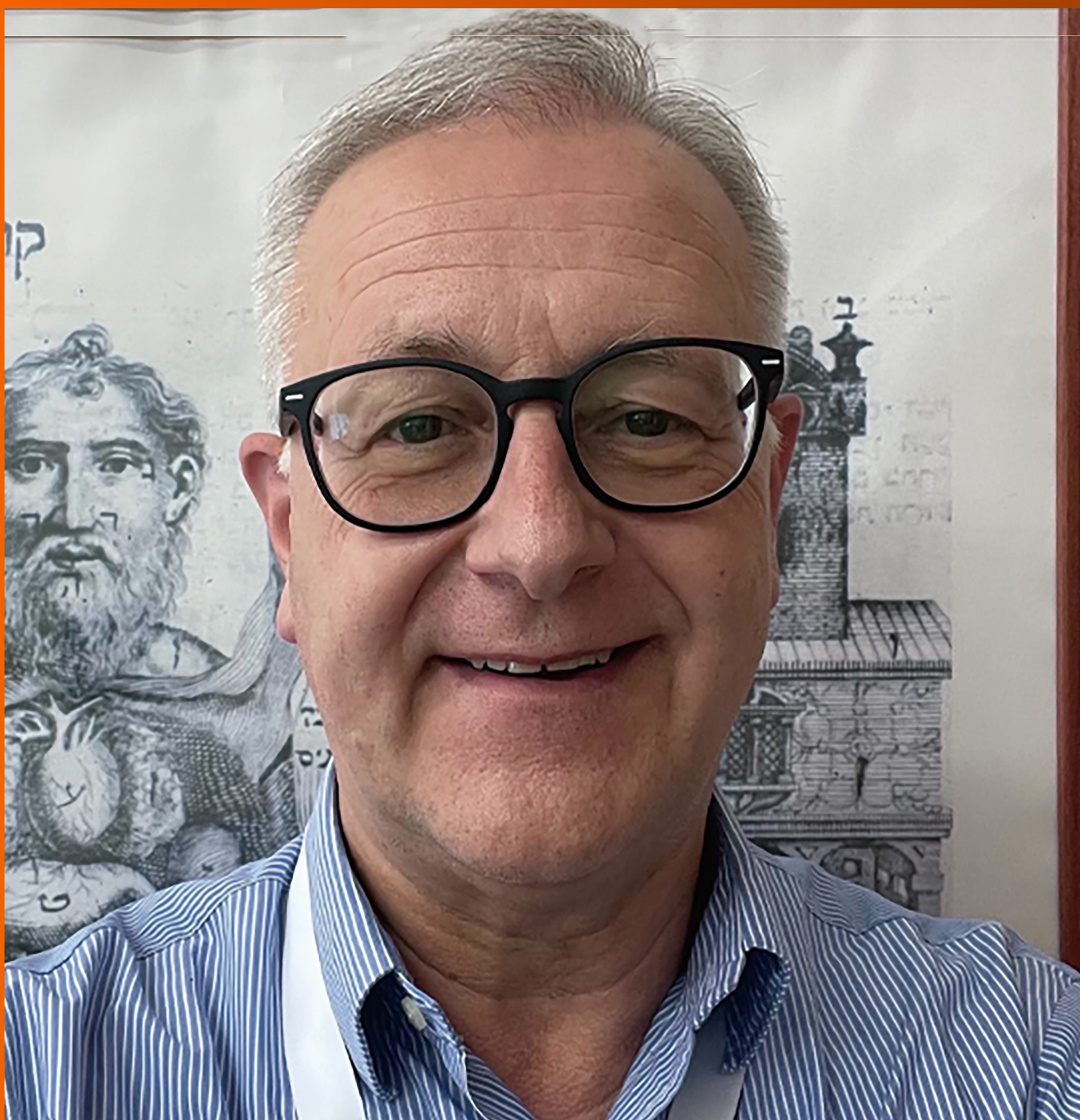


# World Journal of *Hepatology*

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## COVID-19 and the liver: What do we know so far?

Prashant Nasa, George Alexander

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### Abstract

The coronavirus disease 2019 (COVID-19) pandemic has caused unprecedented pressure on public health and healthcare. The pandemic surge and resultant lockdown have affected the standard-of-care of many medical conditions and diseases. The initial uncertainty and fear of cross transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have changed the routine management of patients with pre-existing liver diseases, hepatocellular carcinoma, and patients either listed for or received a liver transplant. COVID-19 is best described as a multisystem disease caused by SARS-CoV-2, and it can cause acute liver injury or decompensation of the pre-existing liver disease. There has been considerable research on the pathophysiology, infection transmission, and treatment of COVID-19 in the last few months. The pathogenesis of liver involvement in COVID-19 includes viral cytotoxicity, the secondary effect of immune dysregulation, hypoxia resulting from respiratory failure, ischemic damage caused by vascular endotheliitis, congestion because of right heart failure, or drug-induced liver injury. Patients with chronic liver diseases, cirrhosis, and hepatocellular carcinoma are at high risk for severe COVID-19 and mortality. The phase III trials of recently approved vaccines for SARS-CoV-2 did not include enough patients with pre-existing liver diseases and excluded immunocompromised patients or those on immunomodulators. This article reviews the currently published research on the effect of COVID-19 on the liver and the management of patients with pre-existing liver disease, including SARS-CoV-2 vaccines.

**Key Words:** COVID-19; Chronic liver disease; SARS-CoV-2; Severe acute respiratory syndrome coronavirus; Liver transplant; Liver and SARS-CoV-2 vaccines; SARS-CoV-2 induced liver injury



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**Core Tip:** Liver involvement in coronavirus disease 2019 (COVID-19) is caused by either viral cytotoxicity or secondary to systemic immune dysregulation. Patients with pre-existing liver disease are at high risk of disease progression, morbidity, and mortality. Chronic liver disease with COVID-19 should be managed as per the standard guidelines, with education on hand hygiene, social distancing, and face masks to reduce hospital admissions. There is no evidence that currently available vaccines for severe acute respiratory syndrome coronavirus 2 will have any complications different from other inactivated vaccines and are recommended for patients with pre-existing liver disease, hepatocellular carcinoma, or liver transplant recipients.

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## INTRODUCTION

*Coronavirus* is an enveloped single-stranded RNA virus belonging to the Coronaviridae family and Orthocoronavirinae subfamily. Severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) respectively caused epidemics in 2003 and 2012. The pandemic of coronavirus disease 2019 (COVID-19) caused by the SARS-CoV-2 was first reported from Wuhan, China on December 31, 2019 in patients with atypical pneumonia[1]. While symptoms are mild in most patients, severe and critical symptoms (in 10%-15% of patients) like hypoxemia ( $SpO_2 < 94\%$ ), acute respiratory distress syndrome, multiorgan failure, or shock; may need hospitalization and respiratory support[2,3]. Older patients, especially those with comorbidities like hypertension, diabetes, chronic liver disease (CLD), and heart disease, are at risk of severe disease and mortality[2,3]. With the rapid spread of COVID-19, there has been significant concern regarding the safe management of patients with pre-existing liver disease (CLD), hepatocellular carcinoma (HCC), and candidates for a liver transplant. This review discusses the current evidence on liver involvement in COVID-19 and its impact on managing patients with CLD, including current recommendations for SARS-CoV-2 vaccines.

## LIVER DYSFUNCTION IN COVID-19

Based on the published literature, 14%-53% of patients with COVID-19 developed hepatic dysfunction, and 2%-11% of the patients were reported to have underlying CLD[4-9]. Hepatic dysfunction characterized by elevated liver enzymes was significantly higher in severe and critical COVID-19 and was associated with poor outcomes[4]. In a meta-analysis of 45 studies, the most common biochemical abnormality of the liver in COVID-19 was hypoalbuminemia (39.8%), followed by elevation of gamma-glutamyl transferase (GGT 35.8%), or aminotransferases [aspartate aminotransferase (AST 21.8%) and alanine aminotransferase (ALT 20.4%)] [10]. The incidence of elevated hepatic enzymes was also higher in COVID-19 patients requiring intensive care unit (ICU) admission as compared to non-ICU patients (62% *vs* 23%) [4]. In another meta-analysis of 128 studies, the most common hepatic abnormality was hypoalbuminemia (61.3%), followed by elevation of GGT (27.9%), ALT (23.3%), and AST (23.4%) in the patients with COVID-19. The degree of the hepatic abnormalities was directly proportional to the severity of the disease[11].

## MECHANISM OF LIVER INJURY

The pathogenic properties of SARS-CoV-2 depend on the binding of viral spike proteins to the host angiotensin-converting enzyme 2 (ACE-2) receptors, which allows the virus to enter the target cells along with priming by the host transmembrane serine protease 2 (TMPRSS2)[12-14]. The ACE-2-TMPRSS2 is expressed in the ileum, liver, lung, nasal mucosa, bladder, testis, prostate, and kidney (in that order)[14-17]. SARS-CoV-2 binding to ACE-2 receptors in the upper respiratory tract is the primary site of replication and entry to the body[14]. ACE-2-TMPRSS2-positive cells in the gastrointestinal tract include enterocytes in the biliary duct or pancreatic duct epithelium and hepatocytes[14,17].

The mechanism of liver injury in COVID-19 is possibly multifactorial. SARS-CoV-2 might induce direct hepatotoxicity (SARS-CoV-2 enters into the liver *via* cholangiocytes or translocation from gut to the liver) or indirect hepatic injury (from systemic inflammation with immune dysregulation, hypoxia from respiratory failure, ischemic damage due to coagulopathy or endotheliitis, right heart failure due to myocarditis, deterioration of pre-existing liver diseases, or drug-induced liver injury)[15] (Figure 1). The liver function abnormalities like increased GGT are consistent with a direct cytotoxic effect of SARS-CoV-2 on cholangiocytes[15,18]. However, the expression of ACE-2 receptors is minimal on hepatocytes suggesting a significant contribution of indirect causes of liver damage rather than direct hepatotoxicity[16,18]. The treatment of severe COVID-19 with antiviral agents, immunomodulators, antibiotics, or supportive agents, may also cause hepatotoxicity. Among those agents, remdesivir, favipiravir, lopinavir/ritonavir combination, corticosteroids, and tocilizumab could increase liver enzyme levels[18-20]. Adjuvant drugs like acetaminophen and antibiotics may also cause hepatotoxicity[20] (Table 1).

## IMPACT OF COVID-19 ON PRE-EXISTING LIVER DISEASE

### COVID-19 with CLD

In a systematic review and meta-analysis of 73 studies, the prevalence of CLD was 3% in 24299 COVID-19 patients[21]. Other studies reported a 3%-11% prevalence of underlying CLD with COVID-19[4-9,22]. The patients with CLD may also be more susceptible to contract SARS-CoV-2 infection[23]. Besides, the presence of CLD increased the risk of severe COVID-19 [pooled odds ratio (OR) 1.48] and overall mortality (pooled OR 1.78)[21,24]. Two other meta-analyses found that pre-existing liver diseases increase the risk of severe COVID-19, decompensation, and mortality[24,25]. From an extensive registry of over 17 million patients from the United Kingdom, COVID-19 was associated with a 2.34 (95% confidence interval: 1.94-2.83) times increased risk of mortality with liver disease[26]. The evidence is conflicting on the increased risk of severe COVID-19 in patients with chronic viral hepatitis[4,27]. However, SARS-CoV-2 infection in patients with chronic hepatitis B could have an increased risk of reactivation. A study of 21 patients with known chronic hepatitis B, SARS-CoV-2 infection was associated with hepatitis B reactivation in three patients[28].

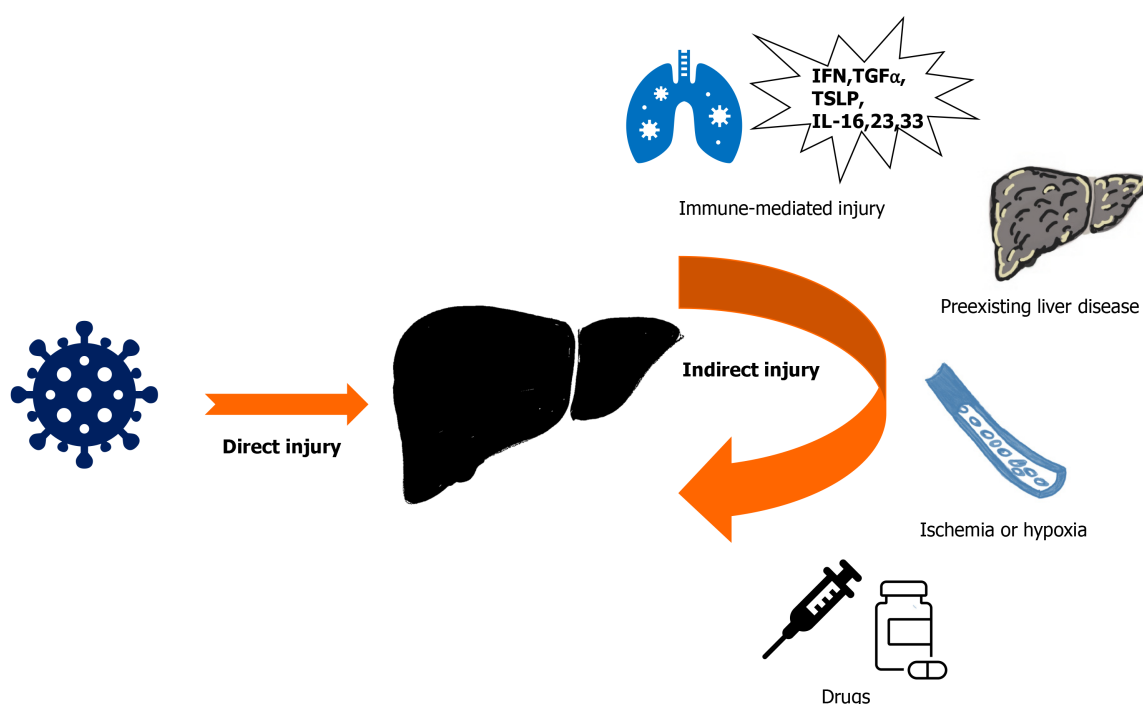
### Fatty liver disease with COVID-19

In a multicenter retrospective study from the United States, CLD and nonalcoholic fatty liver disease (NAFLD) were independent risk factors for ICU admission and invasive mechanical ventilation[29]. NAFLD was also associated with the progression of COVID-19 to severe disease in other studies[30-32]. The Asian Pacific Association for the Study of the Liver COVID-19 Liver Injury Spectrum Study (APCOLIS) study included 228 confirmed COVID-19 patients from 13 Asian countries with pre-existing liver disease. Metabolism associated fatty liver disease (MAFLD) was the commonest (61%) etiology[33]. In a retrospective study, a history of NAFLD/MAFLD was associated with increased odds of admission for COVID-19[34]. Obesity is common in patients with NAFLD and is an independent risk factor for severe COVID-19, invasive mechanical ventilation, and increased mortality[31,35]. However, in a study by Hashemi *et al*[29], the clinical severity of COVID-19 in patients of NAFLD was observed to be independent of obesity. The deleterious interplay of chronic inflammation observed in NAFLD with an acute inflammatory response to SARS-CoV-2 could explain the higher hepatic injury and a worse outcome in metabolically compromised NAFLD patients[36]. In another study, the extent of liver fat was correlated with serum markers of inflammation and oxidative stress[37]. It explains

**Table 1 Impact of drugs currently used for the management of coronavirus disease 2019 on the liver**

Drug	Mechanism of action	Impact on CLD management
Remdesivir	Viral RNA-dependent RNA polymerase inhibitor	Liver toxicity possible; No liver relevant drug-drug interactions
Lopinavir/ritonavir	Protease inhibitors	mTOR inhibitors (sirolimus, everolimus) should not be co-administered; Close monitoring of drug level is required for calcineurin inhibitors (cyclosporine, tacrolimus); The risk of lopinavir-associated hepatotoxicity in patients with very advanced liver disease is low; Patients with decompensated cirrhosis should not be treated
Tocilizumab	Humanized monoclonal antibody targeting interleukin-6 receptor	Patients with decompensated cirrhosis should not be treated Consider risk of HBV reactivation
Methylprednisolone (steroids)	Bind nuclear receptors to dampen proinflammatory cytokines	The risk of other infections ( <i>e.g.</i> , spontaneous bacterial peritonitis) and viral shedding may increase in patients with decompensated liver cirrhosis; Consider antimicrobial prophylaxis; Consider risk of HBV reactivation
Favipiravir	Guanine analogue, RNA-dependent RNA polymerase	Elevation of ALT and AST possible; No data in cirrhosis available

ACE-2: Angiotensin-converting enzyme; CLD: Chronic liver disease; G6PD: Glucose-6-phosphate dehydrogenase; HBV: Hepatitis B virus; mTOR: Mammalian target of rapamycin; SBP: Spontaneous bacterial peritonitis.

**Figure 1 Mechanism of liver injury in coronavirus disease 2019.**

the multifaceted impact of NAFLD on the pathophysiology and clinical course of COVID-19. However, effective treatment for metabolic disease can mitigate the increased risk from NAFLD[29,36].

### COVID-19 and cirrhosis

Patients with cirrhosis are also at increased risk of decompensation with SARS-CoV-2 infection[38]. The presence of cirrhosis was also found to be an independent predictor of mortality in COVID-19[29,31]. In a study from the United States, the risk factors related to higher mortality in COVID-19 and CLD were alcoholic liver disease, decompensated cirrhosis, and HCC[39]. The worse outcomes in patients with cirrhosis can be multifactorial and likely due to cirrhosis-associated immune and inflammation modulation, limited physiological reserves, and increased risk of severe COVID-19[39]. Other large registries of patients with cirrhosis and COVID-19, like SECURE-cirrhosis and COVID-Hep.net, reported a case fatality rate of 38%, which may be as



high as 70% in the Child-Pugh C category[40].

### **Hepatocellular carcinoma**

Patients with malignancy are vulnerable during the COVID-19 pandemic, with an increased risk of SARS-CoV-2 infection[41,42]. The overall prognosis of COVID-19 in cancer patients is poor, with high ICU admissions and mortality[41-43]. A small retrospective study of 28 cancer patients with COVID-19, including 2 HCC patients, had worse outcomes than the general population[43]. The increased risk may be attributed to age, multiple comorbidities, and the presence of cirrhosis. In patients with HCC, COVID-19 may exacerbate existing CLD and complicate the management of cancer.

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## **PRESENTATION OF COVID-19 WITH PRE-EXISTING LIVER DISEASE**

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The SARS-CoV-2 infection in patients with pre-existing liver pathology may increase the risk of decompensation, acute liver injury, or a combination of both. Acute liver injury was the most observed presentation (43%) in CLD patients without cirrhosis, while acute-on-chronic liver failure (11.6%) and decompensation (9%) were more common in patients with cirrhosis[34]. The risk factors for decompensation include comorbid illnesses like diabetes or obesity. The AST/ALT ratio, total bilirubin, and R-value (ALT/ALP ratio) can predict survival in cirrhotic patients[34]. The residual hepatic synthetic function in CLD patients is inversely proportional to liver-related complications with COVID-19. Liver injury has been seen in the third week in CLD patients without cirrhosis and in the first week in cirrhotic patients[34].

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## **COVID-19 AND LIVER TRANSPLANT RECIPIENT**

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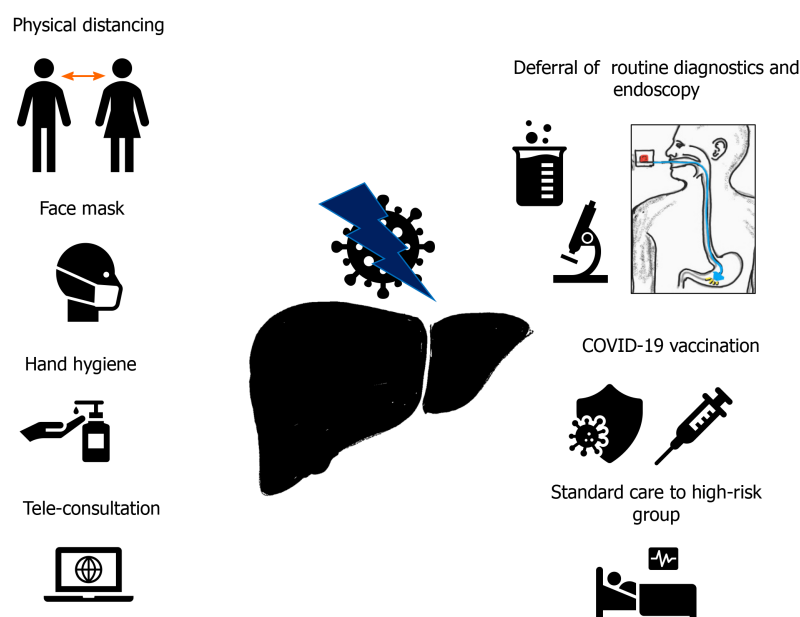
Being an immunocompromised host, liver transplant recipients have an increased risk of acquiring SARS-CoV-2 infection and progression to severe disease. The outcome of COVID-19 in liver transplant patients was evaluated in a prospective study of 111 patients in Spain. Of 96 patients (86.5%) who were diagnosed with COVID-19 requiring hospital admission, 22 patients (19.8%) needed respiratory support, 12 (10.8%) required ICU admission, and the case fatality rate was 18% which was relatively lower than the matched general population despite higher severity of disease[44]. Similar results were found in another multicenter study of 112 patients from the United States. The hospital and ICU mortality rates were 22.3% and 26.8%, respectively, which was lower than the rates in matched patients of CLD without liver transplant[45]. The postulated hypothesis for better outcomes was ongoing immunomodulatory therapies that may ameliorate a harmful immune response (*i.e.* cytokine storm), reducing mortality[45,46]. However, immunosuppressants may delay viral clearance, explaining the severe disease[44]. The factors associated with mortality in liver transplant recipients were new liver injury, younger age, hispanic ethnicity, metabolic syndrome, vasopressor requirement, and antibiotic usage. Moderate liver injury [ALT 2-5 times the upper limit of normal (ULN)] and severe liver injury (ALT more than five times the ULN) was significantly associated ( $P = 0.007$ ) with mortality and ICU admission[45].

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## **MANAGEMENT OF CHRONIC LIVER DISEASE DURING COVID-19 PANDEMIC**

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The COVID-19 pandemic had a considerable impact on the management of CLD. Various factors must be considered and monitored while managing this group of patients. There is a potential threat of cross transmission of SARS-CoV-2 among patients and health care workers (HCWs) during physical assessment and treatment. However, it is imperative to maintain the continuity of care of patients with CLD to reduce the risk of decompensation and hospital admission. The measures recommended for safe and effective management of CLD patients can be divided into general and specific (Figure 2).



**Figure 2** General measures for the safe management of patients with pre-existing liver disease during coronavirus disease 2019 pandemic. COVID-19: Coronavirus disease 2019.

### General measures for all patients

Physical distancing, avoiding closed spaces without a face mask, and hand hygiene are vital pillars of SARS-CoV-2 infection prevention. Education on infection prevention measures should be included with other social measures like abstinence from alcohol and medication compliance. The screening of fever or respiratory symptoms should be performed on all patients and HCWs at the entrance of the hospital premises. Telemedicine, postponing routine outpatient visits, or periodic laboratory testing are other strategies that can be considered, depending on the available resources and patient condition[1]. The patient education must include prophylactic vaccination for streptococcus pneumonia or influenza.

### Specific measures

**Compensated liver disease:** There is no evidence that initial clinical symptoms of SARS-CoV-2 are different in patients with CLD. Patients with NAFLD/MAFLD may suffer from other metabolic comorbidities like diabetes mellitus, hypertension, hyperlipidemia, and obesity, which need optimization and regular monitoring. Experts recommend against the alteration of immunosuppression in autoimmune hepatitis and liver transplant patients to reduce the risk of severe COVID-19[47]. The risk of aerosolization of SARS-CoV-2 during endoscopy must be considered during routine management of esophageal varices. Experts recommended non-endoscopic pathways to assess esophageal varices, especially during periods of high community transmission[47]. Any acute decompensation in patients with known CLD needs exclusion of SARS-CoV-2 coinfection. The potential reactivation of hepatitis B in patients with COVID-19 and chronic hepatitis B mandates monitoring of liver function tests and hepatitis B virus -DNA levels[28].

**Decompensated liver disease:** The care of the patients should follow standard guidelines while reducing direct visits to the healthcare facility (*e.g.*, using telemedicine or telephone consultation) wherever feasible. The standard management of these patients, like prophylaxis for variceal bleeding, spontaneous bacterial peritonitis, or hepatic encephalopathy, should be continued unaltered to prevent further worsening and reduce admissions[47].

