90-1.34	0.996			
Asian	0.54	0.07-4.10	0.550			
Other	1.07	0.97-1.11	0.912			
Type of malignancy						
Melanoma	1.26	0.45-3.51	0.654			
RCC	0.51	0.12-2.28	0.381			
NSCLC	0.53	0.10-2.85	0.456			
Sarcoma	2.38	0.25-22.65	0.451			
Head and neck SCC	1.05	0.89-1.10	0.865			
Other	5.33	0.62-45.68	0.127			
Stage IV malignancy	0.60	0.11-3.26	0.554			
Presence of high grade IMC	0.91	0.32-2.57	0.855			
Type of immune checkpoint inhibitor						
Ipilimumab plus nivolumab	0.95	0.31-2.88	0.922			
Ipilimumab	0.98	0.25-3.77	0.974			
Nivolumab	0.09	0.01-0.83	0.033	0.13	0.01-1.43	0.096
Pembrolizumab	2.74	0.78-9.58	0.114	3.46	0.84-14.19	0.084
Atezolizumab	1.74	0.17-17.73	0.641			
Number of ICI infusions[a]	0.28	0.04-1.82	0.183			
Dose of ICI (mg/kg)	1.88	0.36-9.83	0.457			
Prior ICI use	0.46	0.12-1.80	0.265			
Medical history						
Non-liver, non-upper GI disease[b]	1.67	0.51-5.49	0.397			
Personal history of autoimmune disease[b]	0.78	0.26-2.31	0.648			
Family history of autoimmune disease[b]	0.93	0.20-4.29	0.922			
Prior immune-enhancing therapy[b]	0.55	0.03-9.23	0.678			
Prior interferon-g therapy	1.00	0.99-1.10	0.976			
Vitamin D use	2.45	0.80-7.46	0.116	2.77	0.75-10.20	0.124
Smoking (current or prior)	1.66	0.59-5.65	0.334			
NSAID use	2.55	0.49-13.16	0.265			
Any vaccine	5.33	0.62-45.68	0.127			
Flu vaccine	1.46	0.26-8.19	0.668			

Pneumonia vaccine	1.00	0.99-1.05	0.995
Other vaccine	1.00	1.00-1.01	0.941
Weight at start of ICI (kg)	1.02	0.98-1.05	0.329
Medications			
Steroid at start of ICI	0.98	0.25-3.77	0.974
Steroid duration (d)	1.00	1.00-1.01	0.736
Infliximab use	2.55	0.49-13.16	0.265
Vedolizumab use	1.00	1.00-1.01	0.936

[a]Number of infusions of immune checkpoint inhibitor prior to immune checkpoint inhibitor-mediated colitis diagnosis (cases) or total (controls).
[b]See Supplementary Table 2.
ICI: Immune checkpoint inhibitor; IMC: Immune checkpoint inhibitor-mediated colitis; SD: Standard deviation; RCC: Renal cell carcinoma; NSCLC: Non-small cell lung cancer; SCC: Squamous cell carcinoma; irAE: Immune related adverse event.

DISCUSSION

In our study, development of IMC following ICI use was associated with improved overall survival, although not improved progression-free survival, compared to ICI users without IMC. This is similar to findings at another center demonstrating both improved OS and PFS in patients with IMC[9,10]. We also found that vitamin D supplementation at the start of ICI treatment is a risk factor for developing IMC, in contrast to other research suggesting vitamin D use is associated with lower risk of IMC[11]. Our results, therefore, provide critical additional information on these previous associations and present a need for prospective studies.

Both publications showing improved survival in patients with IMC were retrospective analyses performed at the same center[9,10]. One study noted that ICI class was significantly associated with development of IMC[9], a finding that has been demonstrated several times in retrospective work[8,17,18,20-23]. However, unlike our work, this study did not match control patients to account for this likely confounder, as ICI class has been associated with differences in PFS in some malignancies[24,25]. The second study at this center examined survival in melanoma patients with IMC, compared to our work across multiple malignancies, although frequency matching was performed to account for use of different ICI classes[10]. Since our study is the first to examine survival in patients with IMC at a different center, our work here reinforces that IMC may be associated with increased overall survival and prompts a need for prospective studies.

The only other independent factor in our study positively associated with OS > 12 mo was number of ICI doses. This finding may be due to trivial length-time bias, as patients who survive longer are more likely to receive more doses of ICI. It is also possible that patients who required cessation of ICI due to IMC had worse outcomes, although prior work has suggested that patients still derive equivalent long-term benefit from ICI even if stopped due to irAE[26]. Type of underlying malignancy (sarcoma) was independently associated with OS < 12 mo in our study. These findings are not unexpected, as most advanced soft tissue sarcomas have a median OS of less than one year[27].

In contrast to prior work, we found a positive association between vitamin D supplementation and development of IMC[11]. It is unclear if this is related to low serum vitamin D levels or negative impact of the supplementation itself, as vitamin D levels near the time of ICI initiation were not recorded in most patients. Additionally, the prior report on vitamin D in IMC was in melanoma patients only, which may partially account for discrepancies with our study. As this association did not remain significant in our multivariate analysis, it is possible that another confounding factor may explain the association between vitamin D supplementation and IMC in our study.

In addition to challenging existing findings, we report here on additional novel risk factors for IMC. We are the first to report that prior use of immune-enhancing medications prior to ICI, such as IL-2 or interferon-γ, is significantly and independently associated with decreased risk of IMC. Much more work should be done to evaluate the relationship between these medications and future risk of IMC.

Finally, our study is the first to examine risk factors for severe IMC. In addition to increasing risk for IMC overall, we find that vitamin D supplementation may also be a risk factor for severe IMC. Similarly, our results suggest that the use of ipilimumab may be associated with increased risk of severe IMC, while pembrolizumab may be associated with decreased risk of severe IMC in patients who develop this syndrome. As ipilimumab has previously been associated with increased risk of IMC overall, while anti-PD-1, including pembrolizumab, are associated with lower risk of IMC overall[8,9], these findings emphasize that ICI class may affect severity of IMC.

Our findings may significantly impact clinical practice by identifying novel risks for IMC and severe IMC that clinicians, including oncologists and gastroenterologists, should be aware of, while also potentially providing reassurance to physicians and patients that development of IMC may be a positive

Table 4 Univariate and multivariate predictors of immune checkpoint inhibitor-mediated colitis among patients using immune checkpoint inhibitor (*n* = 128)

Clinical variables	Univariate predictors			Multivariate predictors		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Demographics						
Age (yr)	1.01	0.98-1.04	0.417			
Male	1.00	0.50-2.02	1.000			
Female	1.00	0.50-2.02	1.000			
Race						
White	1.21	0.51-2.88	0.661			
Black	1.00	0.14-7.33	1.000			
Asian	0.79	0.20-3.07	0.730			
Other	0.84	0.27-2.66	0.770			
Type of malignancy						
Melanoma	1.00	0.50-2.00	1.000			
RCC	1.16	0.40-3.42	0.784			
NSCLC	1.00	0.30-3.28	1.000			
Sarcoma	0.82	0.24-2.83	0.753			
Head and neck SCC	0.74	0.16-3.44	0.698			
Other	1.15	0.41-3.18	0.795			
Stage IV malignancy	0.72	0.24-2.22	0.572			
Type of Immune Checkpoint Inhibitor						
Ipilimumab plus nivolumab	1.00	0.49-2.05	1.000			
Ipilimumab	1.00	0.40-2.51	1.000			
Nivolumab	1.00	0.30-3.28	1.000			
Pembrolizumab	1.00	0.47-2.13	1.000			
Atezolizumab	1.00	0.24-4.18	1.000			
Number of Infusions[a]	0.98	0.93-1.02	0.273			
Dose of ICI (mg/kg)	1.23	0.82-1.84	0.327			
Medical History						
Non-liver, non-upper GI[b]	2.11	0.89-5.03	0.091			
Autoimmune disease[b]	2.45	1.04-5.78	0.040	1.87	0.74-4.74	0.186
Prior irAE	7.74	0.92-64.82	0.059			
Family history of autoimmune disease[b]	4.43	0.90-21.74	0.067	3.98	0.74-21.38	0.107
Prior immune-enhancing therapy[b]	0.20	0.04-0.95	0.043	0.19	0.04-1.01	0.052
Prior interferon-γ therapy	0.15	0.018-1.31	0.087			
Vitamin D use	2.51	1.14-5.54	0.022	2.48	1.01-6.07	0.047
Smoking (current or prior)	1.37	0.68-2.74	0.377			
NSAID use	0.89	0.35-2.28	0.811			
Any vaccine	0.49	0.20-1.21	0.123			
Flu vaccine	0.53	0.19-1.45	0.219			
Pneumonia vaccine	0.54	0.15-1.95	0.350			

Other vaccine	1.00	0.06-16.34	1.000
Weight at start of ICI (kg)	1.01	0.99-1.03	0.393

[a]Number of infusions of immune checkpoint inhibitor prior to immune checkpoint inhibitor-mediated colitis diagnosis (cases) or total (controls).
[b]See Supplementary Table 2.
ICI: Immune checkpoint inhibitor; IMC: Immune checkpoint inhibitor-mediated colitis; RCC: Renal cell carcinoma; NSCLC: Non-small cell lung cancer; SCC: Squamous cell carcinoma; irAE: Immune related adverse event.

prognosticator for cancer survival. Neither prior work nor ours found that treatment of IMC, including steroids or infliximab, negatively impacts OS[9,10], and therefore appropriate treatment of IMC should be pursued early on to minimize morbidity and mortality. Both steroid and infliximab use have been suggested to worsen survival in ICI users[12,13], but all current evidence suggests that use of these medications for IMC specifically does not impair cancer outcomes. Our work also cautions against supplementation with vitamin D in ICI users, as this may increase risk of IMC and severe IMC, although carefully designed studies with vitamin D measurements should be performed.

Our work has several strengths. We performed robust cohort matching to minimize confounding effects of ICI class and malignancy. This is also the first study to explore risk factors associated with severe IMC. However, there are limitations to our work. As a retrospective, observational study, it is subject to recall bias and cannot evaluate causation, and may also be subject to immortal time bias (ITB). Patients may have longer exposure to checkpoint inhibitors before developing IMC, compared to patients who do not manifest this irAE, leading to a period where they must survive for long enough to develop IMC and are therefore "immortal"[28]. We found that OS > 12 mo was significantly associated with greater numbers of ICI infusions (Table 2), which is likely due to ITB. However, greater numbers of infusions were not associated with IMC (Table 4). This suggests that the association between OS > 12 mo and IMC is likely independent of the number of ICI infusions, limiting this as a source of ITB in our study.

Other weaknesses of our work include selection of patients based on clinical criteria for IMC, including those who did not undergo endoscopy or other objective testing for intestinal inflammation, and therefore may not have had a true colitis. Like prior work, this is also a single-center study, and our results may not be widely generalizable, particularly since we identified fewer patients compared to prior work and our patient population is highly variable, including individuals with several different underlying malignancies. We did not exclude patients with prior non-GI irAEs in either group, although the presence of these was not independently associated with increased OS in our study. We also have not accounted for other factors which may be potential predictors of ICI response, including tumor PD-L1 expression burden, tumor mutational burden, gut microbial composition, proton pump inhibitor use, and combination treatment with tyrosine kinase inhibitors[29-34].

CONCLUSION

In conclusion, our findings suggest presence of IMC is associated with improved OS in cancer patients when cases were matched closely to controls. We also found that vitamin D supplementation was significantly associated with development of both IMC and severe IMC, while immune-enhancing medications were significantly associated with decreased risk of IMC. Future work should focus on broader populations to resolve the discrepancies raised in our work, and to confirm the association between IMC and increased cancer survival. Closely involving gastroenterologists with the workup and management of IMC will be crucial to ensuring the best care possible for these patients.

ARTICLE HIGHLIGHTS

Research background

Immune checkpoint inhibitor-mediated colitis (IMC) is a common immune-related side effect (irAE) of checkpoint inhibitor treatment for cancer. Prior work has suggested that IMC may be associated with increased survival from cancer.

Research motivation

We sought to determine if IMC was associated with increased overall survival (OS) in a cohort of patients at our institution. These findings could expand existing data on IMC and cancer outcomes and might suggest a common immunological underpinning between the efficacy of checkpoint inhibitors and certain irAEs.

Research objectives
We performed a retrospective case-control study of individuals treated with immune checkpoint inhibitors at our institution who developed IMC, closely matched to a cohort of patients treated with checkpoint inhibitors without IMC. Using univariate and multivariate logistic regression, we determined significant clinical predictors of IMC and the association of presence of IMC on OS.