**Liver transplantation:** The liver transplant recipients are at increased risk of contracting COVID-19, like patients with CLD. The general measures can include teleconsultation to shorten in-hospital stay and interactions with other HCWs. There were attempts to generate international consensus on treatment protocols of liver transplant recipients during this pandemic to reduce the risk of cross-transmission of SARS-CoV-2 and optimize healthcare resources[47]. The immunosuppression in liver transplant recipients may interfere with the immune response against the virus, while

any alteration in the treatment may cause acute graft rejection. Also, the use of various therapeutics to treat COVID-19 and drug-drug interactions with immunomodulators raises concerns of hepatotoxicity. In a prospective cohort study by Colmenero *et al*[44], mycophenolate at doses higher than 1000 mg/d was an independent predictor of severe COVID-19 in 111 liver transplant patients diagnosed with COVID-19. The synergistic effect of mycophenolate and SARS-CoV-2 may deplete peripheral lymphocytes responsible for an aberrant immune reconstitution to SARS-CoV-2[11,48]. In a multicenter study from the United States of COVID-19 in 112 liver transplant patients, new liver injury was associated increased mortality and ICU admission[45].

The close monitoring of liver enzymes in liver transplant patients and COVID-19 is suggested to watch for new liver injury or graft rejection. The immunosuppression regimen preferably should not be altered, except in the case of a mycophenolate-based regimen. Hypothermia is associated with worsening liver functions in severe COVID-19 and should be corrected with appropriate interventions[45].

Candidates for liver transplant: SARS-CoV-2 routine testing should be performed for both the recipient and donor before transplantation. However, a single negative RT-PCR test cannot exclude an asymptomatic infection[47]. During high community transmission or inundated healthcare resources, the transplantation should be offered only to select patients with poor short-term prognosis. It includes acute or acute-on-chronic liver failure, a high Model for End-stage Liver Disease score, or HCC with upper limits of the Milan criteria[45,49]. The diagnostic workup and procedure for the transplant program must be performed rapidly, with a short hospital stay[49].

Hepatocellular Carcinoma: Although the number of patients with HCC in the published COVID-19 studies is minimal, similar infection risk mitigation should be implemented in patients with CLD. The clinical services of cancer patients have been significantly affected by the current coronavirus pandemic, with decreased referral of the patients to the multidisciplinary tumor board (MTB), and treatment delays[50]. The evaluation, treatment and monitoring of HCC should be personalized based on the availability of medical resources and level of infection risk of SARS-CoV-2. Guidelines on the management of liver disease and HCC have been published by various academic societies[47,51]. The recommendations include virtual MTB meetings, prioritizing surgery on a case-to-case basis with preference to patients with low disease burdens and alternative therapies like radiofrequency and microwave ablation in selected patients. Treatment deferral or modification should be based on the best available evidence and availability of resources[52].

## SARS-COV-2 VACCINES

Scientists developed vaccines against SARS-CoV-2 with unprecedented speed. The vaccines have been found effective in reducing the incidence of severe disease, hospitalization, and mortality. Vaccines based on various platforms, like mRNA, nonhuman viral vectors, and inactivated whole SARS-CoV-2 were developed. Despite more than 200000 participants in phase III trials, there is minimal data on efficacy in patients with pre-existing liver diseases. In the BNT162b2 (Pfizer/BioNTech) vaccination study, 217 participants (0.6%) had CLD and only 3 (< 0.1%) had moderate to severe liver disease[53]. Similarly, only 196 liver disease patients (0.6%) were included in the mRNA-1273 (Moderna) trial[54]. Data on patients with pre-existing liver disease is not available from the ChAdOx1-nCoV-19 (Oxford–AstraZeneca) vaccine trial[55]. Patients on systemic immunosuppression were excluded in all phase III trials, undermining the role of vaccines in the liver transplant recipients or patients with autoimmune liver disease on immunosuppressants. However, in the real world, millions are already vaccinated, including patients with liver disease; thus, data on safety and effectiveness are expected to be available soon. The deficiencies of innate or adaptive immune responses and an attenuated response to others vaccines are well recognized in CLD patients. A similar altered response to SARS-CoV-2 vaccines is also suspected. Nevertheless, based on an increased risk of severe disease, and in the absence of any data suggesting harm, the European Association for the Study of the Liver (EASL), the American Association for the Study of Liver Diseases (AASLD), and British Association for the Study of Liver currently recommend the available SARS-CoV-2 vaccines for patients with CLD, and liver transplant recipients[56–58]. Although the vaccines may be less effective in patients with CLD and liver transplant recipients, they still provide protection[58].



## CONCLUSION

Emerging research suggests that liver injury is common in COVID-19 patients and associated with worse outcomes. Patients with CLD and post liver transplant patients are at risk of SARS-CoV-2 infection, with an increased risk of complications and mortality. The management of this vulnerable group of patients should be prioritized based on their clinical condition, strategies to reduce cross transmission, and optimizing limited resources. Liver transplant and HCC management programs should be modified depending on the prevalence of community transmission of SARS-CoV-2. Specific management issues should be considered during the treatment of COVID-19 in patients with pre-existing liver diseases.

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## Direct, remote and combined ischemic conditioning in liver surgery

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### Abstract

Liver ischemia-reperfusion injury is a major cause of postoperative liver dysfunction, morbidity and mortality following liver resection and transplantation. Ischemic conditioning has been shown to ameliorate ischemia-reperfusion injury in small animal models. It can be applied directly or remotely when cycles of ischemia and reperfusion are applied to a distant site or organ. Considering timing of the procedure, different protocols are available. Ischemic preconditioning refers to that performed before the duration of ischemia of the target organ. Ischemic perconditioning is performed over the duration of ischemia of the target organ. Ischemic postconditioning applies brief episodes of ischemia at the onset of reperfusion following a prolonged ischemia. Animal studies pointed towards suppressing cytokine release, enhancing the production of hepatoprotective adenosine and reducing liver apoptotic response as the potential mechanisms responsible for the protective effect of direct tissue conditioning. Interactions between neural, humoral and systemic pathways all lead to the protective effect of remote ischemic preconditioning. Despite promising animal studies, none of the aforementioned protocols proved to be clinically effective in liver surgery with the exception of morbidity reduction in cirrhotic patients undergoing liver resection. Further human clinical trials with application of novel conditioning protocols and combination of methods are warranted before implementation of ischemic conditioning in day-to-day clinical practice.

**Key Words:** Ischemic preconditioning; Ischemia-reperfusion injury; Hepatectomy; Liver transplant; Morbidity; Mortality

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**Core Tip:** The concept of ischemic conditioning seems easy to apply and is an inexpensive method with the potential to protect the liver during hepatic surgery. It covers a wide spectrum of techniques and allows adjustment of the method to the

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particular patient. Unfortunately, despite promising animal studies in preventing ischemia-reperfusion injury by ischemic conditioning, currently there is a lack of sufficient data on its clinical efficacy in humans.

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## INTRODUCTION

Ischemia-reperfusion injury (IRI) remains an important issue in hepatic surgery. IRI is a pathophysiological phenomenon where cellular damage is caused by reperfusion and reoxygenation following an ischemic period[1]. It is the most important pathogenetic factor occurring during the surgical procedure that impairs both functional reserve through loss of remaining hepatocytes and compromising liver capacity to regenerate. Thus, IRI is a major contributor to increased morbidity and mortality following liver resection and transplantation[2,3].

Ischemic preconditioning (IPC) is an adaptive pathophysiological mechanism based on a concept of preparation of the target organ for ischemic conditions in order to decrease the magnitude of IRI[4]. It was first described by Murry *et al*[5] in 1986. In a canine model, the authors demonstrated that short repetitive ischemic episodes protected the heart from subsequent sustained ischemic insult.

IPC can be either applied directly[5] or remotely[6]. Remote IPC (RIPC) is based on a concept of brief cycles of ischemia and reperfusion applied to a distant site or organ in order to exert a protective effect on another organ or site. Considering timing of the procedure, remote ischemic preconditioning (RIPer) refers to that performed over the duration of ischemia of the target organ[7].

Potential mechanisms responsible for the protective effect of tissue conditioning remain poorly understood. Regarding direct conditioning strategies, it is postulated that IPC suppresses cytokine release, enhances the production of hepatoprotective adenosine and nitric oxide and increases ATP availability by slowing the rate of ATP depletion, thus leading to upregulation of the process of cellular ATP production and liver regeneration and reduction of the liver apoptotic response[8,9]. The summary of IRI mechanism and pathways of IPC is illustrated in Figure 1[10]. In remote ischemic conditioning, reduction of hepatocellular injury in the early phase of IRI is achieved by improvement of parenchymal perfusion and oxygenation[11,12]. Interactions between neural, humoral and systemic pathways all lead to the protective effect of RIPC. In particular, these result in inhibition of the inflammatory response and activation of various hepatoprotective subcellular cascades[13].

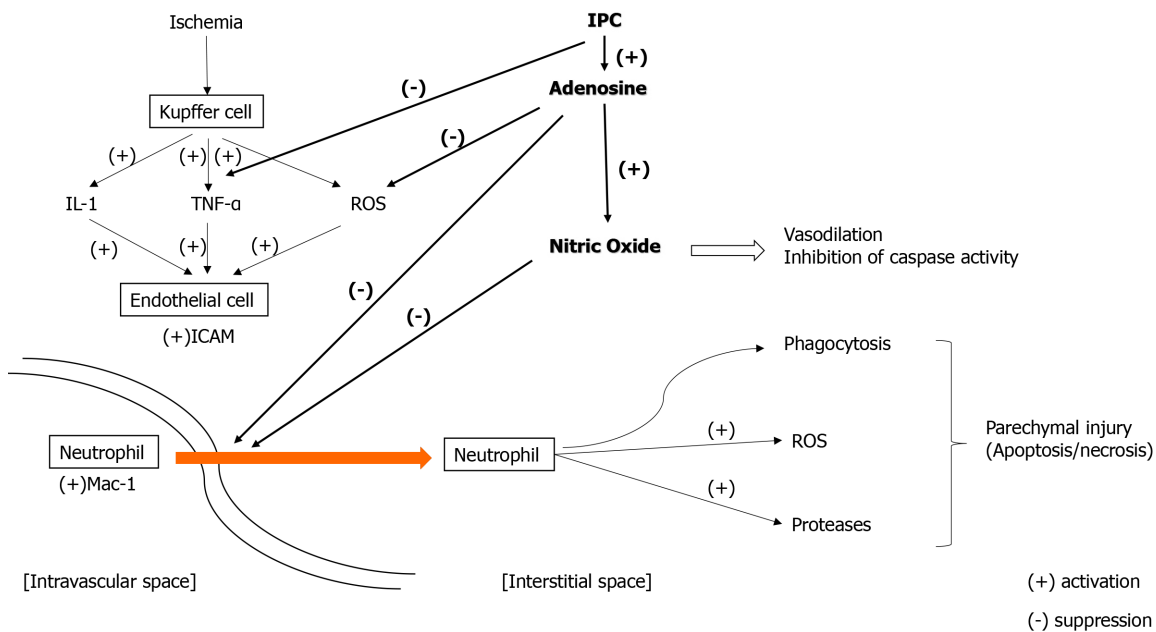
In this review, we focus on clinical application of both, direct and remote, ischemic conditioning methods in hepatic surgery in humans. In the discussed papers we highlight clinical endpoints related to mortality, morbidity, intensive care unit (ICU) stay, hospital stay or intraoperative blood loss (in case of parenchymal resection). Postulated mechanisms of hepatocellular protection diminishing IRI are detailed in the referenced studies.

Hepatic steatosis has been associated with worse outcomes in liver surgery, and it is hypothesized that this is caused by a lower tolerance of steatotic livers to IRI[14,15]. Therefore special emphasis is put on outcomes achieved in patients undergoing liver resection and liver transplantation in humans with steatotic livers.

## DIRECT IPC IN LIVER RESECTION

In 2000, Clavien *et al*[16] published the first non-randomized study on IPC in human liver[16]. Patients were subjected to IPC consisting of 10 min of clamping of the portal triad (Pringle maneuver) followed by 10 min of reperfusion before anatomical left or right hemihepatectomy. Liver cirrhosis, wedge or segmental resections were considered as exclusion criteria. The authors observed lower serum aminotransferase activities and reduced endothelial cell injury in the IPC group. No differences in





**Figure 1 Summary of liver ischemia-reperfusion injury mechanisms and pathways of ischemic preconditioning interventions.** Based on a paper by Montalvo-Jave *et al*[10]. ICAM: Intercellular adhesion molecule; IL-1: Interleukin-1; IPC: Ischemic preconditioning; TNF-α: Tumor necrosis factor-α; ROS: Reactive oxygen species.

mortality, hospital stay or blood loss were detected. These findings were followed by another study by Clavien *et al*[17]. In the randomized controlled trial (RCT), they confirmed previous results and highlighted younger patients and those with liver steatosis as subgroups who derived the most benefits from IPC. Nevertheless, no differences in mortality, hospital stay or blood loss were found. These promising results were followed by a number of studies exploring this field.

Cochrane meta-analysis included four RCTs published until 2008[18]. It assessed IPC followed by continuous clamping (CC) of the portal triad (135 patients) compared with CC alone (136 patients). All the included trials excluded liver resections performed in cirrhotic patients. IPC was achieved by 10 min of clamping followed by 10 min of unclamping, followed by CC in three trials[17,19-21]. In the fourth trial, the duration of initial clamping is likely to be 10 min, although it was not clearly stated. This was followed by 10 min of unclamping followed by CC[22]. The proportion of patients requiring blood transfusion was significantly lower in the IPC group, with no differences in mortality, posthepatectomy liver failure, morbidity, hospital stay or operative time.

Another meta-analysis, conducted by O'Neill *et al*[23], was published in 2013[23]. It comprised all the aforementioned studies and seven RCTs not included in the Cochrane Hepato-Biliary Group study, of which only one included patients with liver cirrhosis[24]. Ten minutes of the Pringle maneuver for IPC with 10 min of reperfusion was the most frequent strategy. In one study, IPC lasted 5 min with 5 min of reperfusion[24] and in another, IPC lasted 10 min with 15 min of reperfusion[25]. CC was used for parenchymal transection in seven studies[17,20-22,24-26], whereas intermittent clamping was used in the remaining four[27-30]. Eight studies that reported blood loss during liver resection found it to be nonsignificantly lower in the IPC group both in intermittent and CC. No differences in mortality, posthepatectomy liver failure, morbidity, operating time, hospital stay, prothrombin time, bilirubin concentration, aspartate aminotransferase (AST) or alanine aminotransferase (ALT) activities were detected (with and without patients with cirrhosis).

Another meta-analysis was published in 2017[31]. The authors focused only on RCTs investigating the role of IPC before CC. Pooled data were analyzed by combining the results of the 13 RCTs. Five trials enrolled both cirrhotic and noncirrhotic patients (91 in the IPC group and 90 in the control group)[21,32-35]. In three trials, IPC was performed through 5 min of inflow occlusion followed by 5 min of reperfusion[32,34,35]. In one study, IPC was done by inflow occlusion for 10 min followed by reperfusion for 15 min before CC[25]. Ten minutes of the Pringle maneuver for IPC with 10 min of reperfusion was used in nine studies[17,19,22,27-30]. In the case of underlying cirrhosis, IPC reduced postoperative morbidity. However, in

patients without cirrhosis, the analysis revealed no significant association between IPC and postoperative morbidity. There were also no differences in morbidity considering ischemia-reperfusion timing (10 + 10 *vs* 5 + 5). Mortality, operative time, total bilirubin concentration, AST or ALT concentration after postoperative day 1, and hospital and ICU stay were similar regardless of IPC.

Three studies focused on patients with steatotic livers in subgroup analyses. Two studies were RCTs[17,25], and one was a prospective nonrandomized study[16]. A total of 29 patients were analyzed as a subgroup (16 in IPC group and 13 in control group). Cutoff for liver steatosis was set as  $\geq 30\%$ , but the type of steatosis (micro- or macrovesicular) was not described. The protocol of IPC was 10 + 10 min in two studies[16,17] and 10 + 15 min in one study[25]. Only peak AST levels were measured as an endpoint in this subgroup comparison. IPC was associated with lower activity of AST after resections in steatotic livers[16,17,25], yet no results on clinical outcomes were provided.

In conclusion, there is currently no evidence supporting direct IPC as a protective strategy against mortality in patients undergoing liver resection, although it may be beneficial for patients with liver cirrhosis with respect to postoperative morbidity. Further investigation of applicability of direct IPC in cirrhotic and steatotic livers is warranted.

## DIRECT IPC IN LIVER TRANSPLANTATION

In 2016, a meta-analysis on IPC in liver transplantation was published by Robertson *et al*[36]. Data from ten studies were analyzed (286 patients in IPC group and 307 patients in control group), four nonrandomized[37-40] and six RCTs[41-46]. Only transplantations of grafts procured from donors after brain death were included in these studies, and no grafts underwent machine perfusion. Grafts were preconditioned in the donor by portal triad clamping for 10 min in all but one study. In one study, IPC lasted for 5 min[46]. Time of reperfusion varied among studies from 10 to 39 min. Authors reported that IPC was associated with lower postoperative mortality, lower incidence of primary graft nonfunction and lower rate of retransplantation. None of these findings were statistically significant. Additionally, AST activity on the third postoperative day, length of ICU stay, length of hospital stay and incidence of acute rejection were all nonsignificantly lower in transplantations with IPC.

In living related liver transplantation, two prospective nonrandomized studies were published[47,48]. The protocol of IPC was 10 + 10 min in both studies. Only right lobes were procured from the donors (32 in IPC group and 32 in control group). There were no differences in graft survival, patient survival, morbidity, hospital stay, histological findings and liver function tests between recipients of IPC and non-IPC liver grafts.

Three studies focused on patients with steatotic donor livers in subgroup analyses. All donors were after brain death (25 in IPC group and 29 in control group). Two studies were RCTs[43,46], and one was a retrospective study[39]. The protocol of IPC was 10 + 10 min in one study[39], 10 + 30 min in second study[43], and in the remaining study IPC lasted for 5 min with ongoing reperfusion[46]. Definitions of significant steatosis varied among studies and comprised presence of any steatosis[39],  $> 15\%$  of macrovesicular steatosis[43] and no specific definition[46]. None of the studies reported results on patient mortality. Clear conclusions cannot be drawn from these studies in terms of impact of IPC on steatotic liver grafts. Morbidity, graft survival, hospital stay, ICU stay and liver function tests seemed to be similar between IPC and non-IPC groups. However, there was a lack of uniform description of severity of hepatic steatosis, and the analyses were limited by small numbers.

In conclusion, there is currently no evidence that direct IPC decreases mortality after deceased and living donor liver transplantation. However, no trial provided data on recipient outcomes after more than 1 year postoperatively, and as such, the long-term effect of IPC on post-transplant outcomes remains to be elucidated. Also, there is insufficient data on IPC impact on steatotic grafts. Therefore, further analysis of this subgroup is warranted.

## REMOTE IPC IN LIVER RESECTION

Only scarce data on remote IPC in liver resection in humans are available (Table 1). In five studies, the total number of 155 patients underwent RIPC with 160 patients serving as controls. Two studies had a third arm, direct IPC, including a total 52

**Table 1 Randomized controlled trials on remote ischemic preconditioning in liver surgery**

Ref.	Intervention (patients, n)	Ischemia-reperfusion	Place of ischemia	Cirrhosis, n	Pringle maneuver	Primary endpoint
Kanoria <i>et al</i> [49], 2017	RIPC (8)	2 × 10 + 10	Lower limb	-	No	Feasibility, safety
	Control (8)	-	-	-		
Rakićet <i>al</i> [50], 2018	RIPC (20)	3 × 5 + 5	Upper limb	-	Yes	Liver function tests
	IPC (20)	15 + 10	Portal triad	-		
	Control (20)	-	-	-		
Teo <i>et al</i> [51], 2020	RIPC (24)	4 × 5 + 5	Upper limb	13	Selectively	Serum ALT
	Control (26)	-	-	19		
Liu <i>et al</i> [52], 2019	RIPC (69)	3 × 5 + 5	Upper limb	56	Yes (in 20 min cycles)	Peak level of total bilirubin
	Control (67)	-	-	51		
Wu <i>et al</i> [53], 2020	RIPC (34)	3 × 5 + 5	Upper limb	23	Yes	Serum ALT and AST
	IPC (32)	10 + 10	Portal triad	26		
	Control (39)	-	-	25		

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IPC: Ischemic preconditioning; RIPC: Remote ischemic preconditioning.

patients. In two studies, liver resection was performed due to colorectal metastases[49,50] and due to primary liver cancers in the others[51-53]. The most common protocol for ischemia-reperfusion was 5 min of upper limb ischemia followed by 5 min of reperfusion in 3 cycles in three studies[50,52,53] and 4 cycles in one study[51]. In the first published pilot randomized feasibility trial, authors applied 2 cycles of 10 min of the lower limb ischemia followed by 10 min of reperfusion[49]. Primary endpoints varied, with serum transaminase activities being the most common. Two studies found significant differences in the early postoperative ALT and AST activities in favor of RIPC[49] and IPC/RIPC over control[50]. In one study, significant differences in postoperative ALT and AST activities on days 1 and 3 in favor of ischemia group (either remote or direct) over control group were observed, but these were absent on postoperative day 7[53]. Analysis of the subgroup of patients with liver cirrhosis was performed in a single study pointing towards no effect of RIPC on ALT activity 24 h posthepatectomy[51]. Mortality, morbidity, blood loss and hospital stay were assessed in three trials, and no differences were found between groups[49,51,52].

Data on hepatic steatosis were provided in only two studies. In one trial, all specimens were evaluated for degree of steatosis[49], with minimal liver steatosis found in both groups. In the second study, etiology of liver cirrhosis was nonalcoholic fatty liver disease in 4 patients (2 in the study group and 2 in the control group)[51]. No further information was given.

In conclusion, there is still insufficient data supporting the use of RIPC in liver resection as protection against IRI in order to improve clinical outcomes.

## REMOTE IPC IN LIVER TRANSPLANTATION

To the authors knowledge, only two studies addressed remote IPC in liver transplantation. In 2017, Robertson *et al*[54] published a pilot randomized controlled feasibility study on orthotopic liver transplantation from deceased donors (after either brain or cardiac death)[54]. Forty patients were randomized to a sham control group (20 patients) or an RIPC group (20 patients). The protocol for ischemia-reperfusion was 5 min of donor lower limb ischemia followed by 5 min of reperfusion in three cycles. Implantation of the liver graft was performed by standard piggy-back and caval replacement techniques. No differences in 90-d mortality, 90-d graft loss, complications, AST activity on the third postoperative day and hospital and ICU stay were detected.

In 2020, Jung *et al*[55] published an RCT on the application of RIPC in living donor liver transplantation[55]. In total, 148 donors were randomized to a sham control

group (73 donors) or an RIPC group (75 donors). The protocol for ischemia-reperfusion was 5 min of donor upper limb ischemia followed by 5 min of reperfusion in 3 cycles. For the recipients, the medical records were retrospectively analyzed. In the donors, no differences in complications, AST, ALT, total bilirubin and international normalized ratio within 7 postoperative days, incidence of delayed recovery of hepatic function and liver regeneration index depending on the use of RIPC were found. However, recipients who received preconditioned grafts had lower AST activity on postoperative day 7 and the maximal AST activity during the first postoperative week. No differences in other laboratory variables, early graft dysfunction, acute kidney injury, graft failure after 12 mo post-transplantation or hospital and ICU stay were detected.

In conclusion, there is no evidence supporting the use of RIPC in deceased and living donor liver transplantations as protection against IRI in order to improve clinical outcomes.

## REMOTE ISCHEMIC PERCONDITIONING, ISCHEMIC POSTCONDITIONING AND COMBINED METHODS OF ISCHEMIC CONDITIONING IN LIVER SURGERY

In search of effective protection against liver IRI, novel concepts are being adapted from experience with other organs. Ischemic postconditioning (IPOS) applies brief episodes of ischemia at the onset of reperfusion following a prolonged ischemia and was first introduced in a rodent heart model[56]. Advantage of IPOS over IPC is that it can be easily applied with precisely controlled timing. Modification of the RIPC technique is RPer, first applied by Schmidt *et al*[7] in the context of myocardial ischemia[7]. In a porcine model, alternating periods of occlusion and perfusion of the limb while the myocardium was under ischemia was examined. Little data exists on the efficacy of these methods alone or in combination in hepatoprotection against IRI.