Research methods
We found that IMC was significantly associated with a higher OS as well as OS greater than 12 mo. In contrast to previous findings, vitamin D supplementation was significantly associated with development of both IMC and severe IMC. However, prior treatment with immune-enhancing medications was significantly associated with decreased risk of IMC.

Research results
In multivariate logistic regression analysis, IMC was significantly associated with a higher OS but not PFS. IMC was significantly associated with OS greater than 12 mo. Vitamin D supplementation was associated with increased risk of IMC.

Research conclusions
Our findings lend strength to the idea that IMC is associated with improved cancer outcomes with checkpoint inhibitor treatment. This may suggest common immunologic underpinnings between IMC and the anti-tumor effects of checkpoint inhibitors. These results also emphasize the importance of involving gastroenterologists with the management of IMC.

Research perspectives
Future research in this area should seek to expand current knowledge of the relationship between IMC and cancer survival. In particular, future work should focus on broadening the type and number of patients treated with immune checkpoint inhibitors and on tracking patients prior to initiating checkpoint inhibitors to determine if this relationship remains significant prospectively.

FOOTNOTES

Author contributions: Weingarden AR and Habtezion A designed the research study; Weingarden AR, Balabanis T, Patel A, and Sharma A performed data collection; Gubatan J analyzed data; Weingarden AR, Gubatan J, Singh S, and Habtezion A wrote and edited the manuscript; all authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Serum metabolic profiling of targeted bile acids reveals potentially novel biomarkers for primary biliary cholangitis and autoimmune hepatitis

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Abstract

BACKGROUND

Primary biliary cholangitis (PBC) and autoimmune hepatitis (AIH) are two unexplained immune diseases. The golden standard for diagnosis of these diseases requires a liver biopsy. Liver biopsy is not widely accepted by patients because of its invasive nature, and atypical liver histology can confuse diagnosis. In view of the lack of effective diagnostic markers for PBC and AIH, combined with the increasingly mature metabolomics technologies, including full-contour metabolomics and target.

AIM

To determine non-invasive, reliable, and sensitive biochemical markers for the differential diagnosis of PBC and AIH.

METHODS

Serum samples from 54 patients with PBC, 26 patients with AIH and 30 healthy controls were analyzed by Ultra-high performance liquid chromatography-tandem mass spectrometry serum metabolomics. The metabolites and metabolic pathways were identified, and the metabolic changes, metabolic pathways and inter-group differences between PBC and AIH were analyzed. Fifteen kinds of target metabolites of bile acids (BAs) were quantitatively analyzed by SRM, and the differential metabolites related to the diagnosis of PBC were screened by receiver operating characteristic curve analysis.

RESULTS

We found the changes in the levels of amino acids, BAs, organic acids, phospholipids, choline, sugar, and sugar alcohols in patients with PBC and AIH. Furthermore, the SRM assay of BAs revealed the increased levels of chenodeoxycholic acid, lithocholic acid (LCA), taurolithocholic acid (TLCA), and LCA + TLCA in the PBC group compared with those in the AIH group. The levels of BAs may be used as biomarkers to differentiate PBC from AIH diseases. The levels of glycochenodeoxycholic acid, glycochenodeoxycholic sulfate, and taurodeoxycholic acid were gradually elevated with the increase of Child-Pugh class, which was correlated with the severity of disease.

CONCLUSION

The results demonstrated that the levels of BAs could serve as potential biomarkers for the early diagnosis and assessment of the severity of PBC and AIH.

Key Words: Primary biliary cholangitis; Autoimmune hepatitis; Biomarkers; Serum metabolic profiling; Bile acids; Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry

Core Tip: Using full-contour metabolomics and SRM, to determine non-invasive, reliable, and sensitive biochemical markers for the differential diagnosis of primary biliary cholangitis (PBC) and autoimmune hepatitis (AIH). We revealed the increased levels of chenodeoxycholic acid, lithocholic acid (LCA), taurolithocholic acid (TLCA), and LCA + TLCA in the PBC group compared with those in the AIH group. The levels of glycochenodeoxycholic acid, glycochenodeoxycholic sulfate, and taurodeoxycholic acid were gradually elevated with the increase of Child-Pugh class, which was correlated with the severity of disease. The levels of BAs could serve as potential biomarkers for the early diagnosis and assessment of the severity of PBC and AIH.

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INTRODUCTION

Primary biliary cholangitis (PBC) and autoimmune hepatitis (AIH) are two unexplained immune diseases[1]. Although advanced methods have been presented for diagnosing PBC and AIH, 5%-10% of PBC patients have anti-mitochondrial antibody-negative, and missed diagnosis or misdiagnosis mainly occurs in clinical practice[2]. For some patients with anti-mitochondrial antibody-positive, rather than significant changes in hepatic histology and function, long-term follow-up revealed that these patients eventually developed to PBC. Thus, early diagnosis of these patients is a clinical challenge. Clinical manifestations of AIH may have similarities to other autoimmune liver diseases, such as drug-induced hepatitis, alcoholic liver disease, inherited metabolic disorders, and hepatitis C virus infection, such as regardless of the cause of liver disease, patients may present with fatigue, abdominal distention, skin and sclera yellow staining, laboratory test show liver dysfunction. Because of the complexity and difficulty of diagnosing, leading to the delayed diagnosis of several AIH patients. Liver biopsy remains the golden standard for the diagnosis of autoimmune liver diseases, while it is an invasive, painful, and costly method that is associated with the possibility of sampling error and variability in interpretation. Therefore, identification of novel and accurate noninvasive biomarkers for the diagnosis and assessment of severity is of great importance.

As one of the emerging 'omics' platforms, metabolomics enables the qualitative and quantitative analyses of metabolites in complex biological samples[3]. As products of cellular adjustment processes, metabolites are regarded as the ultimate readouts that reflect genetic or environmental changes in biological systems[4,5] High-throughput metabolic profiling has been successfully used for the identification of novel diagnostic molecules and disease-related pathways, as well as development of new therapeutic targets for some diseases (*e.g.*, cancer, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, and PBC)[6-10]. Thus, it is essential to identify specific metabolomic markers, and to establish a diagnostic model for AIH or PBC.

In the present study, we aimed to identify serum biomarkers for the differential diagnosis of PBC and AIH using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS). UPLC-QTOF-MS is a newly developed technique that provides rapid and efficient access to detailed information pertaining to the nature of specific components within complex multicomponent mixtures. Compared with traditional high-performance liquid chromatography (HPLC), UPLC possesses the advantages of ultra-high resolution, high-speed scanning, and high sensitivity. Furthermore, bile acids (BAs) are crucial for the diagnosis, follow-up, and prognosis of liver and intestinal disorders, as well as diseases affecting BA metabolism. We applied a targeted metabolomic approach to quantify and compare 15 BA metabolites in PBC/AIH patients with those in healthy controls (HCs). The findings of the present study may reveal potentially novel biomarkers for the diagnosis of PBC and AIH. This study also aimed to compare metabolic profiles between PBC/AIH patients and HCs.

MATERIALS AND METHODS

Patients and study design

A total of 54 PBC and 26 AIH patients who were admitted to the First Hospital of Jilin University (Changchun, China) between May 2009 and November 2013 were respectively recruited in the present study. The study protocol was carefully reviewed and approved by the Institutional Review Board of The First Hospital of Jilin University. All the eligible patients and HCs signed the written informed consent form prior to enrollment. Patients with AIH were diagnosed according to the revised criteria presented by the International Autoimmune Hepatitis Group in 1999[11]. Patients with PBC were diagnosed according to the criteria released by the American Association for the Study of Liver Diseases [12]. Patients taking medication or supplements, or those with gallstones or other factors that might cause cholestatic liver diseases were excluded. In both groups, patients with primary sclerosing cholangitis (PSC), overlap syndromes (*e.g.*, PBC and AIH or AIH and PSC), hepatitis virus infection, human immunodeficiency virus co-infection, hepatocellular carcinoma, or diabetes were excluded. In total, 30 HCs who were admitted to our hospital for physical check-ups were enrolled. These HCs exhibited normal liver functions and had no evidence of disease. No statistically significant differences were found in age and gender among the PBC, AIH, and control groups (Table 1, $P > 0.05$).

Blood samples at the fasting state were collected from the eligible PBC patients, AIH patients, and HCs, in which 1 mL of serum was collected and stored at -80 °C for subsequent metabolic profiling. Participants' baseline characteristics are summarized in Table 1.

Reagents

HPLC-grade acetonitrile was purchase from Merck Inc. (Kenilworth, NJ, United States). HPLC-grade formic acid was obtained from Sigma-Aldrich (St. Louis, MO, United States). These two reagents were used for the preparation of mobile phases in HPLC. Milli-Q water was used, and obtained by filtering distilled water through a Milli-Q system (Millipore, Bedford, MA, United States). The chemical standards for the validation of molecular structure were obtained from Sigma-Aldrich.

Sample preparation and serum metabolic profiling

In the present study, 100 μL of each serum sample was mixed with 400 μL of cold acetonitrile for protein precipitation, followed by centrifugation at 14000 g for 10 min at 4 °C. Then, 400 μL of the supernatant was subsequently collected and lyophilized, and the residue was resolved in 100 μL of 20% acetonitrile. Equal aliquot of each serum sample was pooled together and mixed thoroughly by vortex for 1 min, which was used as the quality control (QC) sample. A QC sample was prepared after preparation of 10 real samples, and QC samples served to assess the repeatability of sample pretreatment and to monitor the stability of the UPLC-QTOF-MS system at the sequence analysis.

The UPLC-QTOF-MS approach was employed to perform serum metabolic profiling of samples obtained from PBC patients, AIH patients, and HCs, as previously described[13]. In brief, 5 μL of the reconstituted solution was carefully injected into the ACQUITY-UPLC system (Waters Corp., Milford, MA, United States) for separation using chromatography. Then, MS signals were acquired *via* the QTOF-MS system (Micromass, Manchester, United Kingdom), which was equipped with an electrospray source operating in both positive and negative ion modes. During the acquisition of MS signals, the m/z scan was set to a range of 100-1000[14]. Then, the AC18 column (2.1 mm × 100 mm, 1.7 μm), which was purchased from Waters Corp., was used for the separation of small molecular compounds at an elution speed of 0.35 mL/min. The gradient was set to 95% formic acid (0.1%, V/V), and maintained for 1 min. Subsequently, elution strength linearly increased to 100% acetonitrile for 22 min, and was kept for 3 min. The total duration was 30 min, which included equilibration for 1 min.

Analysis of BAs

All samples prepared with GCA-d5 as the internal standard and blood samples were resolved in 100 μL

Table 1 Characteristics of enrolled population in the metabolic profiling study

Clinical parameters	PBC (*n* = 54)	AIH (*n* = 26)	Control (*n* = 30)	*P* value (PBC *vs* control)	*P* value (AIH *vs* control)	*P* value (PBC *vs* AIH)
Age (mean, range) (yr), *n*	56 (38-73)	54.6 (17-75)	54.9(34-70)	0.922	0.805	0.890
Sex (Male/Female), *n*	7/47	3/23	4/26	-	-	-
AST (U/L) median, range	113.3 (14-1300)	168.8 (23-961)	23.3 (7-38)	< 0.001	< 0.001	0.423
ALT (U/L) median, range	95.9 (12-734)	153.3 (10-780)	19.0 (7-39)	< 0.001	< 0.001	0.125
ALP (U/L) median, range	292.4 (55-953)	240.1 (46-795)	73.6 (32-116)	< 0.001	< 0.001	0.123
γ-GT (U/L) median, range	299.4 (32-1631)	245.2 (26-957)	21.1 (9-77)	< 0.001	< 0.001	0.377
TBA (μmol/L) median, range	60.0 (2.7-295.7)	126.0 (1.1-1335)	-	-	-	0.696
TBiL (μmol/L) median, range	65.5 (5.6-825.8)	89.1 (6.5-543.9)	10.9 (6.3-19.5)	< 0.001	< 0.001	0.481
DBIL (μmol/L) median range	35.9 (2.4-407.4)	52.9 (2.1-300.8)	3.3 (0.4-6)	< 0.001	< 0.001	0.648
Liver cirrhosis (%)	65 (35/54)	62 (16/26)	-	-	-	-
Liver biopsy (%)	18 (10/54)	53 (14/26)	-	-	-	-
Positive of AMA (%)	81 (44/54)	0 (0/26)	-	-	-	-
Positive of ANA (%)	61 (33/54)	100 (26/26)	-	-	-	-

PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; AST: Aspartatetransaminase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; γ-GT: γ-glutamyl transpeptidase; TBiL: Totalbilirubin; DBiL: Direct bilirubin; TBA: Total bile acid; AMA: Anti-mitochondrial antibody; ANA: Anti-nuclear antibody. All data are presented as median and range. Statistically significant differences between controls and patients were determined by the rank sums Mann-Whitney.

of 25% ACN aqueous solution. The LC-MS parameters were as follows: 20 μL of the reconstituted solution was carefully injected into an ACQUITY UPLC C8 column with a particle size of 1.7 μm (Waters Corp.), and the SRM signals were obtained using an Agilent 6460 Triple Quadruple MS system (Agilent Technologies, Inc., Chicago, IL, United States), which was equipped with an electrospray source operating in the negative ion mode. The column was eluted with 10 mmol/L NH_4HCO_3 (solution A) and acetonitrile (solution B) in a linear gradient, in which the initial gradient was set to 75% solution A. Subsequently, after 9.0 min of elution, the strength was linearly elevated to 90% solution B, which lasted for 4 min. Then, this was returned to the initial gradient after 13.5 min of elution. Along with an equilibration of 1.5 min, the total running time was approximately 15 min. The following MS parameters were set in this study: Gas flow rate, 8 L/min; gas temperature, 350 °C; sheath gas temperature, 400 °C; nebulizer gas pressure, 40 psi; capillary voltage, 3500 V; sheath gas flow rate, 8 L/min; nozzle voltage, 400 V. The precursor and product ion pairs were acquired as follows: Cholic acid (CA) (407.5→407.5), glycocholic acid (GCA) (464.2→74.1), taurocholic acid (TCA) (514.2→80.1), ursodeoxycholic acid (UDCA) (391.4→391.4), glycoursodeoxycholic acid (GUDCA) (448.3→74.1), tauroursodeoxycholic acid (TUDCA) (498.3→80.1), chenodeoxycholic acid (CDCA) (391.4→391.4), glycochenodeoxycholic acid (GCDCA) (448.3→74.1), tauroursodeoxycholic acid (TCDCA) (498.2→80.1), glycochenodeoxycholic sulfate (GCDCS) (528.3→448.3), deoxycholic acid (DCA) (391.2→391.2), glycodeoxycholic acid (GDCA) (448.2→74.1), taurodeoxycholic acid (TDCA) (498.3→80.2), lithocholic acid (LCA) (375.3→375.3), taurolithocholic acid (TLCA) (482.1→80.1), and GCA-d5 (469.2→74.1).

Bioinformatics and statistical analyses

The raw data were imported into Databridge (MassHunter Quantitative Analysis software; Agilent Technologies, Inc.), followed by the peak extraction and alignment on the obtained NetCDF files using XCMS 18.0 software. The alignment parameters were set as follows: The retention time window was 7, the full width at half maximum was 14, and the remaining parameters were set as default. Subsequently, the peaks with the paired m/z, as well as their corresponding peak intensities and retention time were exported into the Excel software. Prior to univariate and multivariate logistic regression analyses, each peak area was initially normalized to the total peak area. In the multivariate logistic regression analysis, the principal component analysis (PCA) in combination with the partial least squares-discriminant analysis (PLS-DA) was conducted by SIMCA-P 11.0 software (Umetrics AB, Umea, Sweden) using the prepared data. After scaling for PCA to unit variance, the data provided an overview of the repeatability of the QC samples. Additionally, the data were Pareto scaled for PLS-DA to assess

the performance of the classification models, and to identify variables for the corresponding model.

In the univariate analysis, data were statistically analyzed by SPSS 18.0 software (IBM, Armonk, NY, United States). The biochemical data and the concentrations of BAs were log-transformed to approximately normalize their distributions. $P < 0.05$ was considered statistically significant. Nonparametric statistical analysis was conducted using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States) for making comparison between two groups.

RESULTS

Patients' baseline clinical characteristics

Patients' baseline clinical characteristics are summarized in Table 1. Previous epidemiological studies have demonstrated that women were more frequently affected by PBC and AIH than men. Consistently, the incidence rates of PBC and AIH were higher in women than in men in our study. Furthermore, to avoid the influences of drugs on the metabolomics analysis, no patient had received any treatment, including traditional Chinese medicine. The mean age of patients with PBC and AIH, and HCs was 56 (range, 38-73), 54.6 (range, 17-75), and 54.9 (range, 30-76) years old, respectively. There were no significant differences in age, parity, and gender among patients with PBC and AIH, and HCs ($P > 0.05$). Besides, 10 cases from the PBC group and 14 cases from the AIH group were newly diagnosed by biopsy. Other cases from the PBC group were diagnosed by M2-positive, and other cases from the AIH group were diagnosed by pathological scores (> 12).

There were 26 cases of Child-Pugh class A, 19 cases of Child-Pugh class B, and 9 cases of Child-Pugh class C in PBC patients. There were 17 Child-Pugh grade A and 9 Child-Pugh grade B patients with AIH. The levels of globulin, transaminases, and specific autoantibodies in the sera are presented in Table 2.

Serum metabolic profiling

A total of 110 serum samples obtained from 54 patients with PBC, 26 patients with AIH, and 30 HCs were analyzed using UPLC-QTOF-MS in both positive and negative ion modes. As shown in Supplementary Figures 1 and 2, a typical base peak chromatogram was detected by MS in positive and negative ion modes, respectively. After the peaks were aligned, 1133 peaks of positive ions and 963 peaks of negative ions were identified using MassLynx and the same acquisition method. The data were transformed into SIMCA-P11 software for PCA. Plots of the PCA scores in positive and negative ion modes are illustrated in Figure 1A, Figure 2A, Figure 3A, Figure 4A, Figure 5A and Figure 6A. Distinct clustering was observed between PBC patients and HCs, and between AIH patients and HCs. No distinct clustering was found between PBC patients and AIH patients. The QC samples were tightly clustered (Figure 1A, Figure 2A, Figure 3A, Figure 4A, Figure 5A and Figure 6A), ensuring the repeatability of the information[13].

Identification of serum metabolites specific to PBC and AIH

To find out the differentially expressed metabolites, *P*-values ($P < 0.05$) in the t-test were combined with variable importance in the projection (VIP) values in the PLS-DA model. The PLS-DA score charts are shown in Figure 1B, Figure 2B, Figure 3B, Figure 4B, Figure 5B and Figure 6B. We also conduct sorting verification on the model to check whether the model is "over-fitting". The results are shown in Figure 1C, Figure 2C, Figure 3C, Figure 4C, Figure 5C and Figure 6C. As can be seen from the sorting test figure, Figure 1C, Figure 2C, Figure 5C and Figure 6C, there is no "over-fitting" in these models. Figure 3C and Figure 4C show that the two models are "overfitted". We only established the discriminant analysis model of multivariate analysis between PBC/Control and AIH/Control, but failed to establish the discriminant analysis model of PBC/AIH. The PBC and AIH samples of the two groups overlaps on the PCA score plot (as shown in Figure 3A and Figure 6A), and the supervised PLS-DA (as shown in Figure 3B and Figure 6B) still failed to distinguish them significantly, indicating that there was little difference in metabolic profile between the two groups of different autoimmune liver diseases.

The METLIN metabolomics database (http://metlin.scripps.edu/) was used to facilitate metabolite annotation through MS analysis. The data of differentially expressed are presented in Tables 3 and 4. Fold-change (FC) was used to indicate changes in potential PBC- and AIH-specific biomarkers, and the chosen FC values were > 2 and < 0.5.

Identification of serum metabolites specific to PBC

As presented in Table 3 the levels of 17 of 26 potential biomarkers identified were elevated in the serum samples of patients with PBC, while the levels of 9 of these 26 potential biomarkers were reduced in the serum samples of patients with PBC compared with those in HCs. Among these biomarkers, the levels of TDCA, GUDCA, tetracosahexaenoic acid, bilirubin, sphinganine, phytosphingosine, L-phenylalanine, L-proline, TCA, LysoPC [18:3 (6Z, 9Z, 12Z)], TUDCA, GCA, LysoPE [0:0/18:4 (6Z, 9Z, 12Z, 15Z)], LysoPE [20:3 (11Z, 14Z, 17Z)/0:0], L-urobilinogen, L-urobilin, and DCA significantly increased in patients with PBC compared with those in HCs ($P < 0.5$). The levels of 12-ketodeoxycholic acid, α-

Table 2 Characteristics of classified enrolled population according to Child-Pugh in the metabolic profiling study

Clinical parameters	PBC-A (*n* = 26)	PBC-B (*n* = 19)	PBC-C (*n* = 9)	AIH-A (*n* = 17)	AIH-B (*n* = 9)
Age (mean, range) (yr), *n*	54 (38, 68)	57.11 (40, 73)	59.33 (51, 67)	52.35 (17, 75)	59.11 (36, 73)
Sex (Male/Female), *n*	6/20	3/16	0/9	2/15	1/8
AST (U/L) median, range	62.77 (20, 210)	182.62 (14, 1300)	113.27 (35, 235)	122.28 (23, 961)	256.78 (64, 472)
ALT (U/L) median, range	74.18 (15, 293)	130.32 (12, 734)	84.51 (17, 236)	100.54 (10, 780)	253.11 (103, 598)
ALP (U/L) median, range	276.41 (75, 53)	352.73 (79, 913)	211.43 (55, 483)	230.33 (57, 795)	258.78 (46, 738)
γ-GT (U/L) median, range	380.18 (40, 631)	281.77 (40, 744)	103.49 (32, 235)	166.46 (26, 654)	393.89 (73, 957)
TBA (μmol/L) median, range	31.04 (2.9, 09.3)	75.16 (2.7, 295.7)	127.37 (23.4, 267.5)	116.46 (1, 1335)	144.29 (15, 379)
TBiL (μmol/L) median, range	19.7 (5.6, 48.8)	81.0 (11.3, 306.5)	181.62 (17.7, 825.8)	48.33 (6.5, 229.5)	166.23 (50.0, 543.9)
DBIL (μmol/L) median, range	9.18 (2.4, 32.0)	48.3 (4.1, 207.9)	99.71 (6.6, 407.4)	27.40 (2.1, 139.3)	101.24 (30.6, 300.8)
Liver cirrhosis (%)	0.38 (10/26)	0.84 (16/19)	1 (9/9)	0.47 (8/17)	0.33 (3/9)
Liver biopsy (%)	0.35 (9/26)	0.05 (1/19)	0 (0/9)	0.64 (11/17)	0.33 (3/9)
Positive of AMA (%)	80 (21/26)	80 (15/19)	89 (8/9)	0 (0/17)	0 (0/9)
Positive of ANA (%)	73 (19/26)	52 (10/19)	44 (4/9)	100 (17/17)	100 (9/9)

PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; AST: Aspartatetransaminase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; γ-GT: γ-glutamyl transpeptidase; TBiL: Totalbilirubin; DBiL: Direct bilirubin; TBA: Total bile acid; AMA: Anti-mitochondrial antibody; ANA: Anti-nuclear antibody. All data are presented as median and range. Statistically significant differences between controls and patients were determined by the rank sums Mann-Whitney.

Table 3 Potential serum biomarkers for primary biliary cirrhosis compared to healthy control in positive and negative ions model

Name	VIP	MZ	Time	PBC/control	
				t-test	Fold change (P/C)
ESI+					
Taurodeoxycholic acid	1.747	500.3033	10.598	0.001	8.146
Glycodeoxycholate	2.175	450.3207	12.146	0.000	4.558
Tetracosahexaenoic acid	1.100	357.2786	9.333	0.045	3.490
Bilirubin	1.511	585.2701	10.218	0.006	3.334
Sphinganine	1.953	302.3052	12.792	0.000	3.285
Phytosphingosine	2.482	318.2999	10.734	0.000	3.039
L-Phenylalanine	1.690	166.0860	2.037	0.002	0.372
L-Proline	1.194	116.0706	0.725	0.030	0.180
12-Ketodeoxycholic acid	1.573	391.2842	15.210	0.004	-0.324
ESI-					
Taurocholic acid	1.493	514.2805	9.146	0.000	6.634
LysoPC [18:3(6Z, 9Z, 12Z)]	1.184	516.3064	13.057	0.001	5.263
Tauroursodeoxycholic acid	1.843	498.2861	10.456	0.000	4.627
Glycocholic Acid	1.866	464.2988	9.957	0.000	3.644
LysoPE [0:0/18:4 (6Z, 9Z, 12Z, 15Z)]	1.316	472.2430	9.798	0.000	3.274
LysoPE [20:3 (11Z, 14Z, 17Z)/0:0]	1.882	500.2947	10.456	0.000	3.225
L-Urobilinogen	1.027	595.3478	11.784	0.003	3.202
L-Urobilin	1.032	593.3315	11.665	0.003	2.411

Deoxycholic acid	1.032	391.2833	11.641	0.003	2.121
α-ketoisovaleric acid	1.040	115.0399	1.831	0.003	-0.348
Pyroglutamic acid	1.252	128.0350	0.931	0.000	-0.392
Lactic acid	1.548	89.0242	0.938	0.000	-0.402
Hypoxanthine	1.384	135.0308	0.886	0.000	-0.431
LysoPE [0:0/20:2 (11Z, 14Z)]	1.335	504.3072	14.333	0.000	-0.453
Ketoleucine	1.392	129.0555	4.110	0.000	-0.486
LysoPE [0:0/22:4 (7Z, 10Z, 13Z, 16Z)]	1.333	528.2850	13.617	0.000	-0.544
MG [0:0/18:4 (6Z, 9Z, 12Z, 15Z)/0:0]	2.060	349.2373	8.969	0.000	-2.181

PBC: Primary biliary cirrhosis; VIP: Variable importance in the projection.

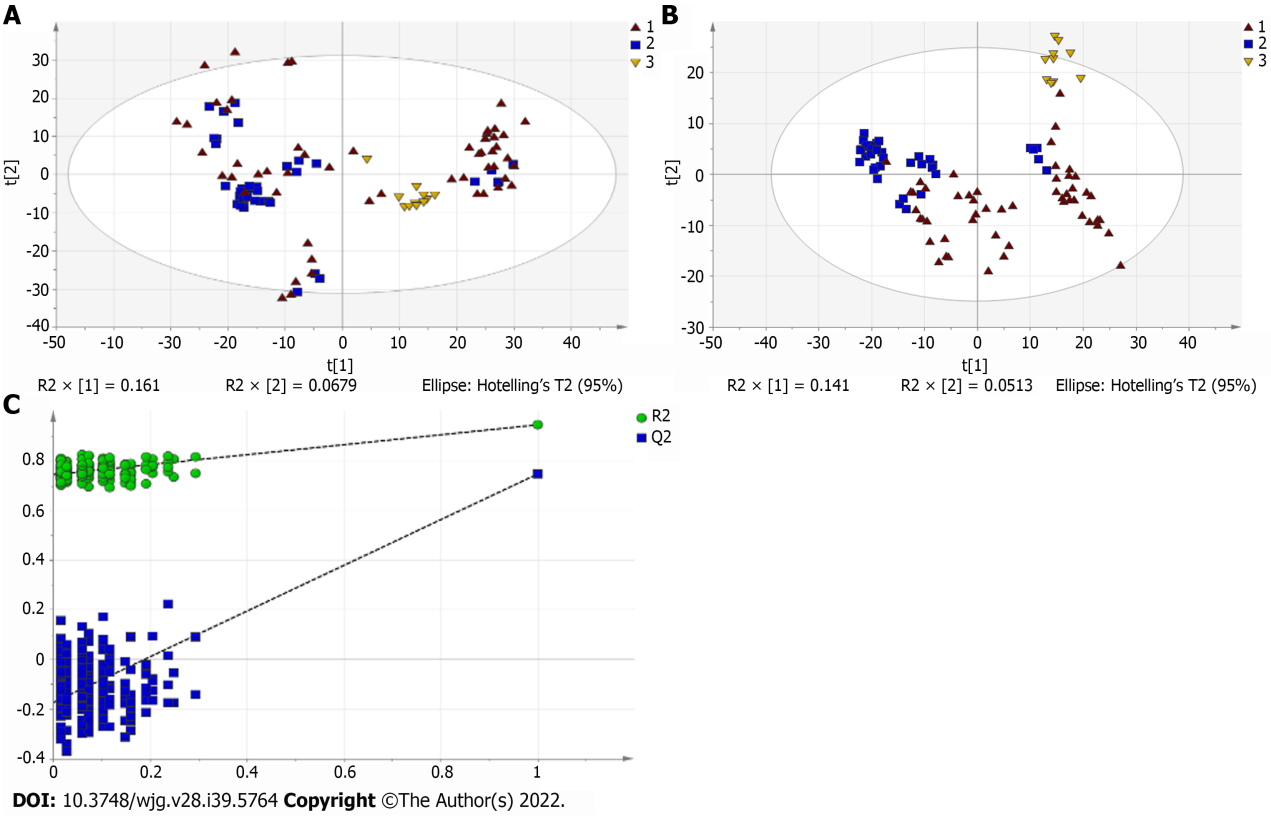

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Figure 1 Multivariate statistical analysis on serum profiling data in positive ions between primary biliary cholangitis and control. A: Plots of principal component analysis (PCA) in positive ion mode. (1) Primary biliary cholangitis (PBC); (2) Control; and (3) Quality control (QC); B: Scatter plots of partial least squares-discriminant analysis (PLS-DA) with a positive model of serum from patients with PBC, autoimmune hepatitis and healthy controls. (1) PBC; (2) Control; and (3) QC; C: Validation plot of the original PLS-DA with a positive model, strongly indicating that the original model is valid and shows signs of overfitting. The permutation test was repeated 200 times in the cross-validation plot.

ketoisovaleric acid, pyroglutamic acid, lactic acid, hypoxanthine, LysoPE [0:0/20:2 (11Z, 14Z)], ketoleucine, LysoPE [0:0/22:4 (7Z, 10Z, 13Z, 16Z)], and MG [0:0/18:4 (6Z, 9Z, 12Z, 15Z)/0:0] in patients with PBC were significantly reduced compared with those in HCs.

Identification of serum metabolites specific to AIH

As shown in Table 4. The levels of 17 of 25 potential biomarkers identified were elevated in the serum samples of the patients with AIH, while the levels of 8 of these 25 potential biomarkers were reduced in the serum samples of patients with AIH compared with those in HCs. Among these biomarkers, the levels of TDCA, GUDCA, L-Urobilin, sphinganine, phytosphingosine, I-Urobilin, bilirubin, stearamide, kynurenine, L-threonine, L-phenylalanine, urea, TCA, LysoPC [18:3 (6Z, 9Z, 12Z)], TDCA, GCA, and LysoPE [20:3 (11Z, 14Z, 17Z)/0:0] significantly increased in patients with AIH compared with those in HCs. The levels of 12-ketodeoxycholic acid, uric acid, pyroglutamic acid, LysoPE [0:0/20:2 (11Z, 14Z)],

Table 4 Potential serum biomarkers for autoimmune hepatitis compared to healthy control

Name	VIP	MZ	Time	AIH/control	
				t-test	Fold change (A/C)
ESI+					
Taurodeoxycholic acid	1.900	500.3033	10.598	0.000	8.791
Glycodeoxycholate	1.912	450.3207	12.146	0.000	5.217
L-Urobilin	1.442	595.3484	7.835	0.007	5.164
Sphinganine	2.308	302.3052	12.792	0.000	4.509
Phytosphingosine	2.344	318.2999	10.734	0.000	4.118
I-Urobilin	1.454	591.3169	7.628	0.006	3.661
Bilirubin	1.876	585.2701	10.218	0.000	3.578
Kynurenine	2.279	209.0919	1.944	0.000	0.592
L-Threonine	1.446	120.0655	0.708	0.007	0.386
L-Phenylalanine	1.551	166.0860	2.037	0.004	0.262
Urea	1.160	61.0395	0.730	0.032	0.113
12-Ketodeoxycholic acid	1.125	391.2842	15.210	0.037	-0.270
Uric acid	1.208	169.0354	1.035	0.025	-0.265
Pyroglutamic acid	2.489	130.0499	1.029	0.000	-0.517
ESI-					
Taurocholic acid	1.605	514.281	9.146	0.000	7.368
LysoPC [18:3 (6Z, 9Z, 12Z)]	1.454	516.306	13.057	0.000	6.614
Tauroursodeoxycholic acid	1.807	498.286	10.456	0.000	5.151
Glycocholic Acid	1.961	464.299	9.957	0.000	3.946
LysoPE [20:3 (11Z, 14Z, 17Z)/0:0]	1.807	500.295	10.456	0.000	3.670
LysoPE [0:0/20:2 (11Z, 14Z)]	1.234	504.307	14.333	0.001	-0.471
Lactic acid	1.489	89.024	0.938	0.000	-0.489
Hypoxanthine	1.380	135.031	0.886	0.000	-0.562
CPA (16:0/0:0)	1.629	391.224	26.118	0.000	-2.159
MG [0:0/18:4 (6Z, 9Z, 12Z, 15Z)/0:0]	1.771	349.237	8.969	0.000	-2.464

AIH: Autoimmune hepatitis; VIP: Variable importance in the projection.

Table 5 Changes of the serum bile acid profile between primary biliary cirrhosis, autoimmune hepatitis and controls

	AIH (mean ± SD)	PBC (mean ± SD)	Control (mean ± SD)	PBC/AIH	AIH/control	PBC/control
				P value	*P* value	*P* value
CA	2.38 ± 0.69	2.26 ± 0.85	1.60 ± 0.51	0.53	< 0.001[b]	< 0.001[a]
GCA	3.00 ± 1.02	2.91 ± 0.91	1.33 ± 0.50	0.69	< 0.001[b]	< 0.001[a]
TCA	2.12 ± 1.23	2.12 ± 0.93	0.03 ± 0.46	0.99	< 0.001[b]	< 0.001[a]
UDCA	2.41 ± 1.03	2.60 ± 1.05	2.05 ± 0.36	0.62	0.2	0.01[a]
GUDCA	2.59 ± 1.17	3.04 ± 1.14	1.64 ± 0.50	0.09	< 0.001[b]	< 0.001[a]
TUDCA	1.48 ± 1.07	1.84 ± 1.09	0.13 ± 0.25	0.15	< 0.001[b]	< 0.001[a]
CDCA	2.88 ± 0.39	3.07 ± 0.48	2.63 ± 0.38	0.046[c]	0.01[b]	< 0.001[a]

GCDCA	3.78 ± 0.80	3.79 ± 0.67	2.93 ± 0.33	0.96	< 0.001[b]	< 0.001[a]
TCDCA	2.73 ± 0.40	2.84 ± 0.44	2.49 ± 0.57	0.27	0.09	0.004[a]
GCDCS	2.39 ± 0.82	2.42 ± 0.70	1.08 ± 0.39	0.95	< 0.001[b]	< 0.001[a]
DCA	2.76 ± 0.35	2.75 ± 0.40	2.76 ± 0.18	0.87	0.7	0.59
GDCA	3.18 ± 0.58	3.14 ± 0.53	2.64 ± 0.32	0.8	< 0.001[b]	< 0.001[a]
TDCA	3.32 ± 0.64	3.16 ± 0.50	2.57 ± 0.19	0.33	< 0.001[b]	< 0.001[a]
LCA	0.94 ± 0.68	1.28 ± 0.66	0.72 ± 0.43	0.04[c]	0.14	< 0.001[a]
TLCA	0.30 ± 0.41	0.48 ± 0.47	0.02 ± 0.06	0.07	< 0.001[b]	< 0.001[a]
LCA + TLCA	1.25 ± 0.18	1.75 ± 0.14	0.74 ± 0.07	0.034[c]	0.031[b]	< 0.001[a]
CDCA/CA	1.30 ± 0.35	1.56 ± 0.65	1.74 ± 0.42	0.26	< 0.001[b]	< 0.001[a]
pBA	3.11 ± 0.45	3.17 ± 0.51	2.67 ± 0.38	0.98	< 0.001[b]	< 0.001[a]
sBA	3.14 ± 0.52	3.19 ± 0.61	2.87 ± 0.17	0.75	0.003[b]	0.002[a]
pBA/sBA	1.02 ± 0.18	1.01 ± 0.17	1.08 ± 0.13	0.85	0.087	< 0.001
sBA	2.77 ± 0.35	2.79 ± 0.40	2.77 ± 0.18	0.81	0.02[b]	0.003[a]
G-BA	4.14 ± 0.70	4.12 ± 0.64	3.16 ± 0.31	0.97	< 0.001[b]	< 0.001[a]
T-BA	3.58 ± 0.54	3.47 ± 0.46	2.89 ± 0.27	0.34	< 0.001[b]	< 0.001[a]
G-BA/T-BA	0.55 ± 0.34	0.65 ± 0.34	0.27 ± 0.30	0.25	< 0.001[b]	< 0.001[a]
total BA	36.31 ± 6.00	37.69 ± 7.40	24.80 ± 3.12	0.41	< 0.001[b]	0.026[a]

[a]$P < 0.05$ Primary biliary cirrhosis (PBC) *vs* control.
[b]$P < 0.05$ Autoimmune hepatitis (AIH) *vs* control.
[c]$P < 0.05$ PBC *vs* AIH
Bile acid (BA) levels are expressed in log10 concentrations. Statistically significant differences in BA concentrations between controls and patients were determined by the rank sums Mann-Whitney test. CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; GCA: Glycocholic acid; GCDCA: Glycochenodeoxycholic acid; GCDCS: Glycochenodeoxycholic sulfate; GDCA: Glycodeoxycholic acid; GUDCA: Glycoursodeoxycholic acid; LCA: Lithocholic acid; TCA: Taurocholic acid; TCDCA: Taurochenodeoxycholic acid; TDCA: Taurodeoxycholic acid; TLCA: Taurolithocholic acid; TUDCA: Tauroursodeoxycholic acid; UDCA: Ursodeoxycholic acid; PBA: Primary bile acid; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis.

lactic acid, pyroglutamic acid, hypoxanthine, CPA (16:0/0:0), and MG [0:0/18:4 (6Z, 9Z, 12Z, 15Z)/0:0] significantly decreased in patients with AIH compared with those in HCs.