In 2012, a mice liver resection study by Song *et al*[57] compared IPC, RIPC (hind limb), IPOS and the combination of IPC with IPOS[57]. The authors found that the combination of direct IPC with IPOS offered additional protection over the solo treatment. In contrast, no additive protection of IPOS was found when applied with RPer in rat liver resection model[58]. In this study, the authors identified RPer as the most promising technique to avoid hepatic IRI, in comparison with IPOS and combination of RPer with IPOS. This was in accordance with other studies on rodent liver resection or transplantation, which confirmed a protective effect of RPer against IRI[59-61]. Combination of different ischemic conditioning techniques in a mouse liver transplantation model was reported by Li *et al*[62]. By comparing IPC and RIPC with a combination of both methods, they found both techniques effective in hepatic IRI protection but no synergistic and additive effect of IPC and RIPC. Another study designed by this group assigned mice to direct IPC (donor), RPer (recipients) and IPC + RPer (donors and recipients were subjected to IPC and RPer, respectively)[63]. By double protection of the graft, first by IPC in donor then by RPer before reperfusion in recipient, they showed that combined treatment brought enhanced attenuation in IRI through additive effects on antioxidation, antiapoptosis, modulation of microcirculation disturbance and inhibition of innate immune response.

The aforementioned protocols have only been tested in animal models. No studies on humans have been published researching the possible application of IPOS, RPer or combined ischemic conditioning. There are currently no ongoing clinical trials on that subject[64].

## CONCLUSION

Direct IPC was not found effective in terms of decreasing mortality after liver resection or transplantation. Its role in specific subgroups of patients remains to be elucidated. Studies on remote IPC in liver resection pointed toward either no beneficial effects or effects limited to moderate reduction of IRI as indicated by serum transaminases and bilirubin concentration. Most studies used protocols with 5 min ischemic periods, which may indicate that this is an insufficient period.

In terms of liver transplantation, RIPC was found to be beneficial only in early graft function from living donors. Those were young, nonsteatotic grafts with relatively short periods of cold and warm ischemia. Other techniques of ischemic conditioning



are yet to be assessed in human clinical trials.

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## Clinical and Translational Research

# Bile acid indices as biomarkers for liver diseases II: The bile acid score survival prognostic model

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## Abstract

### BACKGROUND

Cholestatic liver diseases are characterized by an accumulation of toxic bile acids (BA) in the liver, blood and other tissues which lead to progressive liver injury and poor prognosis in patients.

### AIM

To discover and validate prognostic biomarkers of cholestatic liver diseases based on the urinary BA profile.

### METHODS

We analyzed urine samples by liquid chromatography-tandem mass spectrometry and investigated the use of the urinary BA profile to develop survival models that can predict the prognosis of hepatobiliary diseases. The urinary BA profile, a set of non-BA parameters, and the adverse events of liver transplant and/or death were monitored in 257 patients with cholestatic liver diseases for up to 7 years. The BA profile was characterized by calculating BA indices, which quantify the

#### Institutional review board

**statement:** The study was reviewed and approved by the University of Nebraska Medical Center Institutional Review Board (Approval No. 487-10-EP).

#### Clinical trial registration statement:

This study is registered at <https://www.clinicaltrials.gov/ct2/show/NCT01200082?term=alnouti&draw=2&rank=1>. The registration identification number is [NCT01200082].

#### Informed consent statement:

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

#### Conflict-of-interest statement:

The authors declare that there is no conflict of interests in this study.

#### Data sharing statement:

Technical appendix, statistical code, and data set available from the corresponding author at [[yalnouti@unmc.edu](mailto:yalnouti@unmc.edu)]. Participants gave informed consent for data sharing.

#### CONSORT 2010 statement:

The authors have read the CONSORT 2010, and the manuscript was prepared and revised according to the CONSORT 2010.

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composition, metabolism, hydrophilicity, formation of secondary BA, and toxicity of the BA profile. We have developed and validated the bile-acid score (BAS) model (a survival model based on BA indices) to predict the prognosis of cholestatic liver diseases.

## RESULTS

We have developed and validated a survival model based on BA (the BAS model) indices to predict the prognosis of cholestatic liver diseases. Our results demonstrate that the BAS model is more accurate and results in higher true-positive and true-negative prediction of death compared to both non-BAS and model for end-stage liver disease (MELD) models. Both 5- and 3-year survival probabilities markedly decreased as a function of BAS. Moreover, patients with high BAS had a 4-fold higher rate of death and lived for an average of 11 mo shorter than subjects with low BAS. The increased risk of death with high *vs* low BAS was also 2-4-fold higher and the shortening of lifespan was 6-7-mo lower compared to MELD or non-BAS. Similarly, we have shown the use of BAS to predict the survival of patients with and without liver transplant (LT). Therefore, BAS could be used to define the most seriously ill patients, who need earlier intervention such as LT. This will help provide guidance for timely care for liver patients.

## CONCLUSION

The BAS model is more accurate than MELD and non-BAS models in predicting the prognosis of cholestatic liver diseases.

**Key Words:** Hepatobiliary diseases; Bile acid indices; Death; Liver transplant; Survival model; Prognosis

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**Core Tip:** We have developed survival models based on bile acid (BA) indices to predict the prognosis of hepatobiliary diseases. Our BA models outperformed the model for end-stage liver disease and non-BA models in predicting the occurrence of the adverse events of death and/or liver transplant.

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## INTRODUCTION

Cholestatic liver diseases are hepatobiliary diseases associated with a reducing in bile flow due to impairment in bile production or failure of bile flow into bile duct[1]. Chronic liver diseases account for greater than 41000 deaths in the United States in 2017, making it the 11<sup>th</sup> leading cause of mortality[2]. Most cholestatic diseases progress toward end stage liver failure, which likely requires liver transplantation. Even after liver transplantation, post-surgery complications are common, which may require liver re-transplantation[3].

Biomarkers currently used in the clinic for the diagnosis and prognosis of liver diseases are primarily serum liver enzymes such as aspartate transaminase (AST), alanine transaminase (ALT), and bilirubin. However, these markers have numerous shortfalls including the lack of specificity for liver or bile duct injuries as they can be elevated in hyperthyroidism, adrenal, heart, or muscle disorders. Also, severe cell injury has to occur before their levels increase[4,5]. Multifactorial models with multiple parameters based on these biomarkers are also frequently used and offer advantages compared to the use of their individual biomarker components such as the Child-Turcotte-Pugh score[2].

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More recently, the model for end-stage liver disease (MELD) was developed to predict three-month mortality of patients with end-stage liver disease[5,6]. MELD is calculated based on serum creatinine, bilirubin, international normalized ratio (INR), and Na<sup>+</sup>, which are related to both liver and renal functions. MELD is currently used in many countries to classify patients awaiting transplantation to identify patients with the highest priority for liver transplant (LT)[6]. Since its implementation, MELD led to an intense reduction in the number of people waiting for liver transplant and decreased mortality on the waiting list without affecting post-transplant survival[7]. MELD is also an effective predictor of outcome in other conditions, such as patients have cirrhosis going for surgery and patients with alcoholic hepatitis or fulminant hepatic failure[7]. However, MELD is based on three objective laboratory variables, that are not necessarily liver specific. For example, serum bilirubin can be elevated in cases of hemolysis or sepsis. Serum creatinine can also be elevated from an underlying kidney disease that unrelated to hepatorenal syndrome and is a poor surrogate of renal function in cirrhotic patients[8]. In addition, patients may have an elevated INR which can be secondary to warfarin use. Any of these conditions can increase the MELD score and overestimate the liver disease severity[9]. Furthermore, several studies have shown that patients with cholestatic liver diseases may still have high mortality rates despite having low MELD scores[10,11].

Numerous clinical and preclinical studies have shown up to a 100-fold increase in BA concentrations in urine with various hepatobiliary diseases[12-16]. The impediment in bile flow associated with cholestatic liver diseases cause accumulation of toxic BA in the liver and blood, which can worsen the liver condition that lead to their accumulation and contribute to the unfavorable liver disease prognosis[17]. However, the potential use of BA as a marker for liver diseases have never translated into a widespread use in the clinic[18,19], due to major limitations including the major differences of the physiologic and pathologic effects of the various individual BA and the extremely high inter- and intra-individual variability of BA concentrations.

To this regard, we have developed the concept of "BA Indices", which are ratios calculated from the absolute concentration of individual BA and their metabolites. BA indices offered numerous advantages over absolute BA concentrations including low intra- and inter-individual variability and resistance to the influence of food consumption, age, gender, body mass index (BMI), and moderate alcohol consumption[19-21]. In the 1<sup>st</sup> part of this study, we have demonstrated that BA indices outperformed serum liver enzymes as biomarkers for the diagnosis of cholestatic liver diseases. In this second part of the study, we have developed survival models based on BA indices to predict the prognosis of hepatobiliary diseases. Our BA models outperformed the non-BA and MELD models in predicting the occurrence of the adverse events of death and/or LT.

## MATERIALS AND METHODS

### Study participants

New and existing patients of the University of Nebraska Medical Center (UNMC) hepatology clinic, who were diagnosed with one or multi-hepatobiliary conditions due to chronic hepatitis C ( $n = 63$ ), hepatitis B ( $n = 14$ ), alcoholic liver disease/alcoholic cirrhosis ( $n = 103$ ), primary biliary cholangitis ( $n = 11$ ), primary sclerosing cholangitis ( $n = 13$ ), autoimmune hepatitis ( $n = 24$ ), alpha-1-antitrypsin deficiency ( $n = 5$ ), nonalcoholic fatty liver disease/nonalcoholic steatohepatitis ( $n = 51$ ), carcinoma ( $n = 24$ ), cryptogenic cirrhosis ( $n = 10$ ), polycystic liver disease ( $n = 5$ ), elevated liver function test ( $n = 18$ ), and unknown etiology ( $n = 5$ ), were enrolled in this study. Table 1 shows a summary of our patient population characteristics. A total of 257 patients (121 female and 136 male) between the ages of 19 and 83 years, who were treated for cholestatic liver diseases in UNMC, over the period from November of 2011 to December of 2018, were recruited into the study. All participants were followed up for up to 7 years by collecting urine samples for BA analysis and monitoring non-BA parameters and adverse events including liver transplant, and death from their medical records.

The study was approved by the Institutional Review Board at UNMC and written informed consents were provided for all participating subjects. The registry URL was (<https://www.clinicaltrials.gov/ct2/show/NCT01200082?term=alnouti&draw=2&rank=1>). The clinical trial number was NCT01200082. Thirty milliliters of urine samples were collected from patients on their first visit to the hepatology clinic. All urine samples were stored in -80 °C until analyzed by liquid chromatography-tandem mass

**Table 1 Patient population characteristics**

	Patients	Death	Liver transplant
<i>n</i>	257	27	25
Gender			
Male	136	21	17
Female	121	6	8
Age (yr)			
mean $\pm$ SE	52.2 $\pm$ 0.71	55.9 $\pm$ 1.88	52.9 $\pm$ 2.1
Body mass index			
mean $\pm$ SE	30.7 $\pm$ 0.45	29.65 $\pm$ 1.19	29.11 $\pm$ 0.45
Race			
White	217	26	24
Black	11	0	0
Asian	7	0	0
Hispanic	4	0	1
Others	18	1	0
Non-BA parameters (mean $\pm$ SE)			
Creatinine (mg/dL)	1.02 $\pm$ 0.09		
Albumin (g/dL)	3.53 $\pm$ 0.04		
INR	1.19 $\pm$ 0.02		
Protime (s)	12.01 $\pm$ 0.42		
AST (U/L)	59.9 $\pm$ 4.07		
ALT (U/L)	54.9 $\pm$ 4.26		
Bilirubin (mg/dL)	1.75 $\pm$ 0.15		
AST/ALT	1.28 $\pm$ 0.04		
MELD	10.6 $\pm$ 0.34		
APRI	1.15 $\pm$ 0.11		

BA: Bile acids; INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease; APRI: Aspartate aminotransferase/platelet ratio index.

spectrometry (LC-MS/MS).

### **Non-BA parameters**

The performance of potential biomarkers from the urinary BA profile was also compared with and existing markers of liver function including ALT, AST, serum creatinine, albumin, protime, INR, bilirubin, AST/ALT ratio, and AST/platelet ratio index (APRI). These markers were monitored using the patients' medical records. Bile acid quantification by liquid chromatography–tandem mass spectrometry

Urine samples were extracted using solid phase extraction as described previously[8,22,23]. BA concentrations were quantified by LC-MS/MS, as we described previously.

### **Calculation of BA indices**

BA profile in urine was characterized using BA “indices”, which describe the composition, hydrophobicity, toxicity, and metabolism of total and individual BA as we have described previously[8,22,23].

### **Statistical analysis**

All statistical analysis was performed using the Statistical Product and Service



Solutions software, version 25 (IBM corporation, Armonk, NY, United States) and R software, version 3.6.3 (R Foundation for statistical Computing). A *P* value of 0.05 was considered significant for all the statistical tests described below.

### **Survival model development**

Cox proportional hazards (PH) regression was used to develop survival models to predict the prognosis of hepatobiliary diseases in terms of progressing specifically into the end points/adverse events of death.

For the “death” models, the only endpoint/adverse event recorded was death at 3 and 5 years. We only had 7 and 17 deaths occurring within earlier time points including 1 and 2 years, respectively, which was not enough to develop survival models. Patients who underwent liver transplant (LT) were censored with the date of transplantation. Patients still alive at the end of each period (3 and 5 years) were considered as censored at that time. The term “censored” indicates that the patient was alive at that date and that was the end of the follow-up[22]. Patients dropped off, not due to the occurrence of adverse event, i.e. death, before the end of the follow-up period, were censored at the last day they were seen in the clinic.

In addition to the “death only” models above, we also constructed models to predict death and/or LT. We followed the same approach as the “death” models, with the exception that the endpoint was the occurrence of the adverse events of either death or LT. Patients whom did not have either of the adverse events at the end of each period (3 and 5 years) were censored at that time.

Individual BA and non-BA variables were analyzed as possible predictors of survival in a univariate Cox regression analysis. Values of these variables included in the statistical analysis were obtained at the time of patients’ first visits. Significant variables (*P* < 0.05), which were identified from the univariate analysis were included in the multivariate analysis. To build the multivariate model a backward elimination regression method was used to retain the most significant variables with retention criteria of *P* < 0.05.

### **Model performance, goodness of fit and validation**

Goodness of fit was performed by testing PH assumption for each covariate included in the final Cox model and for the global model as a whole. We used the bootstrapping for model validation.

Receiver operating characteristic (ROC) curve analyses was performed on the scores from the various multivariate Cox models to determine their cut-off values in differentiating patients with *vs* without the adverse event. The cut-off values with optimum specificity and sensitivity were selected and the areas under the ROC curve (AUC) values were calculated.

### **Survival prediction**

The average survival probability [ $S_0(t)$ ] for a patient with an average score were calculated for different time points. To obtain the probability of survival for *t* years [ $S(t)$ ], first the score *e.g.* bile-acid score (BAS) is calculated, and finally  $S(t)$  is calculated using this equation: Survival probability for *t* years:  $S(t) = S_0(t)^{\exp(BAS - BAS_0)}$ .

Where,  $BAS_0$  is the average score from all patients in this study.

Kaplan-Meier plots were used to display survival curves. We have divided patients into two categories of high *vs* low risk and compared their survival with the Log-rank test and Breslow test[22]. We have tried the median cut-off values of the model scores to define high *vs* low risk.

### **Models comparison**

We have used multivariate cox regression analyses to build various models for the prediction of death. The performance of the different models in predicting the occurrence of death within 3- and 5-year periods were compared between the different models using the statistic outcomes from the Bootstrapping, Schoenfeld residuals, AUC, and Kaplan-Meier analyses.

## **RESULTS**

### **Patient population characteristics**

Table 1 shows a summary of the characteristics of the patient population in our study. The demographic variables were (age, BMI, gender, and race). Subjects were divided

into five race groups (White, Black, Asian, Hispanic, and others). During the 7-year follow-up period of 257 patients with cholestatic liver diseases, 27 patients (10.5%) died and 25 patients (9.7%) underwent liver transplantation.

We were interested in predicting the occurrence of adverse events of death within 3- and 5-year periods. During a 3-year follow-up period, 21 patients (8.2%) died and 19 patients (7.4%) underwent liver transplantation. While during a 5-year follow-up period, 25 patients (9.7%) died and 21 patients (8.2%) underwent liver transplantation.

### **Univariate Cox regression analysis for death prediction**

**Supplementary Table 1** shows the results of univariate Cox regression analyses for death prediction by BA Indices. Cox regression detects the risk of death associated with changes in BA indices. Positive regression coefficients imply that the risk of death increases with increasing the values of BA indices, while negative coefficients imply the risk of death increases with a decrease in the values of BA indices. We found correlation between the risk of death and many BA indices ( $P < 0.05$ ).

The hazard ratio (HR) from Cox regressions analysis quantifies the magnitude of the risk of death per unit change in BA indices. Because BA concentrations and indices have different scales and units, we performed the same calculation per 10% and 20% of the mean value of each variable instead of per absolute unit. For example, for a 20% increase in the %CDCA, the risk of death increases 1.26-fold (HR: 1.26;  $P < 0.05$ ).

We performed the same univariate cox regression analysis for demographics and non-BA parameters as well (**Supplementary Table 2**). Notably, the risk of death was significantly higher in males than females from this univariate analysis. Increasing levels of INR, protime, bilirubin, AST/ALT, APRI, and MELD also significantly increased the risk of death, whereas decreasing levels of albumin significantly increased the risk of death.

### **Multivariate Cox regression analysis for death prediction**

In multivariate analysis, a backward elimination regression was used to retain the most significant BA variables. The only BA variables retained in the multivariate model were %CDCA and %Tri-OH, which were independently predictive of survival (**Table 2**). For example, a 20% increase in the %CDCA and %Tri-OH increases the risk of death by 1.34-fold (HR: 1.34;  $P < 0.05$ ) and 1.14-fold (HR: 1.14;  $P < 0.05$ ), respectively. The BAS for individual patients can be calculated from this equation: BAS for death =  $0.039 \times \%CDCA + 0.052 \times \%Tri-OH$ .

For example, for a patient with %CDCA of 20%, and a %Tri-OH of 50%, the BAS would be 3.38.

We performed the same multivariate Cox regression analysis for demographics and non-BA parameters as well. For demographic variables, gender was significant in univariate analysis, but did not retain in multivariate analysis when included in the BA model building. In contrast, gender retained in the multivariate analysis for the non-BA model, but with minimal improvement of model goodness of fit and validation (the Bootstrapping, Schoenfeld residuals, AUC, and Kaplan-Meier analyses). Therefore, we did not include gender in the multivariate Cox models and AST/ALT ratio was the only significant predictive variable of death (**Table 2**). For example, a 20% increase in the AST/ALT, increases the risk of death by 1.36-fold (HR: 1.36;  $P < 0.05$ ). The non-BAS for individual patients can be calculated from this equation: non-BAS for death =  $1.236 \times AST/ALT$ .

In addition, we used the same methodology to develop other models including: (1) mixed BA and non-BA variables including demographics to test how the performance of a global BA- and non-BA mixed model compares to the BA-only and non-BA-only models; (2) MELD variables with coefficients from our data set to create a model with the original MELD variables, but with model coefficients derived from our data set; and (3) original MELD modified with BA and/or non-BA variables including demographics, to test if the performance of the original MELD can be improved by adding significant BA and non-BA parameters from the univariate analysis and vice versa (**Supplementary Table 3**). Overall, none of these strategies produced any statistically significant models neither they did improve the BA or non-BA-only model; therefore, were not further evaluated or validated.

### **Model performance, goodness of fit and validation**

Goodness of fit was performed by testing PH assumption for all the covariates of the final Cox model as well as for the global model as a whole, using a statistical test and a graphical diagnostic based on Schoenfeld residuals. A graphical diagnostic that shows a non-random pattern against time is evidence of violation of the PH assumption. The

**Table 2** Multivariate Cox regression analysis for death prediction

BA indices (%) and non-BA parameters	B-value (regression coefficient)	Standard error	P value	Hazard ratio: Exp (B)		
				1 unit change	10% change	20% change
The BAS model						
%CDCA	0.039	0.010	0.000	1.040	1.159	1.344
%Tri-OH	0.052	0.016	0.001	1.053	1.069	1.142
The non-BAS model						
AST/ALT	1.236	0.303	0.000	3.442	1.165	1.357

Using the regression coefficients from this table: The bile-acids score (BAS) equation is: the non-BAS equation is: BAS: Bile acids score; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

PH assumption is supported by a non-significant relationship between residuals and time. The Schoenfeld residual plots and *P* values supported the validity of the BA and non-BA models (Supplementary Figure 1).

We also used the bootstrapping validation. Bootstrapping validation results for the BA and non-BA models indicate that our regression coefficients were in the range of the 95%CI, *P* values were statistically significant for each covariate, bias values and standard error values were very small (Supplementary Table 4). We can conclude that the Bootstrapping validation results supported the validity of the BA and non-BA models.

Figure 1 shows the ROC curves of the models for death prediction. For 5-year death prediction, the AUC for BAS, non-BAS, and MELD were 0.740, 0.653, and 0.683, respectively. For 3-year death prediction, the AUC for BAS, non-BAS, and MELD were 0.761, 0.664, and 0.715, respectively. Potential cut-off values selected based on the optimum sensitivity and specificity for different models. The ROC-optimum scores for BA, non-BA, and MELD models for death prediction were 2.71, 1.72, and 10, respectively (Table 3).

### Survival prediction

Table 4 presents the estimated survival probability [ $S_0(t)$ ] for a patient with an average  $BAS_0$  of 2.24 (the average BAS from all 257 patients in this study) for different time points. To obtain the survival probability for *t* years [ $S(t)$ ], first  $BAS$  is calculated,  $S_0(t)$  is identified from Table 4, and finally  $S(t)$  is calculated using this equation: Survival probability for *t* years:  $S(t) = S_0(t)^{\exp(BAS - BAS_0)}$ .

Where,  $BAS_0$  is the average BAS from all patients in this study; namely 2.24, while  $BAS$  is the BAS for that particular patient. For the same example patient discussed above, the probability of surviving for at least 3 years is: Survival probability for (3) years =  $0.934^{\exp(3.38 - 2.24)} = 0.81 = 81\%$

The relationship between estimated 5- and 3- year survival probability [ $S(t)$ ] and the BAS in patients with liver disease are shown in Figure 2A. Survival probability decreases as a function of BAS. For example, the 5-year survival probability for patients with BAS of 1.2 (25<sup>th</sup> percentile of the population), 2.1 (50<sup>th</sup> percentile of the population *i.e.* median), and 3.1 (75<sup>th</sup> percentile of the population) are 97%, 93%, and 82%, respectively. Similarly, the 3-year survival probability for patients with the same BAS above, are 98%, 94%, and 85%, respectively.

Table 4 presents the estimated survival probability [ $S_0(t)$ ] for a patient with an average non- $BAS_0$  of 1.58 for different time points. The survival probability for (*t*) years is calculated using this equation: Survival probability for *t* years:  $S(t) = S_0(t)^{\exp(\text{non-BAS} - \text{non-BAS}_0)}$ .

The relationship between estimated 5- and 3- year survival probability [ $S(t)$ ] and the non-BAS in patients with liver disease are shown in Figure 2B. For example, the 5-year survival probability for patients with non-BAS of 1.1 (25<sup>th</sup> percentile of the population), 1.4 (50<sup>th</sup> percentile of the population), and 1.9 (75<sup>th</sup> percentile of the population) are 92%, 90%, and 83%, respectively. Similarly, the 3-year survival probability for patients with the same non-BAS above, are 95%, 91%, and 86%, respectively.

By the end of the study, up to 7 years monitoring of 257 patients with cholestatic liver diseases, 27 patients (10.5%) have died. The Kaplan-Meier estimator was used to estimate subjects' survival free of adverse events over time. We have tried the median

**Table 3 Receiver operating characteristics analysis of bile-acids score, non- bile-acids score, and models for end stage liver diseases for death prediction**

Models	AUC (5-yr)	AUC (3-yr)	(Cutoff value; sensitivity, specificity)
BAS	0.740	0.761	(2.71; 74, 68)
non-BAS	0.653	0.664	(1.72; 67, 66)
MELD	0.683	0.715	(10; 62, 64)

AUC: Areas under the ROC curve; BAS: Bile acids score; MELD: Model for end-stage liver disease.

**Table 4 Estimated survival probability [ $S_0(t)$ ] for death prediction**

t (mo)	5	7	14	24	36	60	76
The BAS							
$S_0(t)$	0.993	0.985	0.971	0.948	0.934	0.916	0.901
The non-BAS							
$S_0(t)$	0.989	0.978	0.958	0.924	0.902	0.876	0.855

BAS: Bile acids score.

of the BAS of the population (2.19) cut-off value to define high *vs* low risk of death (Figure 3A). The estimated mean survival time was 71 mo (5.9 years) for the high-risk group and 82 mo (6.8 years) for the lower risk group based on the median BAS of 2.19 (Table 5). The *P* value of the log rank test and Breslow test were statistically significant (*P* value < 0.05), indicating the median cut-off of BAS, can differentiate low *vs* high risk of death.