Quantification of targeted BAs specific to PBC and AIH

Recently, BAs have been shown to be potentially more effective biomarkers for PBC and AIH. Regarding the most abundant BAs in humans, the following 15 BAs were selected: CA, GCA, TCA, UDCA, GUDCA, TUDCA, CDCA, GCDCA, TCDCA, GCDCS, DCA, GDCA, TDCA, LCA, and TLCA. The levels of these 15 BAs were measured using the UPLC-QTOF-MS. The levels of all BAs in the disease group were higher than those in the control group, and the levels of glycine-bound cholic acid and tauro-bound cholic acid were elevated, shown in Supplementary Table 1.

Furthermore, the levels of BAs in patients with PBC and AIH were compared with the corresponding levels in HCs, show in Table 5. It was revealed that the levels of BAs were elevated in patients with PBC and AIH. The levels of CDCA, LCA, TLCA, and LCA + TLCA in PBC patients were higher than those in AIH patients, in which a significant difference was found in the levels of CDCA and LCA ($P < 0.05$; for TLCA, $P = 0.0767$).The differences in the levels of CDCA, LCA, and LCA + TLCA among the three groups were statistically show in Figure 7. The levels of CDCA, LCA, and LCA + TLCA significantly increased in PBC patients compared with those in AIH patients ($P < 0.05$). Moreover, the CDCA-to-CA ratio decreased in PBC and AIH patients compared with that in HCs.

The receiver operating characteristic curve analysis of BAs with differences between the PBC and AIH groups showed that the area under the curve values of CDCA, LCA, and TLCA were greater than 0.7, and sensitivity was higher than 70%, indicating a high sensitivity, while a low specificity was noted in identification of patients with PBC and AIH (Figure 8 and Table 6). Compared with sensitivity and specificity of the traditional biochemical indicators, such as alanine transaminase, aspartate transaminase, gamma glutamyl transpeptidase, alkaline phosphatase, total bilirubin, direct bilirubin, and total bile acid, sensitivity and specificity of CDCA, LCA and TLCA were higher than the traditional markers of liver injury, which are of great significance for clinical diagnosis and can be further verified by enlarging the sample size. Thus, BAs can be potentially considered as markers for the diagnosis of PBC and AIH.

Table 6 Area under the curve, sensitivity and specificity of difference bile acids, conventional biochemical indicators in primary biliary cirrhosis, autoimmune hepatitis and control group

	AUC	Sensitivity	Specificity
LCA	0.68	0.82	0.50
LCA + TLCA	0.73	0.74	0.46
CDCA	0.74	0.74	0.54
AST	0.54	0.67	0.58
ALT	0.61	0.64	0.61
ALP	0.41	0.56	0.35
GGT	0.43	0.50	0.46
TBiL	0.53	0.59	0.42
DBiL	0.47	0.44	0.62
TBA	0.52	0.52	0.42

CDCA: Chenodeoxycholic acid; LCA: Lithocholic acid; TLCA: Taurolithocholic acid; ALT: Alanine transaminase; AST: Aspartate transaminase; GGT: Gamma glutamyl transpeptidase; ALP: Alkaline phosphatase; TBiL: Total bilirubin; DBiL: Direct bilirubin; TBA: Total bile acid; AUC: Area under the curve.

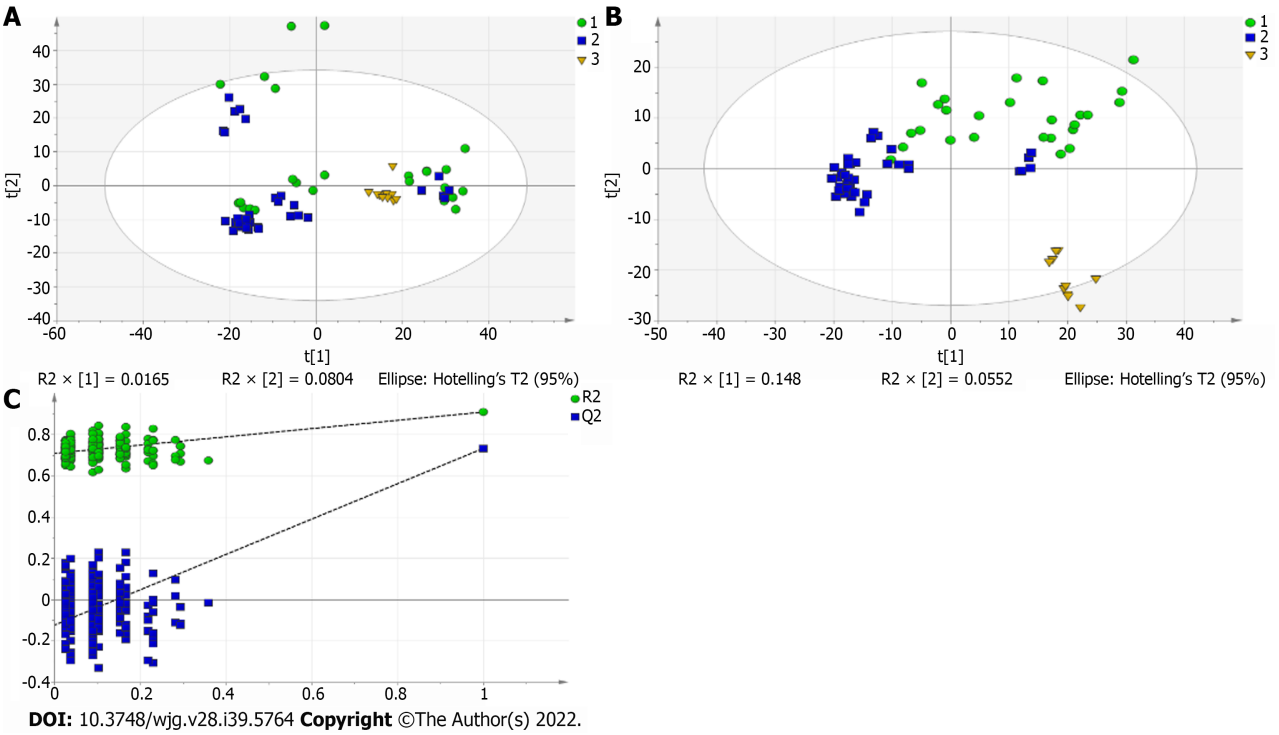

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Figure 2 Multivariate statistical analysis on serum profiling data in positive ions between autoimmune hepatitis and control. A: Plots of principal component analysis in positive ion mode. (1) Autoimmune hepatitis (AIH); (2) Control; and (3) Quality control (QC); B: Scatter plots of partial least squares-discriminant analysis (PLS-DA) with a positive model of serum from patients with primary biliary cholangitis (PBC), AIH and healthy controls. (1) PBC; (2) Control; and (3) QC; C: Validation plot of the original PLS-DA with a positive model, strongly indicating that the original model is valid and shows signs of overfitting. The permutation test was repeated 200 times in the cross-validation plot.

DISCUSSION

In the present study, we established a diagnostic model for PBC and AIH using the UPLC-QTOF-MS. Besides, VIP values from PLS-DA were calculated to describe a quantitative estimation of the discriminatory power of each individual feature. We found changes in the levels of amino acids, BAs, organic acids, phospholipids, sugar, and sugar alcohols in patients with PBC and AIH, and in HCs. These substances are mainly involved in lipid metabolism, BA metabolism, and bilirubin metabolism, which

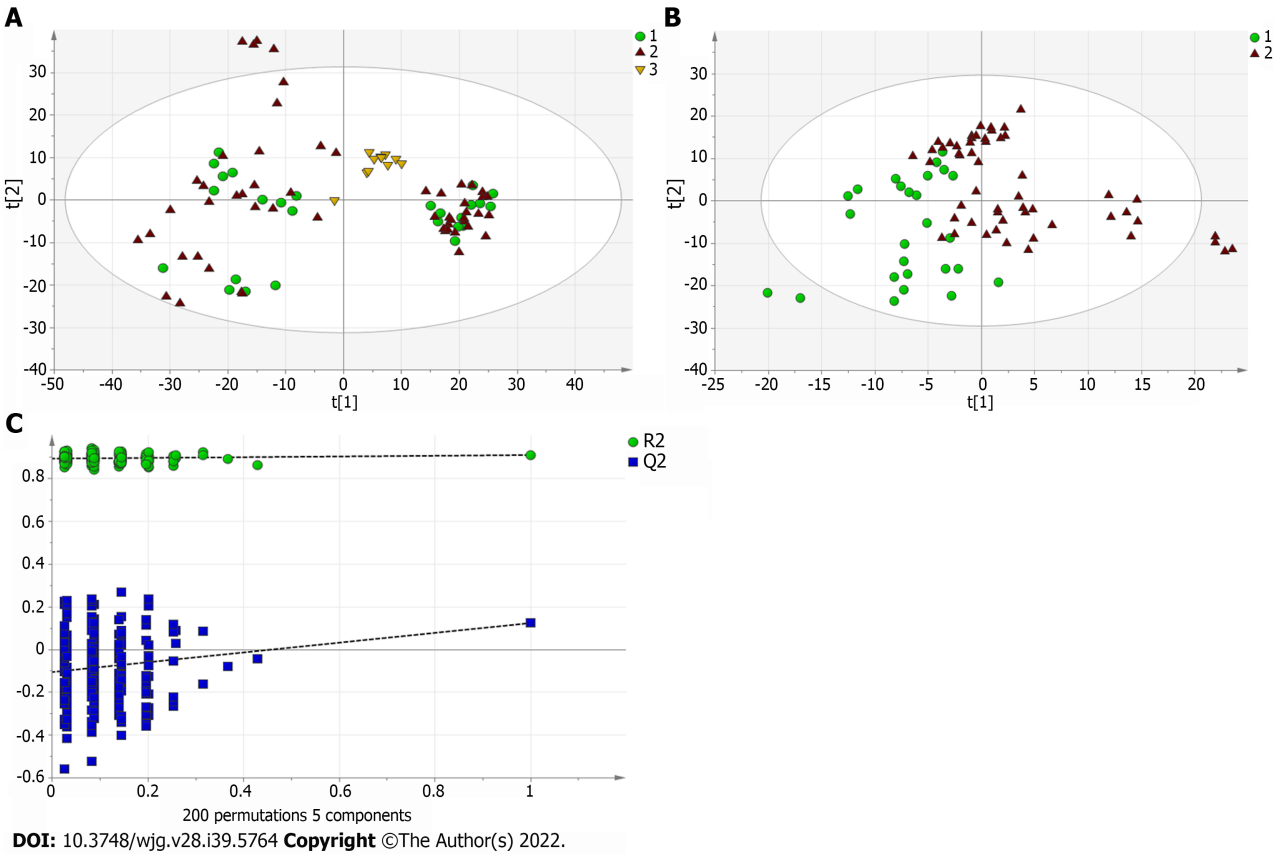

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Figure 3 Multivariate statistical analysis on serum profiling data in positive ions between primary biliary cholangitis and autoimmune hepatitis. A: Plots of principal component analysis in positive ion mode. (1) Autoimmune hepatitis (AIH); (2) Primary biliary cholangitis (PBC); and (3) Quality control; B: Scatter plots of partial least squares-discriminant analysis (PLS-DA) with a positive model of serum from patients with PBC, AIH and healthy controls. (1) PBC; and (2) AIH; C: Validation plot of the original PLS-DA with a positive model, strongly indicating that the original model is valid and shows signs of overfitting. The permutation test was repeated 200 times in the cross-validation plot.

are related to metabolic functions of the liver and inflammatory reactions. These compound classes are also associated with key hepatic metabolic pathways. Importantly, our findings are consistent with those reported previously; for instance, BAs have been identified as a significant factor contributing to PBC[14-16]. When liver injury occurs, intrahepatic clearance rate of BAs decreases and serum BA level increases. BAs have long been used as markers of liver dysfunction. In recent studies, elevated serum levels of BAs have been found to be closely associated with liver diseases[17-21].