Figure 3B shows the Kaplan Meier survival for the high *vs* low risk of death groups based on the median (1.44) for the non-BAS. The estimated mean survival time was 74 mo (6.2 years) for the high-risk group and 79 mo (6.6 years) for the lower risk group based on the median non-BAS of 1.44. The *P* value from the log rank test and Breslow test was insignificant (*P* value > 0.05), indicating the median of non-BAS (1.44) cannot differentiate low *vs* high risk of death (Table 5).

Figure 3C shows the Kaplan Meier survival for the high *vs* low risk of death groups based on the median (11) for the MELD model. The estimated mean survival time was 74 mo (6.2 years) for the high-risk group and 78 mo (6.5 years) for the lower risk group based on the median MELD of 11. The *P* value from the log rank test and Breslow test was insignificant (*P* value > 0.05), indicating the median of MELD (11) cannot differentiate low *vs* high risk of death (Table 5).

### Death and/or LT model

We have developed similar BAS and non-BAS multivariate cox models for the prediction of the adverse events of death and/or LT instead of death only (Supplementary Table 5). Both models were also validated using the same criteria (data not shown). For both 3 and 5-years prediction, AUC was > 0.74 for both models (Supplementary Figure 2 and Supplementary Table 6). Similar to the “death only” models, there were direct relationship between BAS and non-BAS and liver transplant-free survival (Supplementary Figure 3). The estimated mean liver transplant-survival time was 60 mo (4.9 years) for the high-risk group and 79 mo (6.6 years) for the lower risk group based on the median BAS (0.45), which were statistically different (Supplementary Figure 4 and Supplementary Table 7).

## DISCUSSION

We developed a survival model based on BA indices to predict the prognosis of hepatobiliary diseases in terms of progressing into the end point/adverse event of death over a 3- and 5-year period of time. Using the multivariate Cox regression analysis, we have constructed these final models for death prediction: (1) The BAS



Table 5 Kaplan-Meier analysis for survival

Cutoff	Total <i>n</i>	<i>n</i> of events	Estimated mean (mo)	Standard error	95%CI
BAS					
Median cutoff of 2.19					
Low risk < 2.19	128	4	81.68	1.14	79.44-83.93
High risk > 2.19	129	23	70.72	2.5	65.81-75.62
Non-BAS					
Median cutoff of 1.44					
Low risk < 1.44	118	9	78.68	1.70	75.34-82.02
High risk > 1.44	139	18	73.97	2.21	69.64-78.29
MELD					
Median cutoff of 11					
Low risk < 11	133	11	78.06	1.71	74.71-81.42
High risk > 11	124	16	73.91	2.35	69.29-78.52

BAS: Bile acids score; MELD: Model for end-stage liver disease.

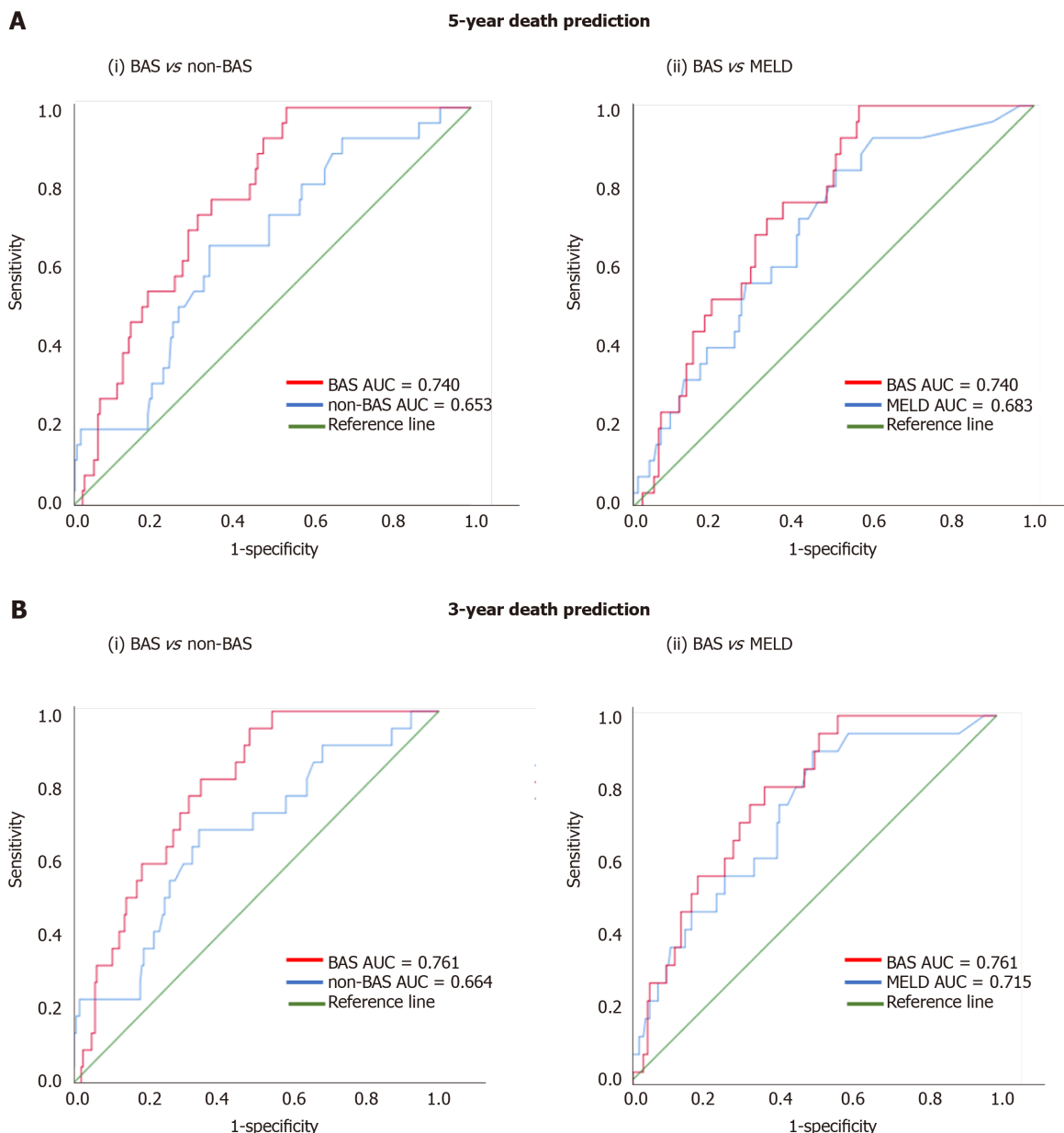
model for death prediction: BAS for death =  $0.039 \times \%CDCA + 0.052 \times \%Tri-OH$ ; (2) The non-BAS model model for death prediction: non-BAS (non-BAS) for death =  $1.236 \times AST/ALT$ . BAS in this population ranged from 0-4, while the non-BAS ranged from 0.44-4.98.

Cholestatic diseases are associated with impaired bile flow to the intestine, which is expected to translate into reduced transformation of primary BA including CDCA and CA into secondary BA by intestinal bacteria. Therefore, accumulation of primary BA in the blood may indicate further impairment in bile flow and worsening of the liver diseases[8,22,23]. This is in agreement with the BAS model, where increased %CDCA and %Tri-OH BA (primarily consists of CA) were the most significant predictors of liver disease prognosis into death. Another interpretation for the accumulation of CDCA could be related to the fact that CDCA is the best substrate for bile salt export pump (BSEP), which is responsible for the efflux transport of BA across the canalicular membrane from hepatocytes into bile. Therefore, loss of BSEP function could be associated with the progression of the liver disease[8,22], which leads to CDCA accumulation in the liver and eventually into the systemic circulation.

Goodness of fit was performed by testing PH assumption using a statistical test and a graphical diagnostic based on Schoenfeld residuals. For death prediction, the PH assumption was met in both BA and non-BA models supporting their validity (Supplementary Figure 1). In addition, we used the bootstrapping method for model validation. Bootstrapping validation results supported the validity of both the BA and non-BA models for death prediction (Supplementary Table 4). Further validation efforts are also ongoing to build internal and eventually external data sets for more rigorous model validation.

We used ROC analysis to compare the accuracy of our prognostic models. The higher the AUC under the ROC curve, the greater the overall accuracy of the marker in distinguishing between groups. For prognostic models, AUC of 0.9 or greater is rarely seen, AUC between 0.8 and 0.9 indicates excellent diagnostic accuracy, and any AUC over 0.7 may be considered clinically useful[23,24]. ROC curves are also used to determine cut-off values which quantify the normal ranges of biomarkers. The selection of optimum cut-off values is a tradeoff between sensitivity and specificity. Accordingly, scores for the BA, non-BA, and MELD models for death prediction of 2.71, 1.72, and 10, respectively, were identified as cut-off values with optimum sensitivity *vs* specificity (Table 3).

For 5-year death prediction, the AUC for BAS was 0.74 compared to 0.65 for non-BAS and 0.68 for MELD models (Figure 1A). Similarly, for 3-year death prediction, the AUC for BAS was 0.76 compared to 0.66 for non-BAS and 0.71 for MELD models (Figure 1B). In addition, BAS sensitivity in death prediction (74% *vs* 67% and 62%) was 7% and 12% higher than non-BAS and MELD, respectively. BAS specificity was also higher than non-BAS and MELD (68% *vs* 66% and 64%). Therefore, ROC analysis show



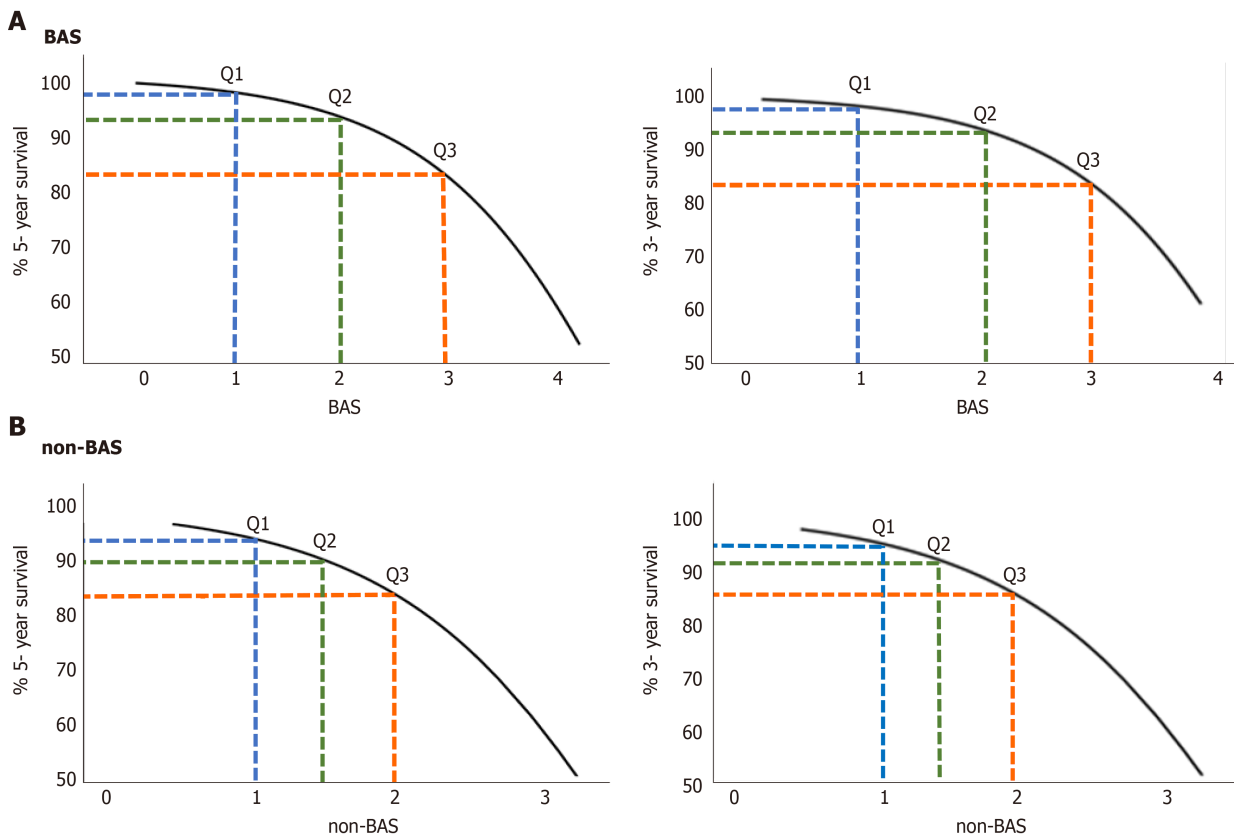
**Figure 1 Receiver operating characteristics curves of bile-acids score, non- bile-acids score, and model for end stage liver diseases for death prediction.** A: The area under the receiver operating characteristic curves (AUC) for bile-acids score (BAS), non-BAS, and model for end stage liver diseases (MELD) for 5-year death prediction; B: The AUC for BAS, non-BAS, and MELD for 3-year death prediction. AUC: Area under the receiver operating characteristic curves; BAS: Bile-acids score; MELD: Model for end stage liver diseases.

that BAS is more accurate and results in higher true-positive and true-negative prediction of death compared to both non-BAS and MELD.

The Cox survival model can be used to predict the survival probability at any time point. The survival probability for  $t$  years  $[S(t)]$  was calculated for every subject using both BAS and non-BAS models, as: Survival probability for  $(t)$  years:  $S(t) = S_0(t) \exp(BAS \cdot 2.24)$ , survival probability for  $(t)$  years:  $S(t) = S_0(t) \exp(non-BAS \cdot 1.58)$ .

Where  $S_0(t)$  presents the estimated survival probability for a patient with an average BAS of 2.24 or non-BAS of 1.58 for different time points (Table 4).

As shown in Figure 2, both 5- and 3-year survival probabilities decrease as a function of both BA and non-BAS. For example, the 3-year survival probability for patients with BAS of 1.2 (25<sup>th</sup> percentile of the population), 2.1 (50<sup>th</sup> percentile of the population *i.e.* median), and 3.1 (75<sup>th</sup> percentile of the population) are 98%, 94%, and 85%, respectively. While, the 3-year survival probability for patients with equivalent non-BAS (25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> population percentiles) are 95%, 91%, and 86%, respectively.



**Figure 2** Estimated 5- and 3-year survival [ $S(t)$ ] from the bile-acids score and non- bile-acids score models. A: The relationship between estimated 5- and 3- year survival probability [ $S(t)$ ] as a function of bile-acids score (BAS); B: The relationship between estimated 5- and 3- year survival probability [ $S(t)$ ] as a function of non-BAS. Q1, Q2, and Q3 are 25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles of the population, respectively. BAS: Bile-acids score.

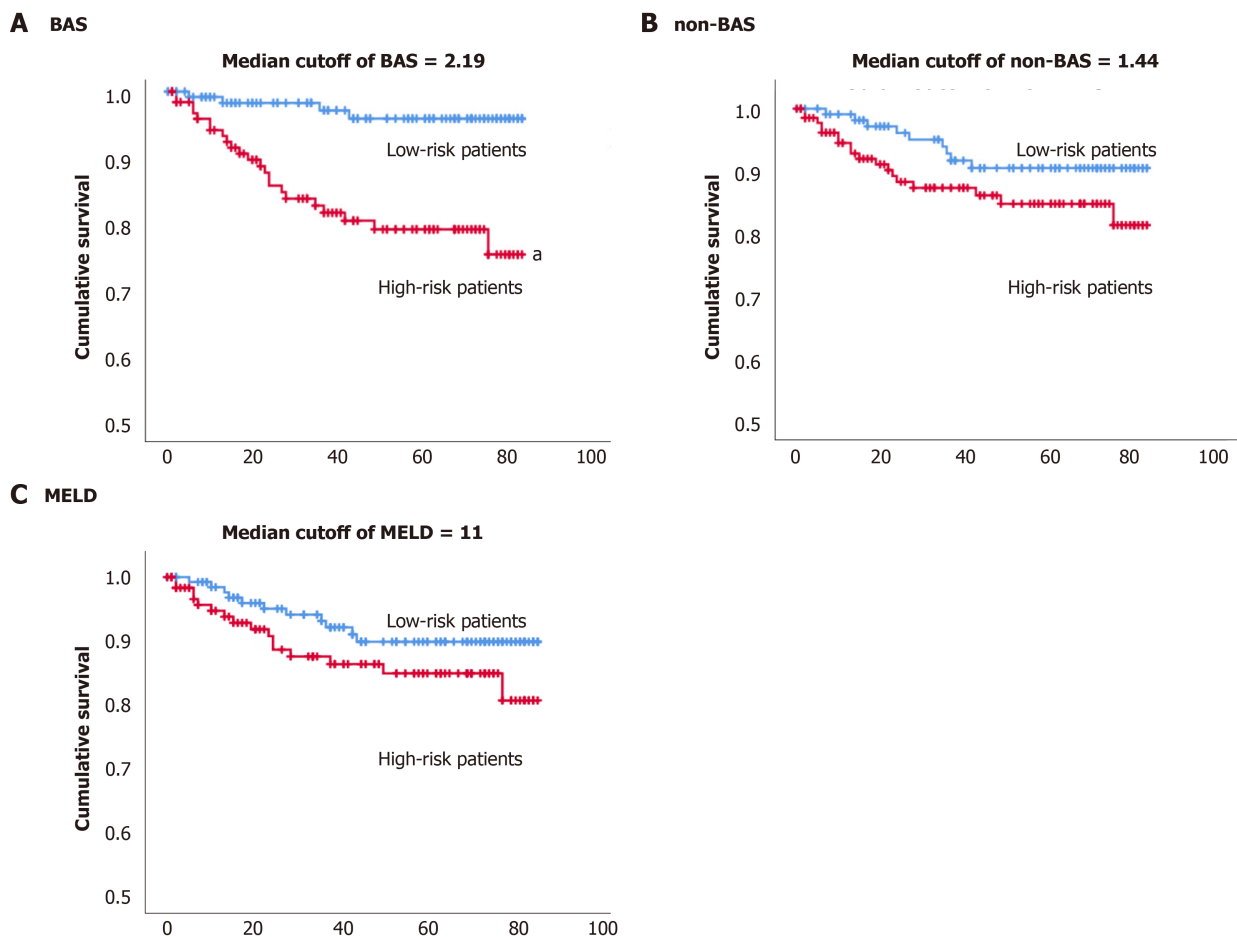
The Kaplan-Meier estimator was used to estimate subjects' survival free of adverse event over time. Median cut-off for BAS (2.19) was able to differentiate low *vs* high risk of death. While the median cut-offs for non-BAS and MELD were not able to differentiate low *vs* high risk of death (Figure 3 and Table 5).

Twenty-three patients with high BAS (> the median BAS of 2.19) died *vs* four patients with low BAS (< the median BAS of 2.19) for the entire study. Therefore, 19 more patients died with high compared to low BAS. In contrast, nine and five more subjects with high non-BAS and high MELD have died compared to low non-BAS and low MELD, respectively. Also, patients with low BAS lived for an average of 82 mo, while patients with high BAS lived for an average of 71 mo since their diagnosis with the liver diseases. Therefore, patients with low BAS lived 11 mo longer than patients with high BAS. On the other hand, patients with low non-BAS or low MELD (< median score), lived, in average, for only five or four months longer, compared to the high non-BAS or high MELD (high score), respectively (Table 5). Consequently, the shortening of lifespan between patients with high *vs* low BAS was 6-7 mo more compared to high non-BAS or high MELD. Also, the number of deaths with high BAS is 2-4-fold higher than that with high non-BAS or high MELD. Therefore, it can be concluded that in this patient population, patients with high BAS are at a much higher risk of death compared to patients with high MELD or high non-BAS.

Similar conclusions can be made regarding the death and/or LT prediction models. Patients with high BAS lived without need for LT 2-5 mo less than patients with high non-BAS or high MELD. Therefore, patients with high BAS are at a higher risk of death and/or LT compared to patients with high MELD or high non-BAS (Supplementary Figures 2-4) and (Supplementary Tables 5-7).

## CONCLUSION

In summary, we have developed and validated a survival model based on BA (the BAS model) indices to predict the prognosis of cholestatic liver diseases. Our results demonstrate that the BAS model is more accurate and results in higher true-positive



**Figure 3 Kaplan-Meier survival plots for high vs low bile-acids score, non- bile-acids score, and models for end stage liver diseases.** <sup>a</sup>*P* value < 0.05 from the Log rank and Breslow tests. A: The median cutoff value of the bile-acids score (BAS) was used to define high vs low risk of death; B: The median cutoff value of the non-BAS was used to define high vs low risk of death; C: The median cutoff value of the model for end stage liver diseases was used to define high vs low risk of death. BAS: Bile-acids score; MELD: Model for end stage liver diseases.

and true-negative prediction of death compared to both non-BAS and MELD models. Both 5- and 3-year survival probabilities markedly decreased as a function of BAS. Moreover, patients with high BAS had a 4-fold higher rate of death and lived for an average of 11 mo shorter than subjects with low BAS. The increased risk of death with high *vs* low BAS was also 2-4-fold higher and the shortening of lifespan was 6-7-mo lower compared to MELD or non-BAS. Similarly, we have shown the use of BAS to predict the survival of patients with and without LT. Therefore, BAS could be used to define the most seriously ill patients, who need earlier intervention such as LT. This will help provide guidance for timely care for liver patients.

## ARTICLE HIGHLIGHTS

### Research background

Most cholestatic diseases progress toward end stage liver failure, which likely requires liver transplantation. Numerous clinical and preclinical studies have shown up to a 100-fold increase in bile acids (BA) concentrations in urine with various hepatobiliary diseases. However, due to their high inter-and intra-individual variability, BA has not been used in clinic as markers for the diagnosis and prognosis of liver diseases. To this end, we have developed the concept of BA indices and utilized it to build a survival model to predict the prognosis of liver diseases.

### Research motivation

Biomarkers currently used in the clinic for the diagnosis and prognosis of liver diseases are primarily serum liver enzymes. Model for end-stage liver disease (MELD)

was developed to predict three-month mortality of patients with end-stage liver disease. MELD is based on three objective laboratory variables that are not necessarily liver specific. The potential use of BA as a marker for liver diseases has never translated into a widespread use in the clinic. To this end, we have developed the concept of BA indices and utilized it to build a survival model to predict the prognosis of liver diseases.

### Research objectives

The objective of this project was to discover and validate prognostic biomarkers of cholestatic liver diseases based on the urinary BA profile. We investigated the use of the urinary BA profile to develop survival models to predict the prognosis of hepatobiliary diseases. One application for BAS could be to define the most seriously ill liver patients, who may need earlier intervention such as liver transplantation.

### Research methods

Sample analysis: Liquid chromatography-tandem mass spectrometry. Statistical analysis: univariate and multivariate Cox proportional hazards regression, testing proportional hazards assumption, receiver operating characteristic curve, survival probability, and Kaplan-Meier plots.

### Research results

The bile-acid score (BAS) model (a survival model based on BA indices) was more accurate and results in higher true-positive and true-negative prediction of death compared to both non-BAS and MELD models. Both 3- and 5-year survival probabilities markedly decreased as a function of BAS. Patients with high BAS had a 4-fold higher rate of death and lived for an average of 11 mo shorter than subjects with low BAS. The increased risk of death with high *vs* low BAS was also 2-4-fold greater and the shortening of lifespan was 6-7-mo lower compared to MELD or non-BAS.

### Research conclusions

We have developed and validated a survival model (the BAS model) based on BA indices to predict the prognosis of cholestatic liver diseases.

### Research perspectives

BAS could be used to define the most seriously ill patients, who need earlier intervention such as liver transplant. This will help provide guidance for timely care for liver patients.

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## Case Control Study

## Gut dysbiosis is associated with poorer long-term prognosis in cirrhosis

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## Abstract

## BACKGROUND

Gut dysbiosis is common in cirrhosis.

## AIM

To study the influence of gut dysbiosis on prognosis in cirrhosis.

## METHODS

The case-control study included 48 in-patients with cirrhosis and 21 healthy controls. Stool microbiome was assessed using 16S ribosomal ribonucleic acid gene sequencing. We used modified dysbiosis ratio (MDR): [*Bacilli* (%) + *Proteobacteria* (%)]/[*Clostridia* (%) + *Bacteroidetes* (%)]. Patients with MDR more the median made up the group with severe dysbiosis, others did the group with non-severe dysbiosis. The follow-up period was 4 years.