The levels of lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) significantly changed in patients with PBC and AIH. To date, no study has used lysophospholipids in the diagnosis of PBC and AIH. Lysophospholipids are biologically active lipids that are involved in a variety of important processes, including cell proliferation, cell migration, angiogenesis, and inflammation[22]. Our results also provided important clues to further explore the pathogenesis of PBC and AIH.

A discriminatory diagnostic model of PBC/AIH could not be established using the UPLC/MS/MS, suggesting that the changes of terminal metabolites in serum samples of patients with PBC and AIH were no special differences. Failure in the establishment of a discriminatory diagnostic model of PBC/AIH could be related to the sample size. Therefore, BAs were quantitatively analyzed according to the differences found between PBC/control and AIH/control groups.

BA is the general term used for a class of bisexual molecules produced by the metabolism of cholesterol. The liver has an effective clearance effect on BAs, and BAs are kept at low levels, confirming the low levels of BAs in the human peripheral blood plasma. In the human liver, cholesterol is metabolized into primary BAs, including CA and CDCA, and then into the intestine, followed by into the corresponding secondary show in Figure 9.

The results of our targeted metabolomic study of BAs showed that the levels of BAs increased in patients with PBC and AIH compared with those in HCs, and the levels of CDCA, LCA, and LCA + TLCA in PBC patients were significantly higher than those in AIH patients. It is noteworthy that CA and CDCA, the two major human BAs, are synthesized from cholesterol in a series of reactions catalyzed by enzymes located in the endoplasmic reticulum, mitochondria, cytosol, and peroxisomes, suggesting that there were significant differences in the levels of BAs in PBC patients, providing clues for the future study on the pathogenesis of PBC. In autoimmune liver diseases, the dysfunction of BA metabolism occurs after liver injury, which may be related to bile stasis after liver injury, especially in

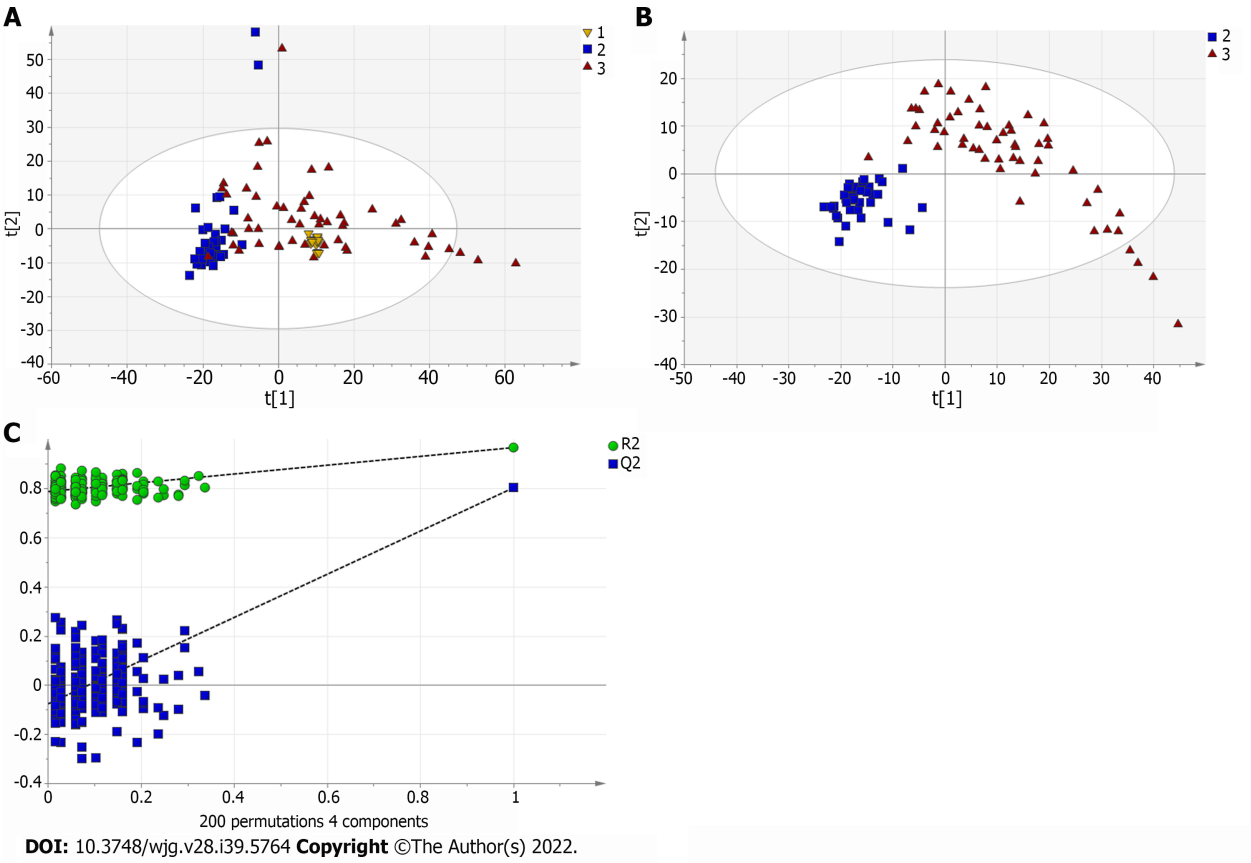

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Figure 4 Multivariate statistical analysis on serum profiling data in negative ions between primary biliary cholangitis and control. A: Plots of principal component analysis in negative ion mode. (1) Primary biliary cholangitis (PBC); (2) Control; and (3) Quality control (QC); B: Scatter plots of partial least squares-discriminant analysis (PLS-DA) with a negative model of serum from patients with PBC, autoimmune hepatitis and healthy controls. (1) PBC; (2) Control; and (3) QC; C: Validation plot of the original PLS-DA with a negative model, strongly indicating that the original model is valid and shows signs of overfitting. The permutation test was repeated 200 times in the cross-validation plot.

PBC, which is more drastic, and is related to the pathogenesis of PBC. After bile duct obstruction and sclerosis, BAs cannot be transported and metabolized normally. Patients may present with jaundice and itchy skin.

Therefore, determination of the levels of BAs in plasma can reflect the synthesis, ingestion, and secretion of hepatocytes. Abnormalities in the levels of BAs not only reflect the extent of liver damage, but also indirectly indicate the conditions of blood-bile barrier in the liver.

To our knowledge, this is the first study that used serum metabolic profiling for diagnosing patients with PBC and AIH.

LCA is an endogenous compound associated with hepatic toxicity during cholestasis. A previous study[23] revealed that LCA induced disruption of phospholipid/ sphingolipid homeostasis through the transforming growth factor-β signaling pathway and serum LPC could be a biomarker for biliary injury.

The hepatic level of LCA was reported to elevate in patients with cholestatic liver disease[24,25]. This result is consistent with our finding, in which the levels of CDCA, LCA, TLCA, and LCA + TLCA were higher in PBC patients than those in AIH patients.

Previous studies[26,27] indicated that the activation of cytochrome P450 is correlated with Farnesoid X receptor (FXR). Mammalian FXR, which is a transcription regulatory factor in bile salt synthesis, is activated by BAs, such as CDCA or LCA[28,29]. The derangements of lipid metabolism are weakened in FXR-null mice compared with those in wild-type mice after LCA exposure[30,31]. As a cholestatic liver disease, the high levels of BAs may induce FXR gene transcription in PBC patients. Therefore, we hypothesized that these pathways may lead to LCA poisoning in PBC patients, and LCA metabolic pathway plays an important role in the incidence of PBC. Lian *et al*[14] used the untargeted metabolomic method of UPLC-MS, and clarified the relationship between LCA level and PBC incidence, as well as the relationship between LCA level and the incidence of lipid metabolism disorders. Our study also revealed the abnormal levels of LPC and LPE in PBC patients.

However, the retention of hydrophobic BAs in pathophysiological conditions, such as cholestatic diseases, plays an important role in liver injury by inducing apoptosis or necrosis of hepatocytes[32]. The retention and accumulation of hydrophobic Bas (*e.g.*, CDCA and DCA)inside hepatocytes during cholestasis have long been implicated as a major cause of liver dysfunction[32].

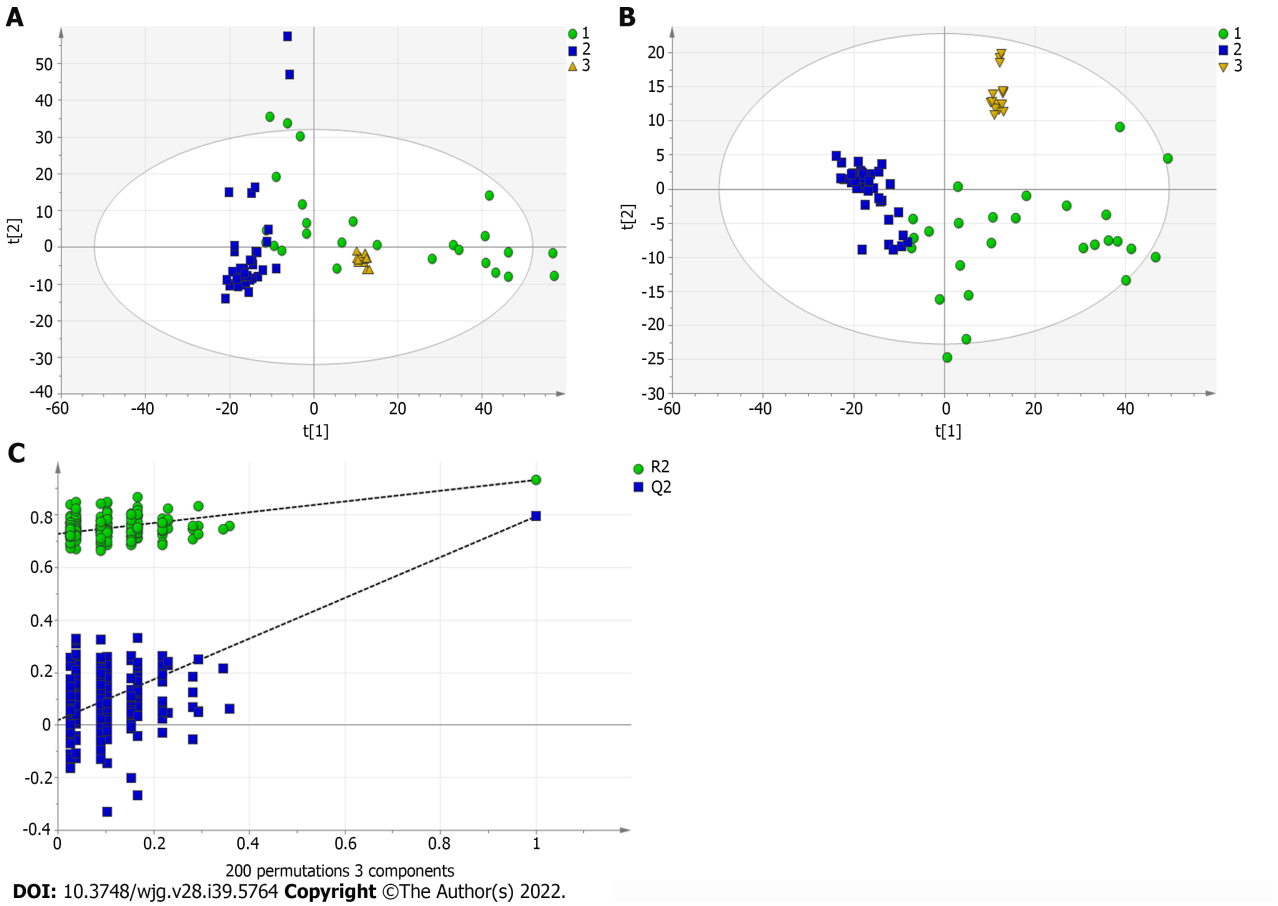

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Figure 5 Multivariate statistical analysis on serum profiling data in negative ions between autoimmune hepatitis and control. A: Plots of principal component analysis in negative ion mode. (1) Autoimmune hepatitis (AIH); (2) Control; and (3) Quality control (QC); B: Scatter plots of partial least squares-discriminant analysis (PLS-DA) with a negative model of serum from patients with Primary biliary cholangitis, AIH and healthy controls. (1) Autoimmune hepatitis (AIH); (2) Control; and (3) QC; C: Validation plot of the original PLS-DA with a negative model, strongly indicating that the original model is valid and shows signs of overfitting. The permutation test was repeated 200 times in the cross-validation plot.