## RESULTS

The mortality rate of patients with severe dysbiosis was significantly higher than that of patients with non-severe dysbiosis (54.2% vs 12.5%;  $P = 0.001$ ). The presence of severe dysbiosis was independent risk factors for death [hazard ratio =  $8.6 \times (1.9-38.0)$ ;  $P = 0.005$ ]. The abundance of *Enterobacteriaceae* ( $P = 0.002$ ), *Proteobacteria* ( $P = 0.002$ ), and *Lactobacillaceae* ( $P = 0.025$ ) was increased and the abundance of *Firmicutes* ( $P = 0.025$ ) and *Clostridia* ( $P = 0.045$ ) was decreased in the deceased patients compared with the survivors. The deceased patients had a higher MDR value than the survivors [ $0.131 \times (0.069-0.234)$  vs  $0.034 \times (0.009-0.096)$ ;  $P = 0.004$ ]. If we applied an MDR value of 0.14 as the cutoff point, then it predicted patient death within the next year with a sensitivity of 71.4% and a

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specificity of 82.9% [area under the curve =  $0.767 \times (0.559-0.974)$ ]. MDR was higher in patients with cirrhosis than in health controls [ $0.064 \times (0.017-0.131)$  vs  $0.005 \times (0.002-0.007)$ ;  $P < 0.001$ ], and in patients with decompensated cirrhosis than in patients with compensated cirrhosis [ $0.106 \times (0.023-0.211)$  vs  $0.033 \times (0.012-0.074)$ ;  $P = 0.031$ ]. MDR correlated negatively with prothrombin ( $r = -0.295$ ;  $P = 0.042$ ), cholinesterase ( $r = -0.466$ ;  $P = 0.014$ ) and serum albumin ( $r = -0.449$ ;  $P = 0.001$ ) level and positively with Child–Turcotte–Pugh scale value ( $r = 0.360$ ;  $P = 0.012$ ).

## CONCLUSION

Gut dysbiosis is associated with a poorer long-term prognosis in cirrhosis.

**Key Words:** Cirrhosis; Dysbiosis; Gut; ROC-analysis; Microbiota; Microbiome; Gut-liver axis

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**Core Tip:** The mortality rate of patients with severe dysbiosis was significantly higher than that of patients with non-severe dysbiosis. The abundance of *Enterobacteriaceae*, *Proteobacteria*, and *Lactobacillaceae* was increased and the abundance of *Firmicutes* and *Clostridia* was decreased in the deceased patients compared with survivors. The abundance of *Bacilli*, *Enterococcaceae* and *Lactobacillaceae* was higher and the abundance of *Clostridia* was lower in those who died during the first year of follow-up compared with those who survived this year. The abundance of *Enterobacteriaceae* and *Proteobacteria* was higher in those who died in 2<sup>nd</sup>-4<sup>th</sup> years of follow-up compared with survivors.

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## INTRODUCTION

Microbiota are stable ecological communities of microorganisms in certain habitats[1]. Recently, the human microbiota has attracted the attention of researchers. Previous studies have shown that its composition varies in different diseases, and it has been hypothesized that the pathology of the human microbiota (dysbiosis) can participate in the pathogenesis of these diseases[2].

As the gut microbiota is the richest human microbiota, most research has been devoted to it. The gut microbiota plays an important role in human life; it digests non-digestible carbohydrates as well as generates vitamins and short-chain fatty acids (butyrate is particularly prominent), which are used as a source of energy by colonocytes. This function is performed by strict anaerobes of the main taxa of normal microbiota, which belong to the *Clostridia* class and *Bacteroidetes* phylum. Nevertheless, the gut microbiota can also play a pathogenic role because it has potential pathogenic bacteria, which belong to the *Bacilli* class (*Streptococcaceae*, *Enterococcaceae*) and *Proteobacteria* phylum (*Enterobacteriaceae*). In addition, facultative anaerobes of the *Bacilli* class and *Proteobacteria* phylum can enter the gut wall, mesenteric lymph nodes, portal, and systemic blood flow. This phenomenon is called bacterial translocation. The gut microbiota is also the main source of endotoxin (lipopolysaccharide), a toxic substance of gram-negative bacteria, primarily *Proteobacteria*[3].

To date, several articles[4-6] have been published that describe alterations of the gut microbiome in cirrhosis. Researchers have shown that the abundance of harmful *Proteobacteria* increases, whereas the abundances of useful *Ruminococcaceae* and *Lachnospiraceae* belonging to the *Clostridia* class decrease in the gut microbiome in cirrhosis.

Analysis of the relationship between gut dysbiosis and the course of cirrhosis is complicated by several problems. The first is the fact that the only reliable method for

analyzing the gut microbiota is sequencing, which is very expensive and requires a rare bioinformatics specialist. Therefore, the study of dysbiosis has not yet transcended the walls of scientific laboratories and entered clinical medicine.

The second problem is the interpretation of obtained data. The researcher acquires a huge amount of redundant data after sequencing. A generalizing indicator should be used to simplify the analysis. Several such indicators have been proposed, including the richness and diversity of microbiota, the *Firmicutes/Bacteroidetes* ratio[7], and the cirrhosis dysbiosis ratio (CDR)[5]. However, these indicators have various disadvantages; first of all, many of them have a weak theoretical basis. Thus, the proliferation of harmful bacteria can lead to an increase in the richness and diversity of microbiota. However, the proliferation of beneficial bacteria can lead to similar changes; therefore, an increase or decrease in these indicators cannot be correctly interpreted. *Firmicutes* is too heterogeneous and represented by useful members of the *Clostridia* class as well as potentially pathogenic members of the *Bacilli* class. In addition, the *Firmicutes/Bacteroidetes* ratio does not take into account *Proteobacteria* that are the main potentially pathogenic bacteria. *Bacteroidetes* has a multifaceted effect on the macroorganism and cannot be considered as only harmful bacteria. Therefore, the *Firmicutes/Bacteroidetes* ratio may be useful for comparing the gut microbiota between countries, between individuals on different diets, or for assessing changes in the microbiota with age, but it cannot show how much better or worse the composition of the microbiota has become.

The CDR proposed by Bajaj *et al*[5] is based on the ratio of “good” to “bad” bacteria. However, it also has some disadvantages. Its values decrease with an increase in the severity of dysbiosis, which can lead to misinterpretation. *Bacteroidaceae* were among the “bad” bacteria, but they play a rather neutral role in the gut microbiome and are widely represented in the microbiomes of healthy individuals, especially in studies from Asian countries[4]. In addition, *Bacteroidaceae*, being strict anaerobes, cannot be subjects of bacterial translocation[8]. At the same time, the list of “bad” bacteria did not include *Bacilli*, which together with *Enterobacteriaceae* are responsible for bacterial translocation[8] and secondary infections[3,9,10] in cirrhosis.

Thus, the development and testing of a pathogenetically-based dysbiosis ratio remains an important task. With this ratio, it will be possible to replace expensive and inaccessible sequencing with polymerase chain reaction (PCR) for selected taxa *via* automatic ratio calculation, which will allow for the introduction of gut dysbiosis tests into clinical practice.

The second important task of studying gut dysbiosis in cirrhosis is to clarify whether its presence affects the prognosis of patients.

Identifying a solution to these two problems is the aim of the present research.

## MATERIALS AND METHODS

### *Theoretical substantiation of the modified dysbiosis ratio*

We used the CDR as a basis but flipped the equation such that the “bad” bacteria were in the numerator and the “good” bacteria were in the denominator. Therefore, our modified dysbiosis ratio (MDR) increased with aggravation of dysbiosis, which was less confusing in its interpretation. We considered *Proteobacteria* and *Bacilli* as “bad” bacteria since they are responsible for bacterial translocation as well as the development of secondary infections[3,8-10] and their contents increase in cirrhosis[4,5]. We used the dominant taxa in healthy individuals, *Clostridia* and *Bacteroidetes*, as “good” bacteria. These taxa are strict anaerobes; therefore, they do not undergo bacterial translocation and do not cause extraintestinal secondary infections in cirrhosis[3,8-10]. *Clostridia* predominate in the American population, where the Western diet is widespread, whereas *Bacteroidetes* are more common in the Asian population, where the Eastern diet is widespread[4,5]. Thus, the total accounting of these taxa is also intended to reduce the effect of diet on the value of the MDR. The abundance of *Clostridia* has been found to decrease with the development of cirrhosis[4,5]. The changes in *Bacteroidetes* abundance in cirrhosis appear to vary across different studies[4-6].

Thus, the pathogenesis- and evidence-based MDR was calculated as follows: [*Bacilli* (%) + *Proteobacteria* (%)]/[*Clostridia* (%) + *Bacteroidetes* (%)].

### *Patients*

In this case-control prospective study, 113 consecutive patients with cirrhosis were admitted to the Department of Hepatology of Clinic for Internal Diseases, Gastroen-

terology and Hepatology at Sechenov University (Moscow, Russia) and screened for inclusion. The study procedures were explained to potential participants, and written informed consent was obtained before enrollment. The present study was approved by the Ethics Committee of Sechenov University in accordance with the Declaration of Helsinki (№03-16).

The inclusion criteria were as follows: diagnosis of cirrhosis verified by histology or clinical, biochemical, and ultrasound findings; and age between 18 and 70 years. The exclusion criteria were as follows: use of lactulose, lactitol, or other prebiotics, probiotics, antibiotics, or metformin in the past 6 wk; alcohol consumption in the past 6 wk; or inflammatory bowel disease, cancer, or any other serious disease. Of the original 113 patients screened for inclusion, 48 met the criteria and were enrolled in the study while 65 were excluded (Figure 1).

A study control group consisted of 21 healthy individuals who visited the clinic for routine health examinations during the same period.

The severity of liver disease was determined using the Child-Turcotte-Pugh (CTP) scoring system[11], in which class A was defined as compensated cirrhosis and classes B and C were defined as decompensated cirrhosis.

### **Gut microbiome analysis**

The morning after admission, a stool sample was taken into a sterile disposable container and immediately frozen at -80 °C[12].

Deoxyribonucleic acid from the stool was isolated using the MagNa Pure Compact Nucleic Acid Isolation Kit I (Roche, Basel, Switzerland) according to the manufacturer's instructions. Libraries for sequencing were prepared by two rounds of PCR amplification. In the first round, specific primers for the v3-v4 region of the 16S ribosomal ribonucleic acid (RNA) gene were used: 16S-F: TCGTCGGCA-GCGTCAG-ATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S-R: GTCTCGTGG-GCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

After amplification, the PCR product was purified using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, United States). Then, a second round of PCR was performed to attach specific adapters and enable multiplexing of the samples. To begin, 5 µL of the first PCR product was added to the reaction after ball cleaning with primers containing Illumina indices (Nextera XT Index v2 Primers; San Diego, CA, United States) and adapter sequences as well as 2 × KAPA HiFi HotStart ReadyMix. The amplification products were also purified using AMPure XP beads (Beckman Coulter). The concentrations of the prepared libraries were then measured using a Qubit 2.0 fluorimeter (London, United Kingdom) and quantitative PCR. The quality of the libraries was assessed using the Agilent 2100 Bioanalyzer (Santa Clara, CA, United States). The libraries were mixed in equal proportions and diluted to the required concentration to be run on a MiSeq (Illumina) device. Pair-end readings of 300 + 300 nucleotides were obtained. Reads were trimmed from the 3'-tail with Trimmomatic (Illumina) and then merged into a single amplicon with the MeFiT tool[13,14]. We did not perform operational taxonomic unit picking; instead, we classified amplicon sequences with the Ribosomal Database Project (RDP) classifier and RDP database[15].

### **Follow-up**

The patients were contacted by phone every 3 mo to confirm that they were alive. If there was no answer, we contacted the patient's relatives by phone to find out if the patient was alive or dead. If it was not possible to contact them, we studied patient electronic medical records in the United Medical Information and Analytical System of Moscow, in which death registration data are entered. The follow-up period was 4 years.

### **Statistical analysis**

Statistical analysis was performed with STATISTICA 10 (StatSoft Inc., Tulsa, OK, United States) and SPSS Statistics (IBM SPSS, Armonk, NY, United States) software. The data are presented as medians (interquartile ranges). Differences between continuous variables were assessed with the Mann-Whitney test because many variables were not distributed normally. Fisher's exact test was used to assess the differences between categorical variables. Survival was assessed using the Kaplan-Meier estimator and Cox regression test. A Cox regression model was constructed to assess the influence of various factors on patient survival and hazard ratios (HRs). Correlations between variables were computed using Spearman's rank correlation. *P* values ≤ 0.05 were considered as statistically significant.



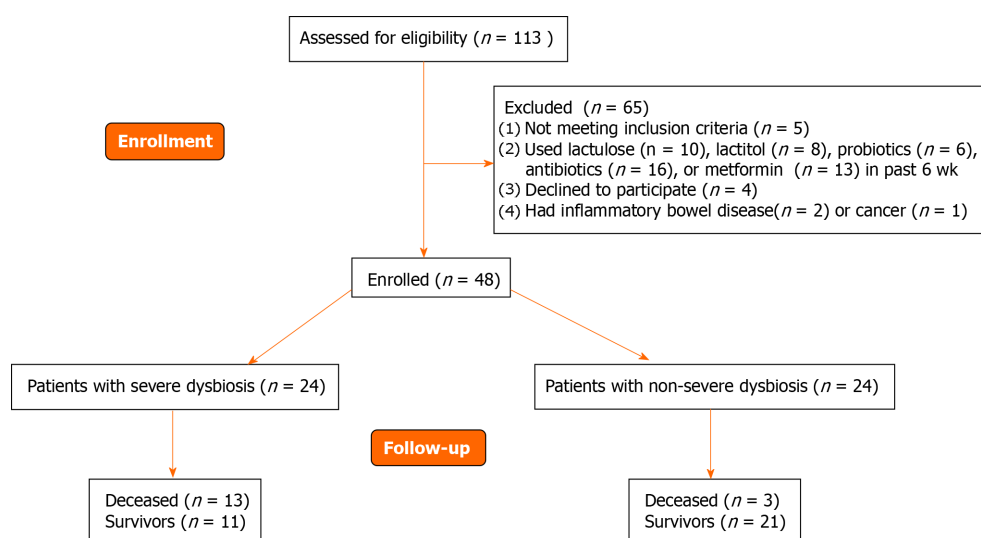


Figure 1 CONSORT 2010 flow diagram.

## RESULTS

Participants with cirrhosis and healthy controls were comparable in age [51 (40-59) *vs* 46 (42-54) years;  $P = 0.489$ ], body mass index [ $24.6 \times (22.7-27.7)$  *vs*  $26.3 \times (25.1-29.0)$  kg/m<sup>2</sup>;  $P = 0.110$ ], and sex distribution (male/female: 23/25 *vs* 8/13;  $P = 0.313$ ).

Seventeen participants with cirrhosis had compensated cirrhosis (CTP class A), and the remaining 31 had decompensated cirrhosis (19 class B and 12 class C). Participants with compensated cirrhosis and decompensated cirrhosis were also comparable in age [49 (38-55) years *vs* 52 (40-59) years,  $P = 0.316$ ], body mass index [ $24.8 \times (21.8-27.8)$  kg/m<sup>2</sup> *vs*  $24.4 \times (22.8-27.7)$  kg/m<sup>2</sup>;  $P = 0.771$ ], and sex distribution (6/11 *vs* 17/14;  $P = 0.160$ ).

The MDR was higher in patients with cirrhosis than in healthy controls [ $0.064 \times (0.017-0.131)$  *vs*  $0.005 \times (0.002-0.007)$ ;  $P < 0.001$ ] and in patients with decompensated cirrhosis than in those with compensated cirrhosis [ $0.106 \times (0.023-0.211)$  *vs*  $0.033 \times (0.012-0.074)$ ;  $P = 0.031$ ]. When taken as the cutoff point, an MDR value of 0.01 made it possible to distinguish patients with cirrhosis from healthy individuals with a sensitivity of 81.3% and a specificity of 90.5% [AUC =  $0.884 \times (0.806-0.962)$ ] (Figure 2). The specificity approached nearly 100% with a cutoff value of 0.02.

If we used the median MDR (0.064) as a cutoff point, then the group of patients with cirrhosis could be divided into patients with severe ( $n = 24$ ) and non-severe ( $n = 24$ ) dysbiosis (Figure 1).

The abundance of useful *Clostridia* was reduced and that of harmful *Bacilli* was increased, whereas the abundance of harmful *Enterobacteriaceae* was not significantly changed in patients with non-severe dysbiosis compared to healthy controls. The abundance of *Clostridia* further decreased, the abundance of *Bacilli* further increased, and the abundance of *Enterobacteriaceae* also increased in patients with severe dysbiosis. Interestingly, an increase in the abundance of *Bifidobacteriaceae* considered beneficial to the gut microbiome was also observed in cirrhosis without significant differences between groups with different degrees of dysbiosis. The abundance of *Bacteroidetes* did not differ significantly between patients with cirrhosis and healthy individuals (Table 1).

There were no significant differences in age, body mass index, sex distribution, and etiology of cirrhosis between patients with severe and non-severe dysbiosis. Patients with severe dysbiosis had lower serum albumin and cholinesterase levels, higher CTP scale values, and higher C-reactive protein levels. Although the incidences of ascites, esophageal varices, and hepatic encephalopathy were higher in patients with severe dysbiosis than in those with non-severe dysbiosis, these differences did not reach the significance level. There were no differences between the groups of patients in red blood cell, white blood cell, and platelet counts; creatinine, sodium, potassium, and glucose levels; and aminotransferase, alkaline phosphatase, and gamma-glutamyl transferase activities (Table 2).

The MDR correlated negatively with prothrombin ( $r = -0.295$ ;  $P = 0.042$ ), cholinesterase ( $r = -0.466$ ;  $P = 0.014$ ) and serum albumin ( $r = -0.449$ ;  $P = 0.001$ ) levels and positively with CTP scale values ( $r = 0.360$ ;  $P = 0.012$ ).

Table 1 Comparison of the gut microbiome at different taxonomic levels between the groups

Taxa	Heath controls (n = 21)	Cirrhosis with non-severe dysbiosis (n = 24)	Cirrhosis with severe dysbiosis (n = 24)	P value, Non-severe dysbiosis vs controls	P value, Severe dysbiosis vs controls	P value, Severe dysbiosis vs non-severe one
<i>Firmicutes</i>	91.8 (89.3-96.4)	89.7 (73.0-93.6)	80.1 (62.7-88.1)	0.074	< 0.001	0.028
<i>Clostridia</i>	88.0 (86.6-91.7)	83.5 (69.8-88.7)	69.8 (57.4-77.2)	0.008	< 0.001	0.001
<i>Ruminococcaceae</i>	33.9 (28.1-41.6)	27.6 (19.2-36.5)	18.8 (7.9-31.7)	0.086	0.002	0.081
<i>Lachnospiraceae</i>	43.8 (37.2-54.6)	37.6 (27.2-60.5)	31.0 (22.1-46.0)	0.488	0.030	0.190
<i>Bacilli</i>	0.1 (0.0-0.2)	0.5 (0.2-1.9)	7.1 (1.3-14.8)	< 0.001	< 0.001	< 0.001
<i>Streptococcaceae</i>	0.03 (0.02-0.10)	0.29 (0.12-0.52)	3.20 (0.38-10.4)	< 0.001	< 0.001	0.002
<i>Lactobacillaceae</i>	0.00 (0.00-0.01)	0.02 (0.01-0.22)	0.47 (0.12-1.50)	0.002	< 0.001	< 0.001
<i>Enterococcaceae</i>	0.00 (0.00-0.00)	0.00 (0.00-0.03)	0.03 (0.01-0.08)	0.067	0.001	< 0.001
<i>Bacteroidetes</i>	5.6 (2.8-8.1)	5.7 (1.8-12.9)	6.1 (3.2-8.2)	0.954	0.829	0.959
<i>Bacteroidaceae</i>	2.5 (0.8-3.4)	1.3 (0.6-4.3)	1.4 (0.2-3.8)	0.991	0.432	0.261
<i>Actinobacteria</i>	0.2 (0.1-0.3)	0.8 (0.3-2.8)	0.7 (0.4-2.9)	< 0.001	< 0.001	0.687
<i>Bifidobacteriaceae</i>	0.1 (0.0-0.2)	0.6 (0.1-2.6)	0.5 (0.2-2.3)	0.002	0.001	0.687
<i>Proteobacteria</i>	0.39 (0.14-0.51)	0.15 (0.10-0.81)	3.57 (1.77-6.65)	0.869	< 0.001	< 0.001
<i>Enterobacteriaceae</i>	0.03 (0.01-0.05)	0.04 (0.02-0.61)	2.70 (1.58-6.24)	0.104	< 0.001	< 0.001

The mortality rate of patients with severe dysbiosis was significantly higher than that of patients with non-severe dysbiosis (54.2% *vs* 12.5%;  $P = 0.001$ ). Moreover, the difference in mortality was insignificant in the first year of follow-up (20.8% *vs* 8.3%;  $P = 0.092$ ) and significant in subsequent years of follow-up (33.4% *vs* 4.2%;  $P = 0.002$ ) (Figure 3).

Deceased patients had a higher MDR value than the survivors [ $0.131 \times (0.069-0.234)$  *vs*  $0.034 \times (0.009-0.096)$ ;  $P = 0.004$ ]. Moreover, this was observed in the deceased in the first year of follow-up [ $0.191 \times (0.035-1.126)$  *vs*  $0.046 \times (0.012-0.115)$ ;  $P = 0.022$ ] as well as in subsequent years [ $0.115 \times (0.074-0.144)$  *vs*  $0.034 \times (0.009-0.096)$ ;  $P = 0.044$ ].

If we took an MDR value of 0.05 as the cutoff point, it predicted patient death within the next 4 years with a sensitivity of 65.2% and a specificity of 81.3%. If we used 0.11 for this, then the sensitivity was 81.3% and the specificity was 62.5% [AUC =  $0.755 \times (0.611-0.899)$ ; Figure 4A].

If we applied an MDR value of 0.14 as the cutoff point, then it predicted patient death within the next year with a sensitivity of 71.4% and a specificity of 82.9% [AUC =  $0.767 \times (0.559-0.974)$ ; Figure 4B].

The presence of severe dysbiosis [HR =  $8.6 \times (1.9-38.0)$ ;  $P = 0.005$ ] and total serum bilirubin level [HR =  $1.005 \times (1.001-1.010)$ ;  $P = 0.015$ ] were independent risk factors for death, unlike serum albumin ( $P = 0.870$ ) and prothrombin ( $P = 0.167$ ) levels, degrees of ascites ( $P = 0.752$ ), and esophageal varices ( $P = 0.230$ ).

In addition, death in the first year of follow-up was significantly determined by serum albumin level [HR =  $0.83 \times (0.71-0.97)$ ;  $P = 0.020$ ], unlike degrees of ascites ( $P = 0.619$ ), dysbiosis ( $P = 0.241$ ), total serum bilirubin ( $P = 0.742$ ) and prothrombin levels ( $P = 0.386$ ), and esophageal varices ( $P = 0.125$ ). However, mortality in subsequent years of follow-up was determined significantly by the degree of dysbiosis only [HR =  $24.8 \times (2.3-269.6)$ ;  $P = 0.008$ ].

The abundances of *Enterobacteriaceae* [ $2.4 \times (1.6-7.6)$  *vs*  $0.4 \times (0.0-1.7)$ %;  $P = 0.002$ ], *Proteobacteria* [ $3.4 \times (1.9-8.2)$  *vs*  $0.6 \times (0.1-2.0)$ %;  $P = 0.002$ ], and *Lactobacillaceae* [ $0.35 \times (0.12-0.81)$  *vs*  $0.06 \times (0.01-0.31)$ %;  $P = 0.025$ ] were increased, and the abundances of *Firmicutes* [ $78.8 \times (62.7-85.6)$  *vs*  $87.1 \times (71.7-93.6)$ %;  $P = 0.025$ ] and *Clostridia* [ $73.0 \times$

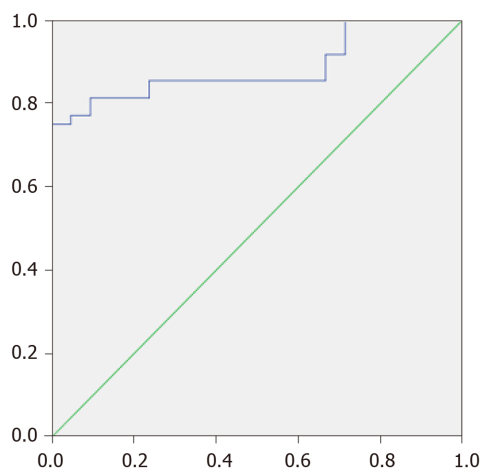
**Table 2** Main indicators of patients with cirrhosis with severe and non-severe dysbiosis

	Severe dysbiosis (n = 24)	Non-severe dysbiosis (n = 24)	P value
Age, yr	51.5 (42.0-59.0)	50.0 (35.0-57.5)	0.392
Body mass index, kg/m <sup>2</sup>	24.6 (22.8-27.7)	24.2 (22.7-27.7)	0.837
Male/female	12/12	11/13	0.500
Etiology of cirrhosis: Alcohol	9	9	0.617
Autoimmune	2	7	0.068
HBV	7	2	0.068
HCV	5	3	0.350
Cryptogenic	1	3	0.304
Child-Turcotte-Pugh score	9 (8-10)	7 (6-9)	0.047
Death	13	3	0.001
Death within the first year of follow-up	5	2	0.092
Death during the following years of follow-up	8	1	0.002
Esophageal varices (present/absent)	20/4	18/6	0.477
Hepatic encephalopathy (overt/minimal/absent)	11/9/4	6/11/7	0.288
Number connection test, seconds	87 (65-118)	79 (59-92)	0.248
Ascites (present/absent)	16/8	11/13	0.122
Spontaneous bacterial peritonitis (present/absent)	0/24	0/24	1.000
Red blood cells, 10 <sup>12</sup> cell/L	3.8 (3.4-4.0)	3.9 (3.6-4.5)	0.370
White blood cells, 10 <sup>9</sup> cell/L	3.8 (2.7-5.3)	3.8 (3.1-5.2)	0.628
Platelets, 10 <sup>9</sup> cell/L	87 (55-120)	76 (60-108)	0.860
Serum total protein, g/L	70 (61-76)	73 (64-78)	0.599
Serum albumin, g/L	31 (28-37)	38 (34-41)	0.009
Serum total bilirubin, μmol/L	47 (31-62)	31 (24-63)	0.375
Prothrombin index (Quick test), %	58 (48-67)	64 (54-71)	0.239
Creatinine, mg/dL	0.69 (0.53-0.87)	0.73 (0.66-0.90)	0.187
Serum sodium, mmol/L	141 (139-144)	141 (138-143)	0.795
Serum potassium, mmol/L	4.3 (4.0-4.7)	4.3 (4.1-4.7)	0.844
Serum glucose, mmol/L	5.1 (4.7-5.6)	5.3 (4.7-6.0)	0.260
Alanine aminotransferase, U/L	36 (25-72)	37 (23-60)	0.804
Aspartate aminotransferase, U/L	54 (41-98)	40 (26-67)	0.219
Gamma glutamyl transferase, U/L	77 (40-148)	76 (36-131)	0.621
Alkaline phosphatase, U/L	221 (188-340)	222 (166-298)	0.542
Cholinesterase, kU/L	2.7 (1.9-3.7)	4.0 (3.6-4.5)	0.031
C-reactive protein, mg/L	10.1 (2.1-16.1)	2.1 (0.3-8.9)	0.032
Splenic length, cm	15.4 (14.0-17.6)	16.1 (13.3-19.2)	0.841

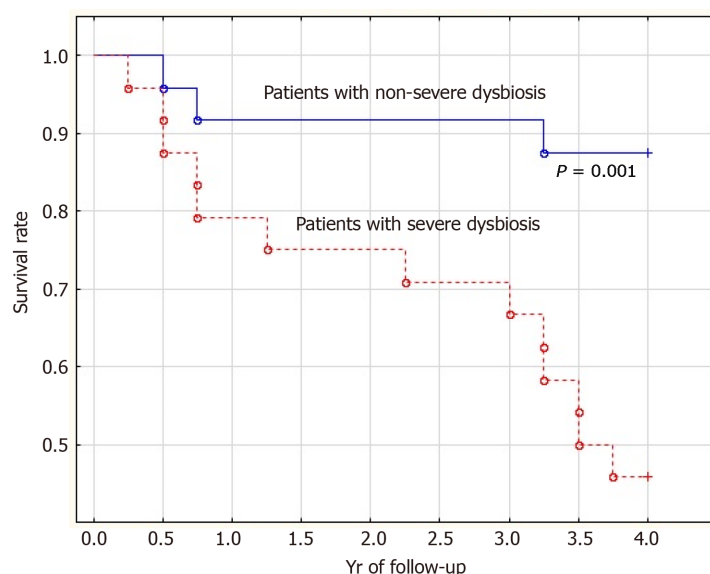
HBV: Hepatitis B virus; HCV: Hepatitis C virus.

(51.9-78.2) vs 80.1 × (68.5-87.2)%;  $P = 0.045$ ] were decreased in the gut microbiome of deceased patients compared to the survivors.

The abundances of *Bacilli* [14.0 × (1.4-18.4) vs 1.1 × (0.3-4.6)%;  $P = 0.017$ ], *Enterococcaceae* [0.09 × (0.04-0.38) vs 0.01 × (0.00-0.04)%;  $P = 0.005$ ], and *Lactobacillaceae* [0.45 × (0.24-1.52) vs 0.09 × (0.01-0.38)%;  $P = 0.021$ ] were higher, and the abundance of *Clostridia* [67.1 × (31.2-78.2) vs 77.5 × (68.5-86.8)%;  $P = 0.047$ ] was lower in those who



**Figure 2** Receiver operating characteristic analysis of modified dysbiosis ratio for distinguish patients with cirrhosis from healthy individuals.

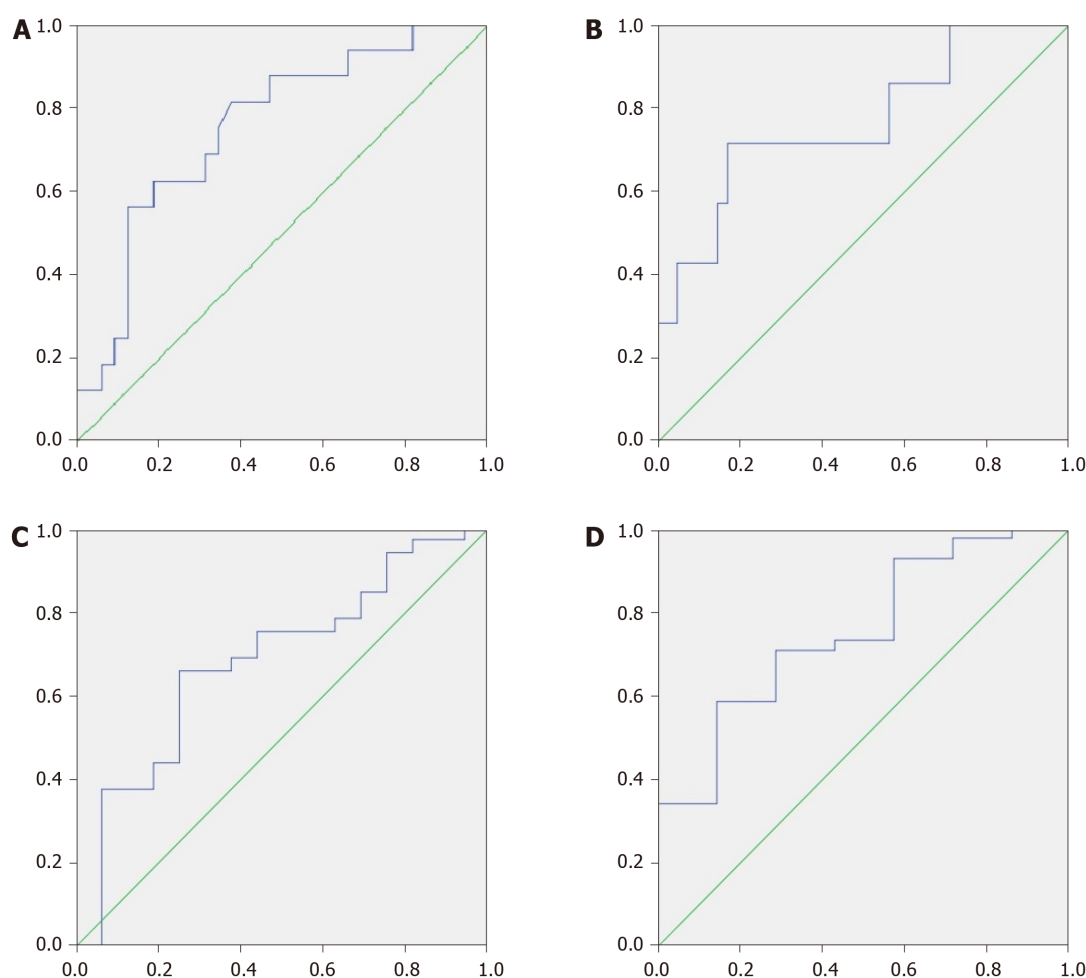


**Figure 3** Survival curve (years) of patients with cirrhosis with severe (dotted line) and non-severe (solid line) dysbiosis.

died during the first year of follow-up compared to those who survived the first year. The abundances of *Enterobacteriaceae* [ $2.2 \times (1.8-6.5)$  vs  $0.4 (0.0-1.7)\%$ ;  $P = 0.009$ ] and *Proteobacteria* [ $3.8 \times (2.5-7.0)$  vs  $0.6 \times (0.1-2.0)\%$ ;  $P = 0.010$ ] were higher in those who died in the second through fourth years of follow-up compared to the survivors. The deceased during the first year of follow-up had higher abundances of *Bacilli* [ $14.0 \times (1.4-18.4)$  vs  $0.5 \times (0.4-4.2)\%$ ;  $P = 0.026$ ] and *Enterococcaceae* [ $0.09 \times (0.04-0.38)$  vs  $0.00 \times (0.00-0.05)\%$ ;  $P = 0.002$ ] than those who died in the next 3 years of follow-up (Figures 5 and 6).

There was no significant difference in the *Firmicutes/Bacteroidetes* ratio between patients with cirrhosis and healthy individuals [ $13.3 \times (7.8-40.9)$  vs  $15.8 \times (11.2-33.1)$ ;  $P = 0.469$ ], the survivors and deceased patients [ $14.0 \times (6.1-51.7)$  vs  $12.7 \times (8.0-26.4)$ ;  $P = 0.938$ ], and patients with compensated and decompensated cirrhosis [ $16.0 \times (7.8-68.7)$  vs  $13.1 \times (7.9-35.6)$ ;  $P = 0.846$ ].

The CDR was significantly lower in patients with cirrhosis than in healthy individuals [ $16.4 \times (7.2-39.0)$  vs  $34.9 \times (23.0-101.1)$ ;  $P = 0.002$ ], in deceased patients than in the survivors [ $10.5 \times (4.5-18.9)$  vs  $19.7 \times (10.7-57.6)$ ;  $P = 0.041$ ], and in decompensated cirrhosis than in compensated cirrhosis [ $13.1 \times (5.0-27.4)$  vs  $22.5 \times (14.1-65.4)$ ;  $P = 0.039$ ]. Using the cutoff value of this ratio equal to 22, we could distinguish between patients with cirrhosis and healthy individuals with a sensitivity of 64.6% and a specificity of 85.7% [AUC =  $0.735 \times (0.620-0.850)$ ]. The CDR was lower in patients who died in the first year of follow-up compared to those who survived the



**Figure 4** Receiver operating characteristic-analysis of modified dysbiosis ratio in predicting death. A: During 4 years; B: During 1 year; and of cirrhosis dysbiosis ratio in predicting death; C: During 4 years; and D: During 1 year.

first year [ $9.4 \times (1.7-15.4)$  vs  $17.7 \times (9.0-54.8)$ ;  $P = 0.035$ ] but did not differ significantly between those who died in the following years and those who survived [ $13.6 \times (7.3-22.5)$  vs  $19.7 \times (10.7-57.6)$ ;  $P = 0.321$ ].

If we used a CDR value of 15 as the cutoff point, then it predicted patient death within the next 4 years with a sensitivity of 68.8% and a specificity of 62.5% [AUC =  $0.684 \times (0.522-0.845)$ ; **Figure 4C**] as well as within the first year with a sensitivity of 85.7% and a specificity of 58.5% [AUC =  $0.753 \times (0.569-0.936)$ ; **Figure 4D**].

## DISCUSSION

Translating scientific developments into clinical practice is a rather difficult task. The study of the gut microbiome in various diseases is becoming mainstream in modern science, but thus far, it has no applications in clinical practice. It is hindered by the high cost of sequencing the fecal microbiome and the shortage of bioinformatics specialists.

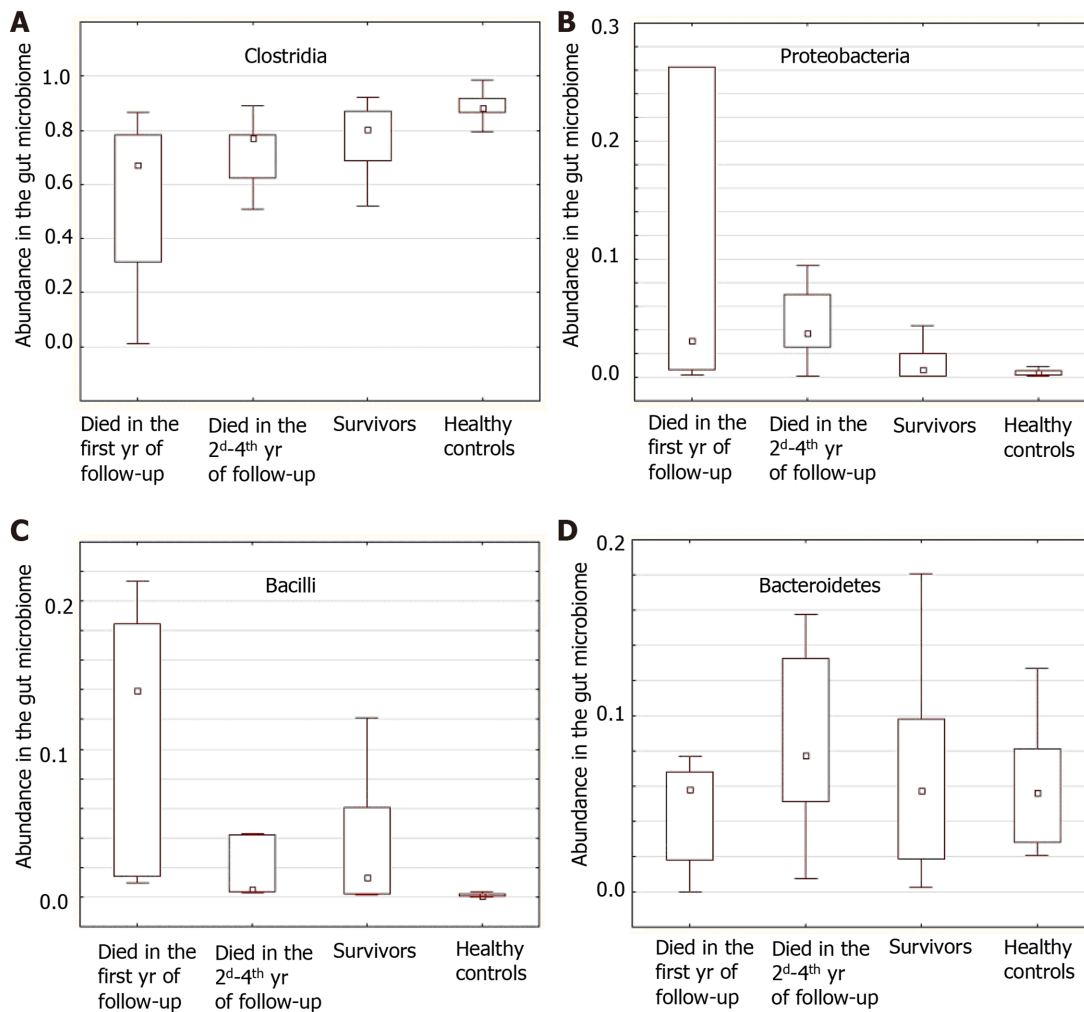
Therefore, an important step in introducing the study of gut dysbiosis into clinical practice is to replace this expensive method with a simpler and more affordable one. PCR is an ideal candidate to determine the content of selected taxa in feces, followed by a comprehensive assessment of the state of the gut microbiome.

The idea to conduct a comprehensive assessment of the state of the gut microbiome in cirrhosis originated with Bajaj and colleagues[5]. However, their CDR can be improved, which was one of the aims of our study.

Here, we modified the CDR to improve its analytical performance and show that it can be used to predict the death of patients.

First, we inverted the CDR equation, placing the abundance of “bad” bacteria in the numerator and the abundance of “good” bacteria in the denominator. Thus, the value





**Figure 5** An abundance of the main taxa in patients who died during the first and the subsequent years of follow-up, survivors and healthy controls. The middle point is the median, the box is the interquartile range, the whiskers are non-outlier range. A: *Clostridia*; B: *Proteobacteria*; C: *Bacilli*; and D: *Bacteroidetes*.

of our MDR increases with the aggravation of dysbiosis, which is more logical. The original CDR decreases with the aggravation of dysbiosis, which can be confusing to interpret.

Our MDR is based on the data regarding the role of various taxa in the pathogenesis of cirrhosis complications and changes in their abundance in cirrhosis. We excluded *Bacteroidaceae* from the list of “bad” bacteria since their role in the pathogenesis of cirrhosis is not clear, and the change in their abundance in the gut microbiome in cirrhosis varies according to different researchers. According to our data, it does not change significantly, according to Chen *et al*[4], it decreases, and according to Bajaj *et al*[5], it increases in compensated cirrhosis and decreases in decompensated cirrhosis, becoming almost the same as that in healthy individuals. On the contrary, in a study by Kakiyama *et al*[6], the abundance of *Bacteroidaceae* decreased with compensated cirrhosis and increased with decompensated cirrhosis. Instead, we added *Bacilli* to the list of “bad” bacteria, which, like *Proteobacteria/Enterobacteriaceae*, are responsible for bacterial translocation and the development of extraintestinal infections in cirrhosis[8-10]. The abundances of both of these taxa increased with cirrhosis according to all studies[4-6], including ours.

As “good” bacteria, we used the higher-level taxon *Clostridia*, which includes all taxa accounted as “good” bacteria in the CDR. The main problem is that the abundance of these taxa is highly dependent on diet[16,17]. Among healthy individuals, it was 90% in the Russian population (our data), approximately 45% in the American population[5], and approximately 30% in the Chinese population[4]. However, if you add to them to the abundance of *Bacteroidetes*, which changes in the opposite direction relative to *Clostridia* and *Firmicutes*[16,17], then the differences were not so large: 95%, 80%, and 90%, respectively. This dependence of the *Bacteroidetes* and

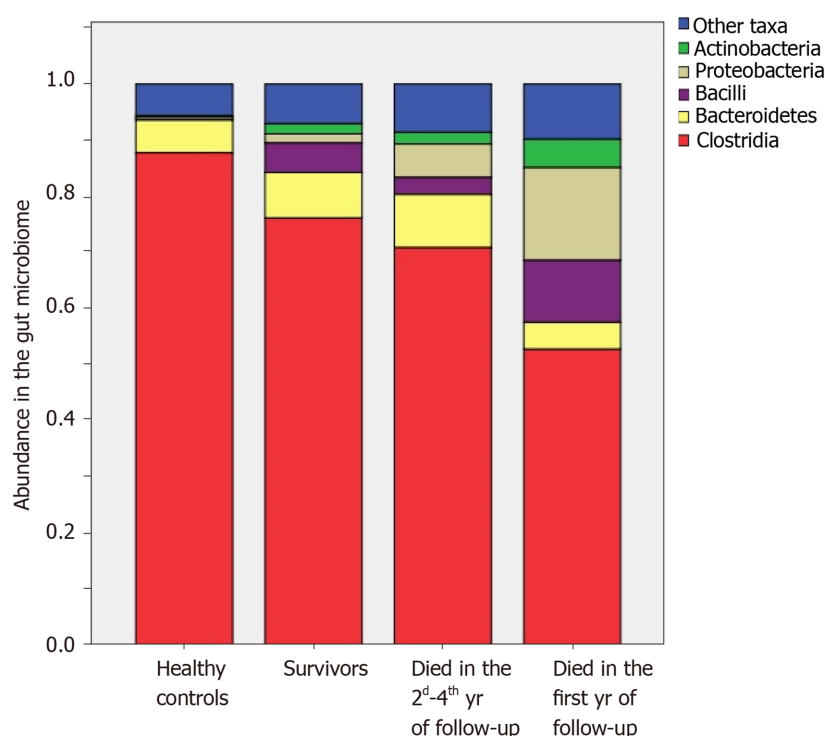


Figure 6 The composition of the gut microbiome in healthy individuals, survivors, and deceased in the first and subsequent 3 years.

*Clostridia* abundances on diet led to the fact that the value of the CDR in our population was more than an order of magnitude higher than in the original study. Thus, the addition of *Bacteroidetes* to the group of “good” bacteria can neutralize the effect of diet on MDR and allow it to be used in different populations.

In our study, we were able to show that despite the change in the order of values, the CDR retained its main characteristics: it was higher in healthy individuals, lower in patients with compensated cirrhosis, and minimal in patients with decompensated cirrhosis.

Both the CDR and MDR were useful in assessing the prognosis of patients with cirrhosis, but the analytical characteristics of our modification were higher. In particular, the MDR, unlike the CDR, made it possible to assess the long-term (more 1 year) prognosis of patients.

Interestingly, the different taxa included in the MDR had different effects on prognosis. *Clostridia* and *Bacilli* mainly determined the medium-term prognosis (death within a year), and *Proteobacteria* and *Enterobacteriaceae* determined the long-term prognosis (death over the subsequent 3 years). This finding may be due to the fact that *Bacilli* provide a more powerful translocation of living bacteria, which leads to faster death, whereas *Enterobacteriaceae* act mainly by translocating their endotoxin, which leads to a more delayed death.

Thus, we were able to show that the gut microbiome in cirrhosis can be comprehensively and reliably evaluated using targeted analysis of the most significant taxa, which will allow for replacing expensive and poorly available sequencing with cheaper and more affordable PCR for four indicators (*Proteobacteria*, *Bacilli*, *Clostridia*, and *Bacteroidetes*) that does not require interpretation by rare bioinformatics specialists.

This will be a big step forward in introducing the achievements of fundamental hepatology into clinical practice, as it will give doctors an instrument for assessing the state of the gut microbiome in their patients as well as determining how it is affected by drugs that are prescribed for the correction of dysbiosis. This reality reinforces the strength of our study.

In addition, our study is the first to describe the effect of gut dysbiosis on the prognosis of patients with cirrhosis, thereby confirming existing hypotheses about the important role of the gut-liver axis in the course of cirrhosis[3,18-21]. This is its second strong point.

The limitation of our study is its small sample size, which did not prevent us from obtaining significant results. It should also be noted that patients with severe hepatic encephalopathy (grades 2-4) are typically not admitted to our clinic, so these patients were not included in our study. The question of whether our results can be transferred

to this cohort of patients remains open. Since patients with infections received antibiotics before admission, which could change the composition of the gut microbiota, we excluded them from the study. None of the included patients developed infectious complications of cirrhosis during hospitalization. Thus, patients with infectious complications of cirrhosis were not included in our study, and it is not clear whether the results can be generalized to them. A larger study involving non-included patient populations should be provided to confirm the findings.

New studies are needed to evaluate how various methods (*e.g.*, probiotics, prebiotics, antibiotics, and fecal transplantation) can correct dysbiosis by analyzing the MDR and how this correction can improve the prognosis of patients with cirrhosis.

## CONCLUSION

In conclusion, we were able to improve the CDR as well as show that gut dysbiosis is associated with poor prognosis in cirrhosis. Thus, we have developed a methodological apparatus and scientific basis for the correction of gut dysbiosis in such patients.

## ARTICLE HIGHLIGHTS

### **Research background**

Gut dysbiosis is common in cirrhosis.

### **Research motivation**

The aim is to study the influence of gut dysbiosis on prognosis in cirrhosis.

### **Research objectives**

The objectives include the development and test of a modified dysbiosis ratio (MDR) to distinguish between patients with cirrhosis and healthy controls, patients with compensated and decompensated cirrhosis, deceased and surviving patients.

### **Research methods**

The case-control study included 48 in-patients with cirrhosis and 21 healthy controls. Stool microbiome was assessed using 16S ribosomal ribonucleic acid gene sequencing. We used MDR:  $[Bacilli (\%) + Proteobacteria (\%)]/[Clostridia (\%) + bacteroidetes (\%)]$ . Patients with MDR more its median made up the group with severe dysbiosis, others did the group with non-severe dysbiosis. The follow-up period was 4 years.

### **Research results**

The mortality rate of patients with severe dysbiosis was significantly higher than that of patients with non-severe dysbiosis. The presence of severe dysbiosis was independent risk factors for death. The deceased patients had a higher MDR value than the survivors. MDR was higher in patients with cirrhosis than in health controls and in patients with decompensated cirrhosis than in patients with compensated cirrhosis.

### **Research conclusions**

Gut dysbiosis is associated with a poorer long-term prognosis in cirrhosis.

### **Research perspectives**

A larger study involving non-included patient populations should be provided to confirm the findings. New studies are needed to evaluate how various methods (*e.g.*, probiotics, prebiotics, antibiotics, and fecal transplantation) can correct dysbiosis by analyzing the MDR and how this correction can improve the prognosis of patients with cirrhosis.