The hydrophobicity of BAs is an important determinant of the toxicity and protection of BAs. Under normal conditions, the levels of BAs undergoing further biotransformations to dianionic glucuronidated or sulfated derivatives are negligible, although they may become important in cholestasis[33].

Several mechanisms may be involved in the cytotoxicity associated with the most hydrophobic BAs in cholestatic liver diseases[32]. BAs could disrupt cell membranes through their detergent action on lipid components[34] and promote the generation of reactive oxygen species that, in turn, oxidatively modify lipids, proteins, and nucleic acids, and eventually cause hepatocyte apoptosis[35].

As shown in Figure 9 CDCA/LCA/TLCA are all related to the decomposition and hydrolysis of bacteria in the intestine. CDCA is decomposed into LCA through bacteria in the intestine, and then, synthesizes TLCA through the intestinal bacteria. Intestinal bacteria may play a key role in this process. Therefore, we can hypothesize that dysfunction of intestinal bacteria may increase the incidence of autoimmune liver diseases, including PBC. Lv *et al*[36] and Zheng *et al*[37] found that the interaction of intestinal microflora with metabolism and immunity is crucial for the occurrence or development of PBC.

The Child-Pugh scoring system was used to classify PBC and AIH patients according to their Child-Pugh scores, show in Supplementary Table 2 and the levels of BAs in these patients with Child-Pugh scores were statistically show in Table 7. It was found that the levels of GCA, TCA, GCDCA, GCDCS, TDCA, and tauro-conjugated BAs were gradually elevated with the increase of Child-Pugh scores. The levels of BAs in PBC patients with Child-Pugh class C were significantly different from those in PBC patients with Child-Pugh class A ($P < 0.05$). The levels of BAs in AIH patients with Child-Pugh class B were significantly different from those in PBC patients with Child-Pugh class A ($P < 0.05$). The levels of GCA, GCDCS, and TDCA significantly differed in PBC and AIH patients with Child-Pugh class B (Figure 10). These BAs are all conjugated BAs, suggesting that the levels of conjugated BAs are elevated in patients with severe liver injury. The determination of BA level can not only reflect liver damage, but also indicate the degree of liver damage, which is similar to the results of our previous study on drug-induced liver injury (DILI)[38]. The increase in the levels of GCA, TCA, TUDCA, GCDCA, GCDCS, and TDCA was corresponded to a higher degree of DILI. Tang *et al*[15] used UPLC/Q-TOF-MS to analyze

Table 7 Changes of the serum bile acid profile between primary biliary cirrhosis and autoimmune hepatitis in different grade of Child-Pugh

	AIH-A/PBC/A	AIH-B/PBC-B	AIH-A/AIH-B	PBC-A/PBC-B	PBC-B/PBC-C	PBC-A/PBC-C	AIH-A/control	AIH-B/control	PBC-A/control	PBC-B/control	PBC-C/control
	P value	*P* value	*P* value	*P* value	*P* value	*P* value	*P* value	*P* value	*P* value	*P* value	*P* value
CA	0.75	0.34	0.24	0.67	0.82	0.50	< 0.001	< 0.001	< 0.001	< 0.001	0.03
GCA	0.79	0.03	< 0.001	0.07	0.43	0.02	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
TCA	0.79	0.03	< 0.001	0.0522	0.71	0.0567	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
UDCA	0.77	0.26	0.07	0.70	0.30	0.38	< 0.001	0.52	<0.001	0.05	< 0.001
GUDCA	0.60	0.33	0.40	0.58	0.21	0.06	< 0.001	0.13	< 0.001	< 0.001	< 0.001
TUDCA	0.97	0.22	1.00	0.10	0.29	0.02	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
CDCA	0.26	0.09	0.07	0.30	0.44	0.78	< 0.001	0.47	< 0.001	< 0.001	< 0.001
GCDCA	0.89	0.19	0.01	0.06	0.17	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
TCDCA	0.82	0.20	0.31	0.34	0.92	0.26	0.05	0.48	0.02	0.01	0.04
GCDCS	0.33	0.04	< 0.001	0.15	0.24	0.01	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
DCA	0.71	0.26	0.02	0.13	0.66	0.38	0.24	0.01	0.64	0.21	0.45
GDCA	0.50	0.08	0.09	0.37	0.09	0.11	< 0.001	< 0.001	< 0.001	0.01	< 0.001
TDCA	0.69	0.03	< 0.001	0.01	0.33	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
LCA	0.29	0.13	0.57	0.82	0.24	0.33	0.08	0.51	< 0.001	< 0.001	< 0.001
TLCA	0.07	0.88	0.05	0.13	0.74	0.29	0.01	< 0.001	< 0.001	< 0.001	< 0.001
CDCA/CA	0.46	0.04	0.04	0.66	0.50	0.27	< 0.001	< 0.001	< 0.001	0.06	0.26
Primary bile acid	0.75	0.81	0.96	0.42	0.70	0.96	< 0.001	0.02	< 0.001	< 0.001	< 0.001
Secondary bile acid	0.92	0.80	0.20	0.33	0.20	0.45	< 0.001	0.45	< 0.001	0.15	< 0.001
Secondary/primary	0.61	0.99	0.32	0.27	0.23	0.44	0.30	0.05	0.06	0.01	0.78
Secondary bile acid	0.89	0.91	0.08	0.05	0.24	0.79	0.13	0.08	0.09	0.12	0.35
Glycoconjugates	0.53	0.16	< 0.001	0.21	0.16	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Tauroconjugted	0.48	0.08	< 0.001	0.02	0.42	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Glyco/tauro	0.21	0.56	0.23	0.63	0.07	0.18	0.01	< 0.001	< 0.001	< 0.001	< 0.001
Total BA	0.62	0.72	0.05	0.26	0.19	0.01	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Bile acid (BA) levels are expressed in log10 concentrations. Statistically significant differences in BA concentrations between controls and patients were determined by the rank sums Mann-Whitney test. CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; GCA: Glycocholic acid; GCDCA: Glycochenodeoxycholic acid; GCDCS: Glycochenodeoxycholic sulfate; GDCA: Glycodeoxycholic acid; GUDCA: Glycoursodeoxycholic acid; LCA: Lithocholic acid; TCA: Taurocholic acid; TCDCA: Taurochenodeoxycholic acid; TDCA: Taurodeoxycholic acid; TLCA: Taurolithocholic acid; TUDCA: Tauroursodeoxycholic acid; UDCA: Ursodeoxycholic acid; PBA: Primary bile acid; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis.

the metabolic groups of blood and urine in 32 pairs of PBC patients and HCs. It was found that the BA level increased with the PBC progression, while the higher accuracy of our findings was confirmed. Elevated levels of BAs are correlated with severity of a variety of diseases. BAs can be used as a factor to judge the severity of the disease and as a basis for the diagnosis of the disease. It is necessary to further expand the sample size for research.

CONCLUSION

A discriminatory diagnostic model for PBC and AIH using UPLC-QTOF-MS was established. Besides, differential metabolomics analysis was conducted using the PLS-DA model to screen the differentially expressed substances in the different groups. The changes in the levels of BAs, LPC, LPE, bilirubin, and

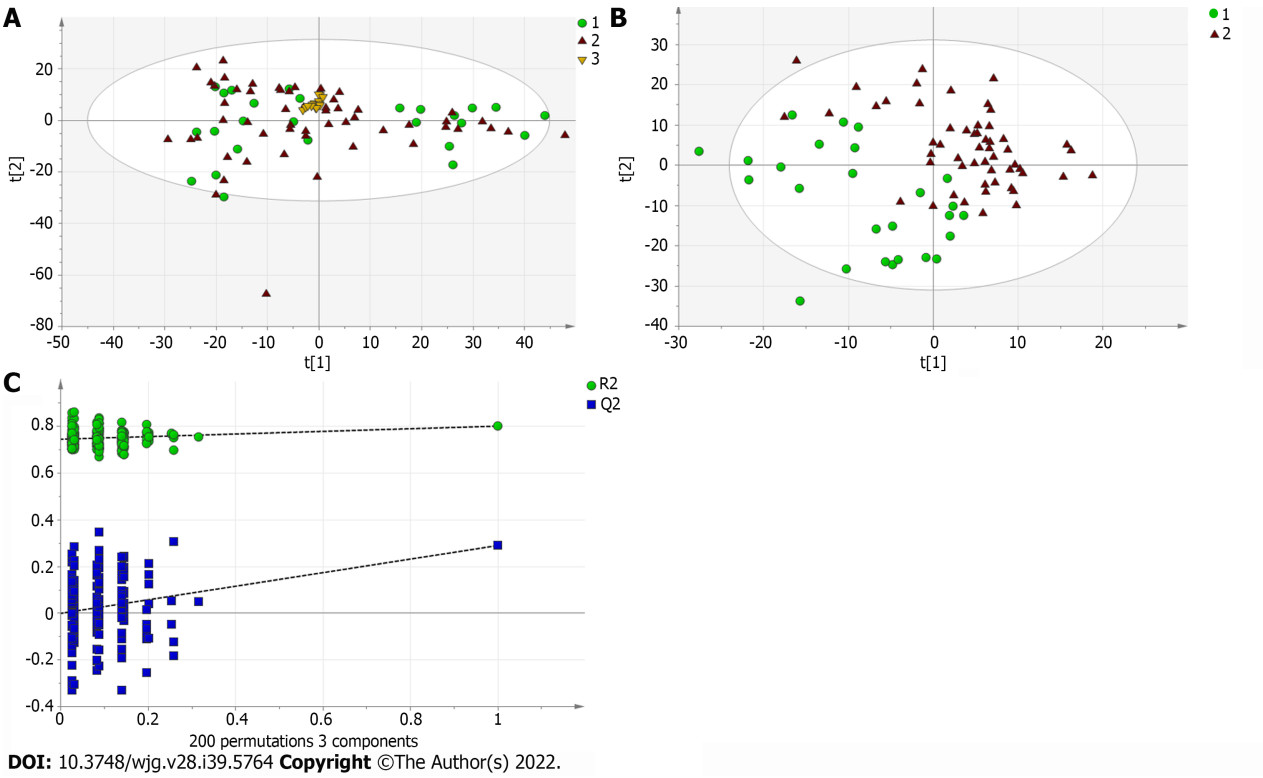

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Figure 6 Multivariate statistical analysis on serum profiling data in negative ions between primary biliary cholangitis and autoimmune hepatitis. A: Plots of principal component analysis in negative ion mode. (1) Autoimmune hepatitis (AIH); (2) Primary biliary cholangitis (PBC); and (3) Quality control; B: Scatter plots of partial least squares-discriminant analysis (PLS-DA) with a negative model of serum from patients with PBC, AIH and healthy controls. (1) AIH; and (2) PBC; C: Validation plot of the original PLS-DA with a negative model, strongly indicating that the original model is valid and shows signs of overfitting. The permutation test was repeated 200 times in the cross-validation plot.