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## Retrospective Study

## Combination of type IV collagen 7S, albumin concentrations, and platelet count predicts prognosis of non-alcoholic fatty liver disease

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Written informed consent was obtained from all the patients.

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**Abstract****BACKGROUND**

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease and affects approximately 25% of the general global adult population. The prognosis of NAFLD patients with advanced liver fibrosis is known to be poor. It is difficult to assess disease progression in all patients with NAFLD; thus, it is necessary to identify patients who will show poor prognosis.

**AIM**

To investigate the efficacy of non-invasive biomarkers for predicting disease progression in patients with NAFLD.

**METHODS**

We investigated biomarkers associated with mortality in patients with NAFLD who visited the Kawasaki Medical School General Medical Center from 1996 to 2018 and underwent liver biopsy and had been followed-up for > 1 year. Cumulative overall mortality and liver-related events during follow-up were calculated using the Kaplan-Meier analysis and compared using log-rank testing. We calculated the odds ratio and performed receiver operating characteristic curve analysis with logistic regression analysis to determine the optimal cut-off value with the highest prognostic ability.

**RESULTS**

We enrolled 489 patients who were followed-up for a period of 1-22.2 years. In total, 13 patients died (2.7% of total patients enrolled); 7 patients died due to liver-related causes. Poor prognosis was associated with liver fibrosis on histological examination but not with inflammation or steatosis. Blood biomarkers associated with mortality were platelet counts, albumin levels, and type IV collagen 7S

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levels. The optimal cutoff index for predicting total mortality was a platelet count of  $15 \times 10^4/\mu\text{L}$ , albumin level of 3.5 g/dL, and type IV collagen 7S level of 5 mg/dL. In particular, only one-factor patients with NAFLD presenting with platelet counts  $\leq 15 \times 10^4/\mu\text{L}$ , albumin levels  $\leq 3.5$  g/dL, or type IV collagen 7S  $\geq 5$  mg/dL showed 5-year, 10-year, and 15-year survival rates of 99.7%, 98.3%, and 94%, respectively. However, patients with two factors had lower 5-year and 10-year survival rates of 98% and 43%, respectively. Similarly, patients with all three factors showed the lowest 5-year and 10-year survival rates of 53% and 26%, respectively.

## CONCLUSION

A combination of the three non-invasive biomarkers is a useful predictor of NAFLD prognosis and can help identify patients with NAFLD who are at a high risk of all-cause mortality.

**Key Words:** Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Platelet count; Albumin; Type IV collagen 7S; All-cause mortality

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**Core Tip:** We investigated biomarkers associated with mortality in non-alcoholic fatty liver disease (NAFLD) patients who underwent liver biopsy. Blood biomarkers associated with mortality were platelet count, albumin levels, and type IV collagen 7S levels. In particular, 5-year and 10-year survival rates were reduced for patients with all three factors: platelet counts below  $15 \times 10^4/\mu\text{L}$ , albumin levels below 3.5 g/dL, and type IV collagen 7S levels more 5 ng/dL. In summary, the combination of the three non-invasive biomarkers is a useful predictor of NAFLD prognosis and helps identify patients with NAFLD who are at high risk of death from all causes.

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease and affects approximately 25% of the general global adult population[1]. The development of NAFLD is associated with lifestyle-related diseases, such as obesity, type 2 diabetes, hypertension, and dyslipidemia. Cardiovascular disease is the leading cause of death among NAFLD patients[2,3]. However, liver-related diseases are also a major cause of death among patients with NAFLD, and liver-specific and all-cause mortality rates are higher for these patients than for the general population NAFLD, and liver-specific and all-cause mortality rates are higher for these patients than for the general population[1]. The incidence of liver-specific and all-cause mortality among patients with NAFLD is generally 0.77 and 11.77 per 1000 years, respectively, while it is 15.44 and 25.56 per 1000 years, respectively, for patients with non-alcoholic steatohepatitis (NASH)[1].

The prognosis of NAFLD patients with advanced liver fibrosis is known to be poor[1,4-8]. Progression of liver fibrosis in patients with NAFLD is associated with mortality from various non-liver-related causes[6].

Liver biopsy is typically performed for diagnosing advanced fibrosis in patients with other liver diseases, such as NASH; however, it is not a practical tool for the diagnosis of NAFLD. In addition, the limitations of liver biopsies, such as invasiveness, poor patient tolerance, sampling variability, and high costs, are well known. Thus, there is increasing interest in developing and validating non-invasive methods for measuring liver stiffness, such as imaging and elastography techniques

based on ultrasonography or magnetic resonance imaging[3,4,9-14]. However, a limitation of these methods is that the images are visualized using an instrument that is not available in many institutions. Therefore, serum biomarkers that can assess the progression of liver fibrosis in patients with NAFLD may serve as important tools for identifying patients with advanced fibrosis. Some biomarkers of interest, such as procollagen type III N-terminal propeptide, type IV collagen 7S, hyaluronic acid, and Mac-2 binding protein [WFA(+)-M2BP] levels, and cytokeratin-18 have been used for identifying patients with NAFLD with advanced fibrosis. Other studies have used different biomarker scores, such as the BARD score, NAFLD fibrosis score, FIB-4 (fibrosis-4) index, aspartate aminotransaminase (AST) to alanine aminotransaminase (ALT) ratio, AST to platelet ratio index, FibroTest, and Enhanced Liver Fibrosis score, for the assessment of liver fibrosis[3,4,11,13,14-24]. However, none of these scores predict the prognosis of NAFLD patients. Hence, we aimed to investigate the efficacy of non-invasive biomarkers for predicting disease progression in patients with NAFLD.

## MATERIALS AND METHODS

### Patients

We retrospectively identified patients with NAFLD who underwent liver biopsy at the Kawasaki Medical School General Medical Center from 1996 to 2018 (Table 1). The exclusion criteria were as follows: history of other liver diseases including hepatitis B virus or hepatitis C virus infections, autoimmune liver diseases, drug-induced liver injury, metabolic liver diseases, or history of alcohol intake (men,  $\geq 30$  g/d and women,  $\geq 20$  g/d). Blood tests were performed before the liver biopsy, and we examined the prognostic factors based on the blood test results. The study protocol complied with the guidelines of the 1975 Helsinki Declaration and was approved by the Institutional Research Ethics Committee. Written informed consent was obtained from all the patients.

### Clinical, biochemical, and histological parameters

We investigated the mortality rate and causes of death among the enrolled patients. We also investigated the development of any complications during the follow-up period. The start date of the follow-up period was defined as the date of liver biopsy and the end date of the follow-up period was defined as the date of last follow-up for surviving patients or the date of death for patients who died during the follow-up period. All NAFLD patients visited our hospital once every 3-6 mo. The following clinical parameters were included in the analysis: age at diagnosis of NAFLD; sex; body mass index calculated as weight (in kg) divided by height (in meters squared); and the presence of diabetes mellitus, hyperlipidemia, and dyslipidemia. We also included the following biochemical parameters in the analysis: platelet count, levels of albumin, total bilirubin, AST, ALT, gamma glutamyl transpeptidase, total cholesterol, cholinesterase, serum iron, ferritin, leptin, adiponectin, and high-sensitivity C-reactive protein, and homeostasis model assessment insulin resistance. The FIB-4 index was calculated as follows:  $\text{age (years)} \times \text{AST (U/L)} / \text{platelet count} (\times 10^4 / \mu\text{L}) \times \sqrt{\text{AST (U/L)}}$ [13,16,17]. Type IV collagen 7S and procollagen III peptide (P-III-P) were used as indicators of liver fibrosis.

### Liver biopsy and histological analysis

All liver biopsies were performed using 16G or 17G biopsy needles with ultrasound guidance or using 14G needles with laparoscopic guidance. The histological examinations were performed by two experienced liver pathologists who were blinded to the patient details. The histological parameters included fibrosis, inflammation, steatosis, hepatocyte ballooning, and the NAFLD activity score (NAS) system[25]. The individual histological features of NAFLD were assessed using the following NAS system proposed by the NASH Clinical Research Network (NASH CRN): lobular inflammation (0-3), steatosis (0-3), and hepatocellular ballooning (0-2)[26,27]. The liver fibrosis stages were assessed according to Brunt's criteria.

### Statistical analysis

The cumulative all-cause mortality and liver-related events during follow-up were assessed using the Kaplan-Meier method and compared using the log-rank test. The Kaplan-Meier analysis included the following variables: steatosis grade, ballooning

**Table 1 Clinical and histological characteristics of the patient population (n = 489)**

Characteristics	Values
Age	50.1 (14-82)
Male sex, %	54.6
Body mass index, kg/m <sup>2</sup>	26.9 (20.8-49.5)
Fibrosis stage, 0/1/2/3/4	65/173/111/122/18
Grade, 0/1/2/3	45/204/178/62
Steatosis, 0/1/2/3	13/158/228/90
NAFLD activity score, < 4/≥ 5	265/224
ALT, IU/L	69 (2-563)
AST, IU/L	43 (13-312)
γ-GTP, IU/L	60 (12-736)
Total bilirubin, mg/dL	0.8 (0.04-2.7)
Total cholesterol, ng/dL	198 (102-317)
Cholinesterase, IU/L	205 (90-337)
Platelet count, × 10 <sup>4</sup> /μL	20.8 (6.6-44.7)
Albumin, g/dL	4.5 (2.5-5.4)
HOMA-IR	2.9 (0.7-22.4)
Iron, μg/dL	119 (13-295)
Ferritin, ng/dL	149 (3.9-983)
Leptin, ng/dL	9.3 (1.1-59.3)
Adiponectin, μg/mL	5.5 (2.0-27.5)
High-sensitivity CRP, mg/dL	0.117 (0.01-1.92)
P-III-P, U/mL	0.7 (0.28-3.8)
Type IV collagen 7S, ng/mL	4.1 (1.9-15)
Hyaluronic acid, ng/mL	28 (9-619)
Fibrosis-4 index	1.29 (0.17-1.29)

NAFLD: Non-alcoholic fatty liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GTP: Gamma-glutamyl transpeptidase; HOMA-IR: Homeostatic model assessment of insulin resistance; CRP: C-reactive protein; P-III-P: Procollagen-III peptide.

grade, NAS category, fibrosis stage, albumin, platelet counts, type IV collagen 7S levels, and FIB-4 index. We also calculated the odds ratio and performed receiver operating characteristic (ROC) curve analysis with logistic regression analysis to determine the cutoff values with the highest predictive ability. The optimal cut-off value was determined based on the Youden index. The prognostic performance of the optimal cutoff value was expressed as the diagnostic specificity, sensitivity, positive predictive value, and negative predictive value, using area under the ROC (AUROC) curve analysis. In univariate (unadjusted) and multivariate (adjusted) analyses, the hazard rate ratio estimates (relative risk) for outcomes were calculated using Cox proportional hazard regression analysis to control for the effect of potential risk factors (confounders) while considering the different follow-up durations. A *P* value < 0.05 was considered significant. All statistical analyses were performed using JMP (version 14.2, SAS system, United States). The statistical methods of this study were reviewed by Akiyoshi Izumi from Asahigawaso Rehabilitation and Medical Center, Okayama.

## RESULTS

### Survival rate

In total, 489 patients were enrolled in the present study; the 5-year survival rate was 98.5%, and the 10-year, 15-year, and 20-year survival rates were 95.4%, 91.9%, and 91.9%, respectively. The follow-up period varied between 1 and 22.2 years (Figure 1). In total, 13 (2.7%) patients died; of these, 7 patients died of liver-related causes [hepatocellular carcinoma (HCC) was observed in 1 patient; Table 2]. The complications that developed during the follow-up period were HCC ( $n = 12$ ), other organ cancers ( $n = 13$ ), and cerebrovascular disorders ( $n = 9$ ).

### Liver histological findings

Patients presenting with progression of advanced liver fibrosis after liver biopsy had increased mortality. The 5-year and 10-year survival rates of patients with NASH CRN Stage 4 disease were 81% and 41%, respectively. However, the degree of inflammation or steatosis was not associated with poor prognosis. The optimal area under the curve for albumin was 3.8 and 3.5 with specificities of 47% and 39%, sensitivities of 95% and 99%, positive predictive values of 98% and 98%, and negative predictive values of 21% and 56% (Figure 2).

### Blood test factors

A univariate Cox hazard model was used for analyzing factors associated with mortality at the time of diagnosis of the NASH Clinical Research Network. We found that the ALT levels, platelet counts, albumin levels, and levels of liver fibrosis markers (P-III-P, type IV collagen 7S and FIB-4 index) were significantly associated with mortality (Table 3).

Survival curves were created using the following biomarkers: type IV collagen 7S, platelet count, albumin, and FIB-4 index. ALT was not included as a biomarker because the levels frequently varied. To investigate the predictive performance of these biomarkers with respect to NAFLD mortality, an optimal COI for type IV collagen 7S level, platelet count, albumin level, and FIB-4 index was determined based on the ROC curve analysis of all 489 patients with NAFLD. As shown in Figure 3A-D, the cutoff values for the platelet count, albumin level, type IV collagen 7S concentration and the FIB-4 index were set at  $15 \times 10^4$ , 3.8 g/dL, and 3.5 mg/dL, 5.0 ng/mL, and 1.3 and 2.61, respectively.

At the time of NASH diagnosis, patients with albumin levels  $< 3.5$  mg/dL, platelet counts  $< 15 \times 10^4$ , type IV collagen 7S levels  $\geq 5$  ng/dL, and FIB-4 indexes  $\geq 2.67$  clearly showed reduced survival (Figure 4A-D). Furthermore, we investigated the prognosis by combining type IV collagen 7S, which had a high AUROC among liver fibrosis markers (type IV collagen 7S, P-III-P, and FIB-4 index), the albumin level, and platelet count. Albumin level  $< 3.5$  mg/dL, platelet count  $< 15 \times 10^4/\mu\text{L}$ , and type IV collagen 7S levels  $\geq 5$  ng/dL were examined individually and in combination. The 5-year, 10-year, and 15-year survival rates for patients with only one factor were 99.7%, 98.3%, and 94%, respectively. However, survival rates were low for patients who presented with more than one factor. For these individuals, the 5-year and 10-year survival rates were 98% and 43%, respectively. For those who presented with two factors, the 5-year and 10-year survival were 53% and 26%, respectively, and for those presenting with three factors (Figure 5).

## DISCUSSION

To the best of our knowledge, this study is the first study to evaluate the predictors of the prognosis of NAFLD based on the results of a blood test. We found that a combination of three non-invasive biomarkers, namely, platelet count, albumin level, and type IV collagen 7S level, is a useful predictor of NAFLD prognosis. The major causes of death in patients with NAFLD are cardiovascular events, organ cancers other than liver cancer, and liver-related disease. Among Japanese patients with NAFLD, the reported mortality rates associated with NAFLD are low during the follow-up period. The causes of death are more likely to be cancers of other organs and cerebral cardiovascular events than liver-related pathologies[28].

The most important predictor of outcomes among patients with NAFLD is the progression of liver fibrosis[1,5-7]. Angulo *et al*[6] retrospectively analyzed the long-term outcomes of 619 patients diagnosed with NAFLD in the United States, Europe, and Thailand during 1975-2005[6] and reported that only liver fibrosis, among various



**Table 2 Summary of the causes of death**

	<i>n</i> (%)
All deaths	13 (2.7)
Liver-related events	7 (1.4)
HCC + liver failure	3
HCC only	1
Liver failure	3
Cerebrovascular disease	1 (0.2)
Non-liver cancers	4 (0.8)
Pancreatic cancer	2
Bile duct cancer	2
Infection	1 (0.2)

HCC: Hepatocellular carcinoma.

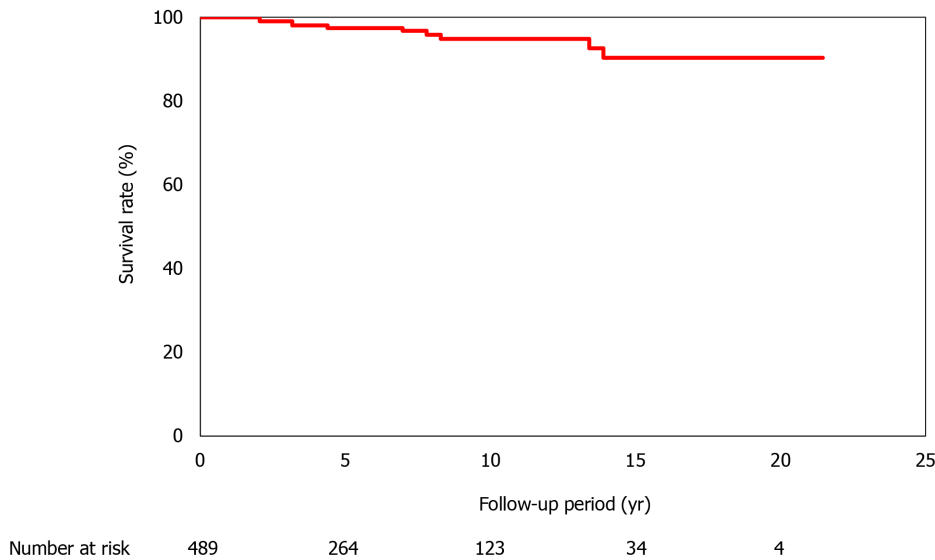
**Table 3 Factors associated with mortality among the patients with non-alcoholic fatty liver disease (*n* = 489)**

	AUROC	Odds ratio	95%CI	<i>P</i> value
AST	0.57	1.00	0.99-1.02	0.9841
ALT	0.71	0.97	0.95-0.99	0.0026
γ-GTP	0.521	1.00	0.99-1.01	0.4259
Platelet count	0.748	0.78	0.69-0.88	< 0.0001
Total bilirubin	0.588	1.10	0.55-1.39	0.3208
Total cholesterol	0.580	0.99	0.98-1.01	0.2
Iron	0.553	1.01	1.02	0.1801
Albumin	0.815	0.093	0.04-0.20	< 0.0001
Ferritin	0.527	1.00	1.00-1.00	0.7651
Leptin	0.565	1.00	0.94-1.06	0.7441
HOMA-IR	0.731	1.04	1.009-1.06	0.0182
P-III-P	0.786	5.58	2.27-11.6	0.0014
Type IV collagen 7S	0.863	1.48	1.28-1.67	< 0.0001
Fibrosis-4 index	0.914	1.799	1.44-2.23	< 0.0001

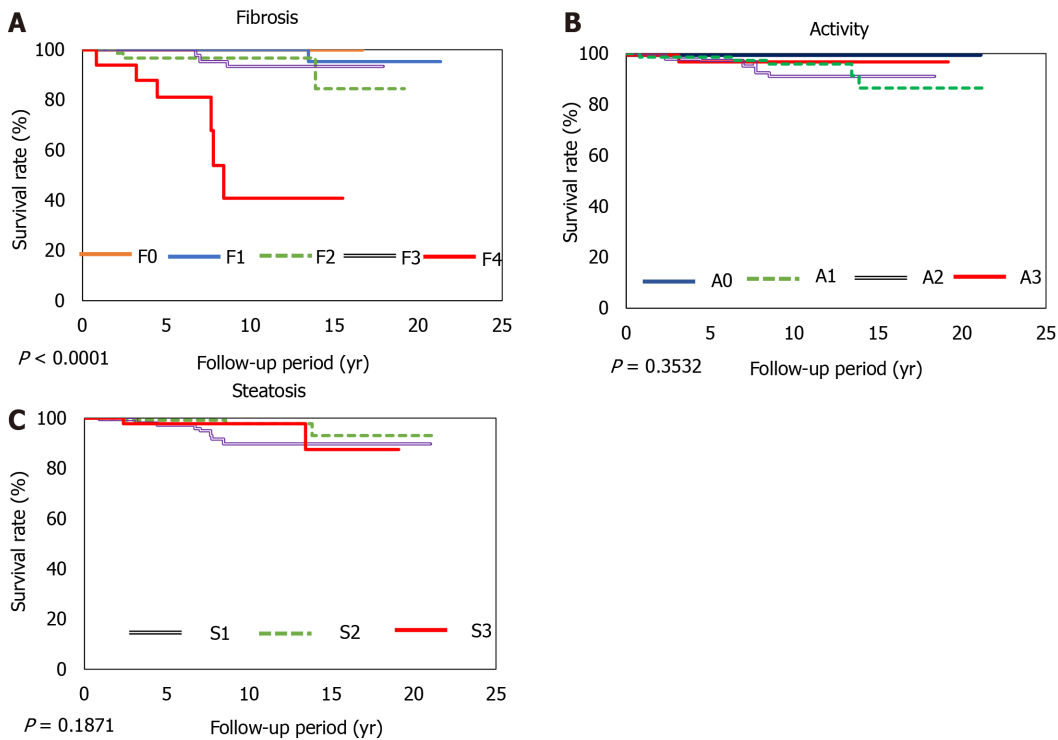
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; γ-GTP: Gamma-glutamyl transpeptidase; HOMA-IR: Homeostatic model assessment of insulin resistance; P-III-P: Procollagen-III peptide; AUROC: Area under the receiver operating characteristic curve.

longitudinal histological features, was associated with disease prognosis. Only liver fibrosis was independently associated with long-term all-cause mortality, liver transplantation, and liver-related events. Meta-analyses have also reported that liver fibrosis is an important risk factor for liver-related mortality[1,7]. Compared with NAFLD patients without fibrosis, NAFLD patients with fibrosis were at an increased risk of all-cause mortality, and the risk increased as fibrosis progressed[7]. In our study, patients with advanced liver fibrosis, especially cirrhosis, also showed poor prognosis; however, an association with inflammation, steatosis, or ballooning was not noted. Our findings further confirm that the progression of fibrosis markedly affects the prognosis of patients with NAFLD.

Several biomarkers can be used to evaluate liver fibrosis in patients with NAFLD[3,4,11,13,14-25,29]; however, previous studies have not examined disease prognosis using blood biomarker levels recorded at the time of NAFLD diagnosis



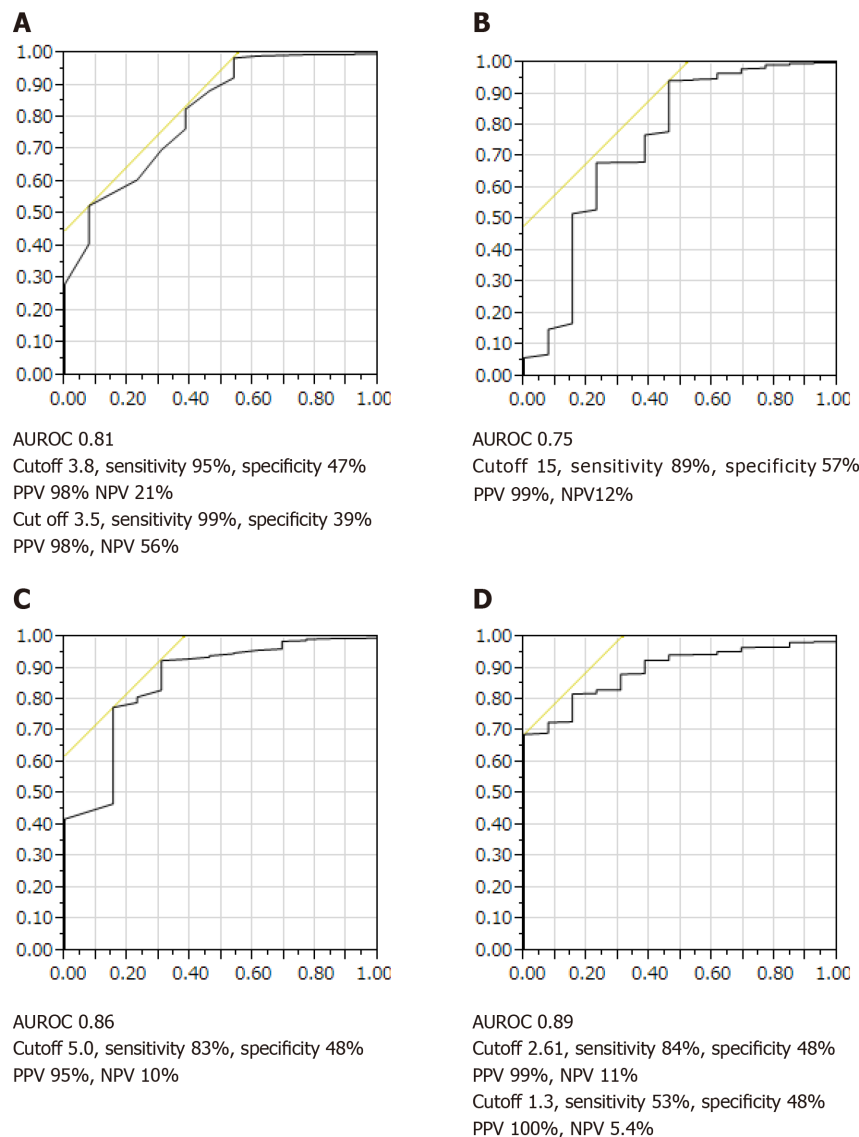
**Figure 1** Survival of the 489 patients with non-alcoholic fatty liver disease. The follow-up period varied between 1 yr and 21.2 yr, and all-cause mortality was considered. The survival rates are 98.5% at 5 yr, 95.4% at 10 yr, 91.9% at 15 yr, and 91.9% at 20 yr.