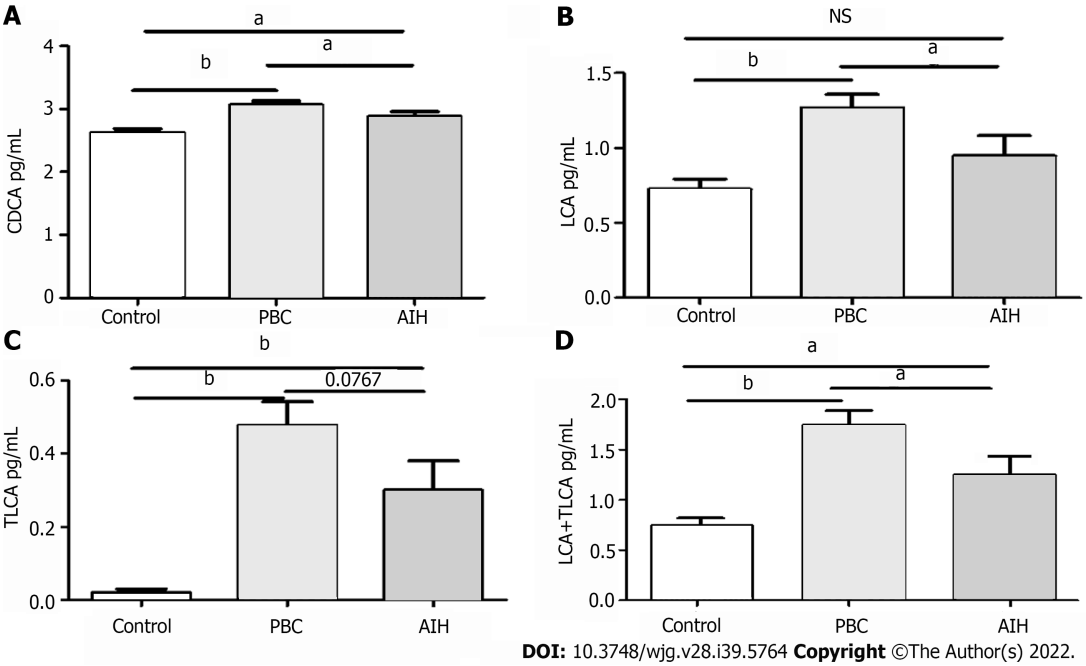

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Figure 7 Comparative analysis of alterations in serum bile acid levels in patients in the mild and severe injury groups, and in healthy controls. A: Chenodeoxycholic acid; B: Lithocholic acid (LCA); C: Taurolithocholic acid (TLCA); D: LCA + TLCA; $^{a}P < 0.05$; $^{b}P < 0.0001$; NS: Not significant.

phytosphingosine in PBC and AIH patients and HCs were compared.

The levels of CDCA, LCA, TLCA, and LCA + TLCA significantly increased in the PBC group compared with those in the AIH group. These results suggested that the levels of BAs can be used as a marker to differentiate PBC from AIH, and the results may be advantageous to study the pathogenesis

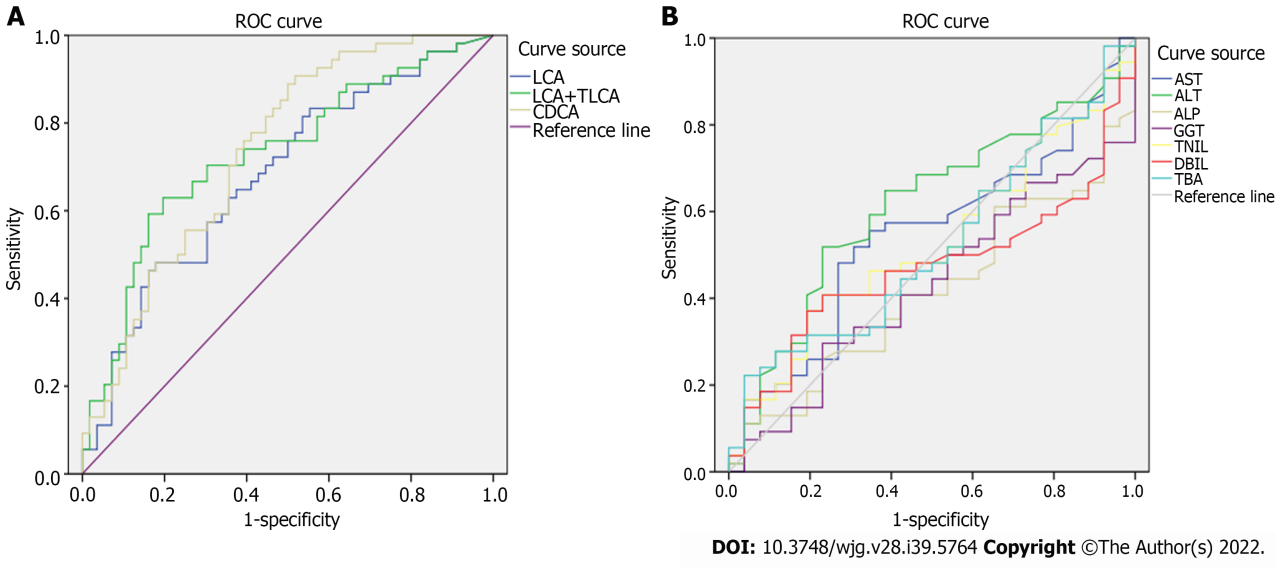

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Figure 8 Receiver operating characteristic curve analysis. A: Lithocholic acid (LCA), sum of LCA and taurolithocholic acid, chenodeoxycholic acid; B: Common clinical biochemical indicators.

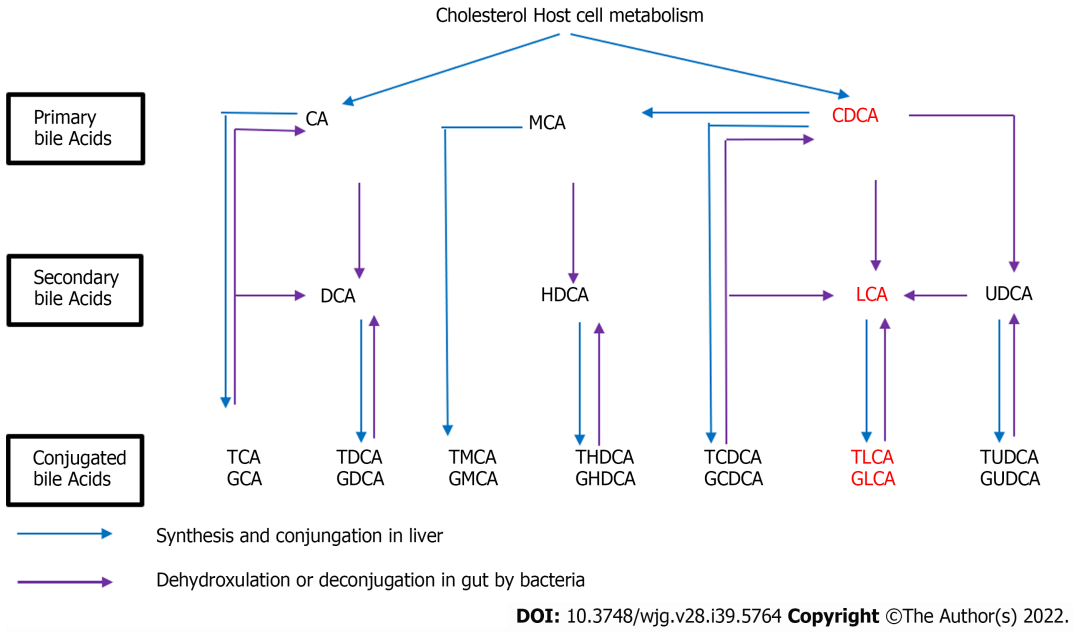

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Figure 9 Cholesterol host cell metabolism. CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; TCA: Taurocholic acid; GCA: Glycocholic acid; TDCA: Taurodeoxycholic acid; GDCA: Glycodeoxycholic acid; TCDCA: Taurochenodeoxycholic acid; TLCA: Taurolithocholic acid; TUDCA: Tauroursodeoxycholic acid.

of PBC/AIH in the future.

In conclusion, this study revealed that in patients with PBC and AIH, there were significant differences in serum levels of BAs. However, due to the existence of some limitations (*i.e.*, the small sample size, the lack of staging methods for PBC and AIH, and phenotypic information), further study with a larger sample size is required to eliminate the above-mentioned limitations and to confirm the findings.

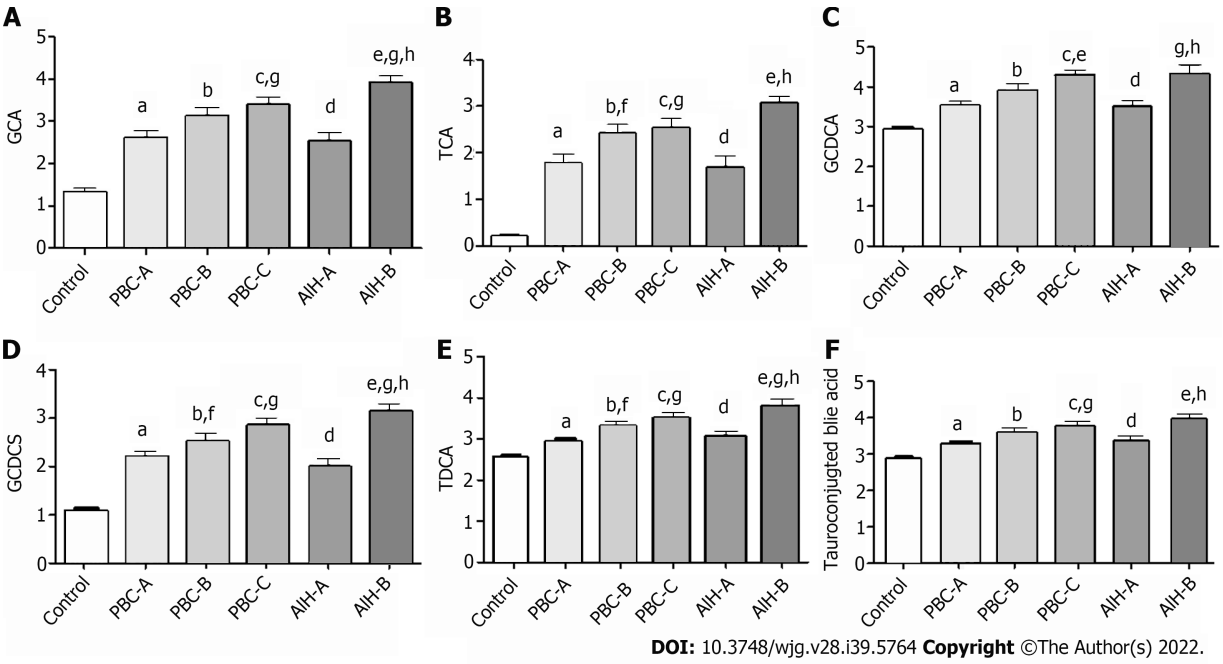

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Figure 10 Bile acid levels are expressed in log10 concentrations. Statistically significant differences in bile acid concentrations between controls and patients were determined by the rank sums Mann-Whitney test. Primary biliary cholangitis (PBC)-A *vs* control, $^{a}P < 0.05$; PBC-B *vs* control, $^{b}P < 0.05$; PBC-C *vs* control, $^{c}P < 0.05$; autoimmune hepatitis (AIH)-A *vs* control, $^{d}P < 0.05$; AIH-B *vs* control, $^{e}P < 0.05$; PBC-A *vs* PBC-B, $^{f}P < 0.05$; PBC-A *vs* PBC-C, $^{g}P < 0.05$; AIH-A *vs* AIH-B, $^{h}P < 0.05$; PBC-A *vs* AIH-A. A: Glycocholic acid; B: Taurocholic acid; C: Glycochenodeoxycholic acid; D: Glycochenodeoxycholic sulfate; E: Taurodeoxycholic acid; F: Tauroconjugted bile acid. GCDCA: Glycochenodeoxycholic acid; GCDCS: Glycochenodeoxycholic sulfate; TDCA: Taurodeoxycholic acid; GCA: Glycocholic acid; TCA: Taurocholic acid.

ARTICLE HIGHLIGHTS

Research background

Primary biliary cholangitis (PBC) and autoimmune hepatitis (AIH) are two unexplained immune diseases. It is difficult to identify and Liver biopsy should be done.

Research motivation

Avoid liver perforation and relieve the pain of patient, to improve the diagnostic rate of PBC and AIH.

Research objectives

To determine non-invasive, reliable, and sensitive biochemical markers for the differential diagnosis of PBC and AIH.

Research methods

Metabolomics technologies, including full-contour metabolomics and target.

Research results

We revealed the increased levels of chenodeoxycholic acid, lithocholic acid (LCA), taurolithocholic acid (TLCA), and LCA + TLCA in the PBC group compared with those in the AIH group. The levels of glycochenodeoxycholic acid, glycochenodeoxycholic sulfate, and taurodeoxycholic acid were gradually elevated with the increase of Child-Pugh class, which was correlated with the severity of disease.

Research conclusions

The levels of bile acids could serve as potential biomarkers for the early diagnosis and assessment of the severity of PBC and AIH.

Research perspectives

It is necessary to further expand the sample size for research and search for the mechanism for the changes.

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FOOTNOTES

Author contributions: Niu JQ contributed to conception and design of the research, review and editing; Ma ZH, Sun LC, and Li P contributed to investigation and wrote the manuscript; Ma ZH, Wang XM, and Wu RH contributed to acquisition of data and statistical analysis; Hao DL contributed to funding acquisition; all authors have read and approve the final manuscript.

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Informed consent statement: All the eligible patients and HCs signed the written informed consent form prior to enrollment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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