**Figure 2** Survival rates according to the grading of fibrosis, inflammation, and steatosis. The overall survival rates for stage 4 liver fibrosis are 81% at 5 yr and 41% at 10 yr. A: Fibrosis (F0-4); B: Inflammation (A0-3); C: Steatosis (S1-3).

using liver biopsy.

NAFLD may progress rapidly in some patients and slowly in other patients. Singh *et al*[5] performed a systematic review and meta-analysis of 11 paired biopsy cohort studies that included 411 patients with > 2145 person-years of follow-up data and reported that approximately 30% of the patients developed advanced fibrosis and 70% of the patients remained stable or the stage of fibrosis in these patients improved. Furthermore, the annual fibrosis progression rates were 0.07 stages for patients with NAFLD and 0.14 stages for patients with NASH. Nasr *et al*[30] conducted a biochemical, clinical, and histological analysis of 129 patients with NAFLD who were enrolled between 1988 and 1993 in a prospective cohort study and followed them for 19.8 years. They reported that end-stage liver disease developed in 12 (9.3%) patients and advanced fibrosis developed in 34% of the patients. Furthermore, among the 113

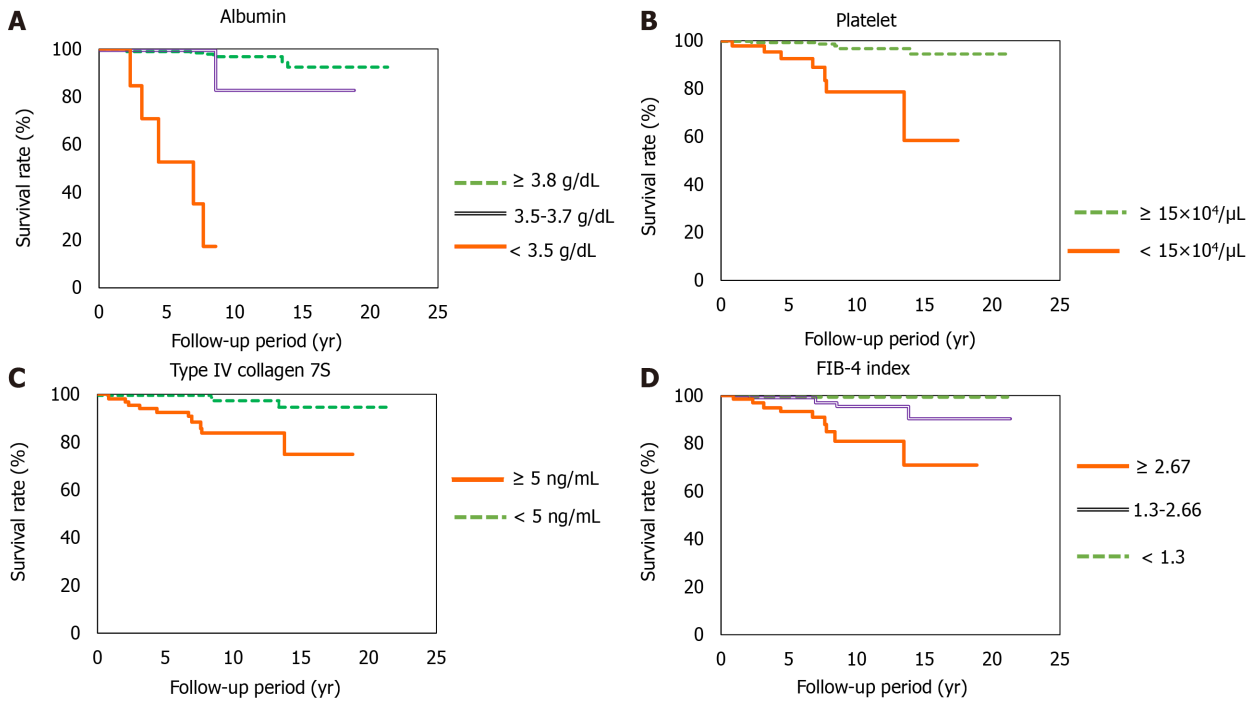


**Figure 3 Receiver-operating characteristic curves for survival among patients with non-alcoholic fatty liver disease.** A: Albumin concentration; B: Platelet count; C: Type IV collagen 7S concentration; D: Fibrosis-4 index. AUROC: Area under the receiver operating characteristic curve, PPV: Positive predictive value, NPV: Negative predictive value.

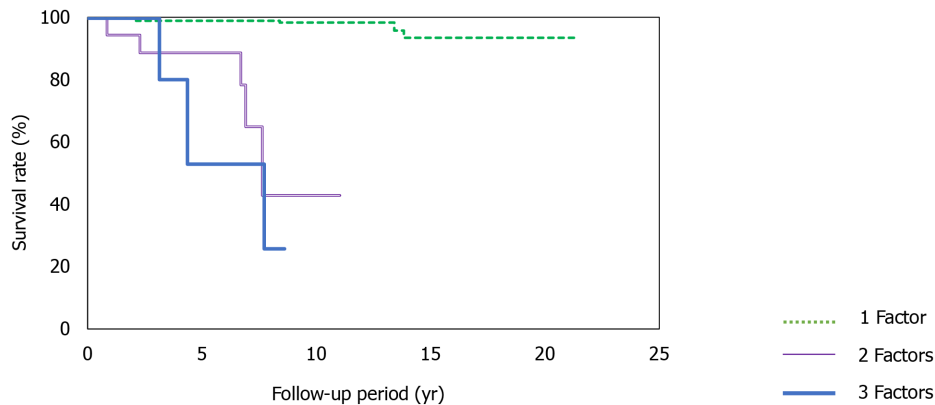
patients with low baseline fibrosis (stage 3), 16% of the patients developed advanced fibrosis. No differences in clinical, histological, or biochemical variables were observed between patients who developed liver fibrosis and those who did not. These studies did not examine the association of *PNPLA3* polymorphisms with menopause. Although the difference in the progression of NASH and NAFLD is not clear, racial differences and genetic factors, including *PNPLA3* expression[31], weight gain, onset and deterioration of diabetes[32], sex differences, and menopausal factors, affect prognosis[33].

It is necessary to consider the various factors that affect disease progress in each case of NAFLD. Although several studies have reported on the evaluation of biomarkers and elastography methods that can predict the progression of liver fibrosis[3,4,11,13,14-25], non-invasive biomarkers that can easily predict the prognosis of NAFLD have not been identified to date.

Our results indicate that patients with NAFLD who present with a combination of albumin level  $< 3.5$  g/dL, platelet count  $< 15 \times 10^4/\mu\text{L}$ , and type IV collagen 7S level  $\geq 5$  ng/mL show poor prognosis. In particular, the 10-year survival rate was only 43% for patients who presented with all three factors. We observed that type IV collagen 7S was a more useful indicator of advanced liver fibrosis than other biomarkers (Table 3). Yoneda *et al*[24] reported that the type IV collagen 7S level is a more useful marker of prognosis for patients with advanced fibrosis associated with NASH than for patients with mild fibrosis. Furthermore, a scoring system that uses type IV collagen 7S and AST levels, named the CA index, has been reported to predict NASH and fibrosis



**Figure 4 Survival rates.** A: Albumin concentration (albumin  $\geq 3.8$  g/dL vs 3.5-3.7 g/dL;  $P < 0.001$ , albumin  $\geq 3.8$  g/dL vs  $< 3.5$  g/dL;  $P < 0.0001$ , albumin 3.5-3.7 g/dL vs  $< 3.5$ ;  $P < 0.0001$ ); B: Platelet count (platelet  $\geq 15 \times 10^4/\mu\text{L}$  vs  $< 15 \times 10^4/\mu\text{L}$ ;  $P < 0.0001$ ); C: Type IV collagen 7S concentration (type IV collagen 7S  $\geq 5$  ng/mL vs  $< 5$  ng/mL;  $P < 0.0001$ ); D: Fibrosis-4 index (Fibrosis-4 index  $\geq 2.67$  vs 1.3-2.67;  $P < 0.001$ , Fibrosis-4 index 1.3-2.67 vs  $< 1.3$ ;  $P < 0.0001$ , Fibrosis-4 index  $\geq 2.67$  vs 2.67;  $P < 0.0001$ ). FIB: Fibrosis.



Number at risk

0 Factor	353	189	90	24	4
1 Factor	107	58	29	10	0
2 Factors	22	13	4	0	0
3 Factors	7	4	0	0	0

**Figure 5 Survival rates according to positivity for the different biomarkers.** Patients with only one risk factor have relatively good survival rates at 5 yr (99.7%), 10 yr (98.3%), and 15 yr (94%). However, patients with two risk factors have lower survival rates at 5 yr (98%) and 10 yr (43%), and patients with all three risk factors have even lower survival rates at 5 yr (53%) and 10 yr (26%) (1 factor vs 2 factors,  $P < 0.0001$ ; 1 factor vs 3 factors,  $P < 0.0001$ ; 2 factors vs 3 factors;  $P < 0.05$ ).

associated with NAFLD with sufficient accuracy, thus allowing for convenient diagnosis and screening of NASH and associated fibrosis[21]. The same index was found to be useful in 400 Japanese patients from 18 institutes with biopsy-proven NAFLD and advanced liver fibrosis due to CA or FA fibrosis. The CA index is a combination of AST and type IV collagen 7S levels, and the FM fiber index includes type IV collagen 7S and hyaluronic acid levels and vascular cell adhesion[25]. The type

IV collagen 7S level is useful for determining advanced fibrosis in patients with NASH and was found to be more sensitive and specific than other fibrosis markers assessed in our study.

Albumin is also an important biomarker for predicting the prognosis of HCC in patients with NAFLD. Kawaguchi *et al*[34] analyzed the factors affecting survival by performing a random forest analysis for 247 NAFLD-HCC patients diagnosed between 2000 and 2014 and recruited from 17 medical institutions in Japan. The results showed that the best prognostic profile for patients with NAFLD-HC comprised treatment for HCC and serum albumin levels > 3.7 g/dL.

There are some limitations of this study. We did not classify prognosis according to all-cause mortality; moreover, the study population comprised patients from a single center. Nevertheless, it is significant that the study followed a long-term course of up to 20 years.

In our study, the platelet count, albumin level, type IV collagen 7S level, and the FIB-4 index were important prognostic factors at the time of diagnosis of NAFLD. Our findings suggest that these factors should be recorded in patients with NAFLD at the time of diagnosis to determine future treatment strategies.

Studies conducted in the future should focus on assessing these biomarkers further and examining long-term prognosis using Fibroscan and magnetic resonance elastography. Further research is also needed to confirm these findings in other populations.

## CONCLUSION

This study may prove useful in clinical practice because simple predictors of NAFLD progression, namely, albumin level, platelet count, and type IV collagen 7S level, were identified; all these parameters can be easily assessed in daily practice.

## ARTICLE HIGHLIGHTS

### Research background

Non-alcoholic steatohepatitis has few symptoms until it progresses; thus, it is necessary to identify non-alcoholic fatty liver disease (NAFLD) patients who will show poor prognosis.

### Research motivation

The limitations of liver biopsies, such as invasiveness, poor patient tolerance, sampling variability, and high costs, are well known. Thus, there is increasing interest in developing and validating non-invasive methods for measuring liver stiffness. However, many current methods involve instruments that are not available in many institutions.

### Research objectives

Serum biomarkers that can assess the progression of liver fibrosis in patients with NAFLD may serve as important tools for identifying patients with advanced fibrosis. We aimed to investigate the efficacy of non-invasive biomarkers for predicting disease progression in patients with NAFLD.

### Research methods

We investigated biomarkers with predictable prognosis for NAFLD patients who underwent liver biopsy. All patients were followed-up for > 1 year.

### Research results

The combination of three non-invasive biomarkers involved in NAFLD prognosis comprised platelet counts, albumin levels, and type IV collagen 7S. Our results indicate that patients with NAFLD who present with a combination of albumin levels < 3.5 g/dL, platelet counts <  $15 \times 10^4/\mu\text{L}$ , and type IV collagen 7S levels  $\geq 5 \text{ ng/mL}$  show poor prognosis. In particular, the 10-year survival rate was only 43% for patients who presented with all three factors.



### Research conclusions

The combination of platelet count, albumin level, and type IV collagen 7S was useful in further predicting the prognosis of NAFLD.

### Research perspectives

Studies conducted in the future should focus on assessing these biomarkers further and examining long-term prognosis.

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## Retrospective Study

## Surgical treatment outcomes of primary hepatic sarcomas: A single-center experience

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**Institutional review board**

**statement:** The study was reviewed and approved by the IRB of Samsung Medical Center (IRB number 2020-09-077).

**Informed consent statement:**

Acquiring participant's consent seems to be realistically impossible and does not influence integrity of research. And there would be no reasons that participant would deny providing his or her consent; research involves no more than minimal risk to the patients. Therefore, the IRB of Samsung Medical Center approved that the participant's consent can be

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## Abstract

**BACKGROUND**

Primary hepatic sarcoma is a rare tumor originated from mesenchymal tissue. There are various pathologic types of primary hepatic sarcoma and the treatment outcome of this tumor was usually disappointing. Unlike hepatocellular carcinoma, outcome of primary hepatic sarcoma is not well-known due to its rarity. However, with development of medical technology, surgical treatment may lead to better survival.

**AIM**

To investigate the surgical outcomes of primary hepatic sarcoma, we gathered and analyzed the cases of a single institute.

**METHODS**

From August 2001 to September 2016, a total of nine patients were surgically treated for primary hepatic sarcoma after exclusion of cases with open and closure, early loss to follow-up and sarcomatoid hepatocellular carcinoma and sarcomatoid cholangiocellular carcinoma. Baseline characteristics, tumor characteristics such as tumor pathology, size and number, surgical and adjuvant treatments were reviewed. Tumor recurrence, and patient survival were analyzed with retrospective approach.

**RESULTS**

The enrolled participants included five patients with angiosarcoma and four patients with undifferentiated sarcoma. All patients experienced tumor recurrence at a median of 52 post-operative days. Only two patients survived and the 5-year survival rate was 29.6%. One patient with angiosarcoma who received central

waivered.

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hepatectomy for primary tumor and received radiofrequency ablation for recurrent tumor still lives for 11 years. One patient with undifferentiated sarcoma received Rt. lobectomy for primary tumor followed by chemotherapy and radiation therapy still lives around 30 mo even though she got additional operation for recurrent tumor. Two patients who received living donor liver transplantation due to angiosarcoma died. Only adjuvant therapy was associated with survival gain ( $P = 0.002$ ).

## CONCLUSION

Patients with primary hepatic sarcoma may gain survival benefit with surgical resection followed by adjuvant therapy, even though the outcome remains relatively poor.

**Key Words:** Liver; Angiosarcoma; Undifferentiated sarcoma; Operation; Survival; Recurrence

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**Core Tip:** This is a retrospective study to analyze the outcomes of primary hepatic sarcoma. A total of nine patients were included, five of them are with angiosarcoma and four are with undifferentiated sarcoma. While all patients experienced tumor recurrence, one patient with angiosarcoma and another patient with undifferentiated sarcoma still survive for 11 years and 30 mo respectively, after receiving effective local treatment for recurrent tumors. Although the outcome of primary hepatic sarcoma is known to be poor, surgical treatment with appropriate adjuvant therapy may support the long-term survival.

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## INTRODUCTION

Sarcoma is a malignant tumor that usually arises from mesenchymal tissue. Most sarcomas originate in the extremities, and the prognosis of sarcoma in these sites is well known due to its prevalence[1]. Primary hepatic sarcoma is a rare and aggressive tumor with poor outcomes. Most malignant tumors in the liver are hepatocellular carcinomas (HCC); hepatic sarcomas represent less than 1%[2]. Hepatic sarcoma has various pathologic types, including angiosarcoma, undifferentiated (embryonal) sarcoma, leiomyosarcoma, and epithelioid hemangioendothelioma, among others. For most of these tumors, the cause is still unknown, and there are usually no specific symptoms until abdominal pain presents due to the effect of the mass increasing in size[3,4]. The various types, the rarity, and the difficulty in diagnosis of primary hepatic sarcoma results in various prognoses, thereby making it difficult to set a treatment plan. The aim of our study is to analyze the outcomes of primary hepatic sarcoma following surgical resection in a single institute.

## MATERIALS AND METHODS

### Patient selection

From August 2001 to September 2016, a total of 43 patients underwent surgical treatment for primary hepatic sarcoma at Samsung Medical Center, South Korea. These patients were selected by searching the word “sarcoma” in liver pathological report through all time of our institute. Open and closure, inadequate medical chart, and sarcomatoid HCC or cholangiocellular carcinoma cases were excluded. Six early-follow up loss patients who were treated with resection in our center and then



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transferred to other hospitals were also excluded. Four patients who did not want additional therapy and did not come to outpatient clinic were also excluded. Data was collected by retrospective approach.

Patient age, sex, and pre-operative blood tests such as total bilirubin, aspartate transaminase, alanine aminotransferase, alpha fetoprotein, and CA 19-9 were evaluated. Operation types were reviewed, and pathological diagnosis, tumor size and number were evaluated. Disease recurrence was evaluated using computed tomography or positron emission tomography scan. Patient death was the primary end point.

### **Operation and adjuvant therapies**

Operation type was decided by tumor size, location and liver function and the target of operation was R0 (microscopically margin-negative) resection. However, patients with R1 (macroscopically no remnant tumor, but margin-positive microscopically) and R2 (seen remnant tumor) resection were also included in the study population. Patients usually received chemotherapy when the pathologic report was diagnosed as primary hepatic sarcoma, unless the patient was in poor general condition and could not endure chemotherapy. For cases of recurrence or metastasis of cancer at the vertebrae, radiation therapy (RT) was performed. Resectable recurrent tumors were excised surgically. For small recurrences in the liver, radiofrequency ablation (RFA) could also be performed.

### **Statistical analysis**

Baseline characteristics and tumor markers were compared using the Mann-Whitney test. Cox proportional hazards regression analysis and the Kaplan-Meier method were used to analyze disease-free survival, overall survival and corresponding risk factor. The independent variables were age, sex, tumor size and number, tumor markers and adjuvant therapy. Disease free-survival and overall survival was compared according to pathologic type of sarcoma and adjuvant therapy due to known different prognosis of each sarcoma type. Statistical analysis was executed using IBM SPSS-24 statistical program (IBM Institute, NY, United States).

## **RESULTS**

### **Baseline characteristics**

After exclusion of open and closure, early loss to follow-up, inadequate medical chart, and sarcomatoid HCC or cholangiocellular carcinoma among 43 patients, total nine patients with pure hepatic sarcomas were involved in this study (Figure 1). According to pathological diagnoses, we divided the patients into an angiosarcoma group ( $n = 5$ ) and an undifferentiated sarcoma group ( $n = 4$ , Table 1). Baseline characteristics of each group showed no statistical differences in any variables including tumor size, number of tumors, and tumor markers (Table 2). Median size of the largest tumor was 13 cm. Median age of the patients was 57, and only one patient was pediatric (a 2-year-old female).

### **Angiosarcoma group**

Among the five patients with angiosarcoma, only one patient survived. Median survival duration was 13 mo, and median disease-free survival was 53 d. Two patients (a 57-year-old male and a 52-year-old male, case 1 and 3, respectively) could not receive adjuvant chemotherapy due to poor general condition and expired relatively early (66 d and 127 d, respectively). The pediatric patient (case 2) underwent living donor liver transplantation (LDLT) for angiosarcoma and was diagnosed with multiple bone metastasis at the extremities on post-operative day 53. She received six cycles of ifosfamide/carboplatin/etoposide chemotherapy and expired at post-operative 31 mo. Case 5, a 60-year-old male, received LDLT and experienced recurrence at the liver and metastasis at the vertebra and rib at post-operative 11 mo. He received palliative RT on the bone metastases and expired at post-operative 13 mo.

Case 7, a 62-year-old male who received central hepatectomy for a 2 cm angiosarcoma on segment 8, is still alive after 11 years (Figure 2A). The pathologic resection margin had no cancer cells, and the tumor was the infiltrative type without invasion to any other organs. After operation, the patient experienced recurrence on segment 2 with a 0.9 cm tumor. Successful RFA was done, and he has been cancer-free for six years.

Table 1 Characteristics of the study population

Case	Sex	Age	Pathology	Size (cm)	Operation, R Status	Adjuvant therapy	Recurrence (mo)	Follow-up (mo)	Outcomes
1	M	57	HAS	13.5	Lt. lobectomy, R0		1.7	2.2	Dead
2	F	2	HAS	21	Living donor LT, R0	CTx <sup>1</sup>	1.8	31.2	Dead
3	M	52	HAS	7.3	Rt. Trisectionectomy, R0		1.2	4.2	Dead
4	F	48	UDS	13	Rt. lobectomy, R0	CTx <sup>2</sup>	1.6	68.2	Dead
5	M	60	HAS	2.4	Living donor LT, R0	RT	11.0	13.4	Dead
6	F	53	UDS	25	Rt. Trisectionectomy, R2		0	1.3	Dead
7	M	62	HAS	2	Central hepatectomy, R0	RFA	59.9	135.1	Alive
8	F	60	UDS	11.5	Rt. lobectomy, R0	CTx, RT, Exc <sup>3</sup>	14.6	29.9	Alive
9	M	60	UDS	24	Rt. lobectomy and Rt. Npx, R0	CTx <sup>4</sup>	1.2	9.9	Dead

<sup>1</sup>ICE (ifosfamide/carboplatin/etoposide) 6 cycles.

<sup>2</sup>VIP (etoposide/ifosfamide/cisplatin) 5 cycles, AI (doxorubicin/ifosfamide) 5 cycles, docetaxel/gemcitabine 2 cycles.

<sup>3</sup>VIP (etoposide/ifosfamide/cisplatin) 6 cycles, RT on vertebral metastasis, abdominal wall metastatic tumor excision.

<sup>4</sup>AI (doxorubicin/ifosfamide) 4 cycles, docetaxel/gemcitabine 1 cycle. HAS: Hepatic angiosarcoma; UDS: Undifferentiated sarcoma; Npx: Nephrectomy; CTx: Chemotherapy; RT: Radiation therapy; RFA: Radiofrequency ablation; Exc: Excision.

Table 2 Characteristics of the groups

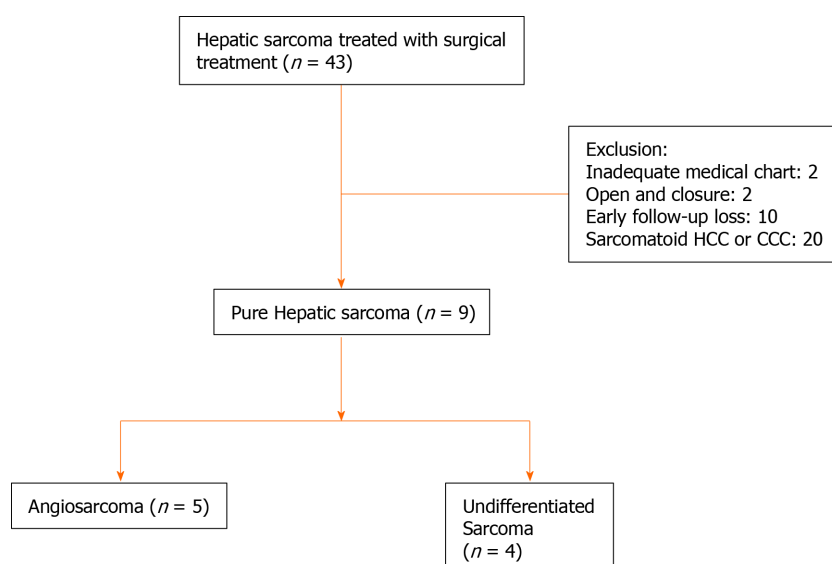
	Total (n = 9)	Angiosarcoma (n = 5)	Undifferentiated sarcoma (n = 4)	P value
Age (range) <sup>1</sup>	57 (2-62)	57 (2-62)	56 (48-60)	0.999
Sex, male (%)	5 (55.6)	4 (80)	1 (25)	0.120
Largest tumor size (cm) <sup>1</sup>	13.0	7.3	18.5	0.142
Tumor number <sup>1</sup>	1.0	2	1	0.371
AFP <sup>1</sup>	2.8	2.6	2.8	0.999
CA19-9 <sup>1</sup>	13.2	3.2	1970	0.180
Recurrence (%)	9 (100)	5 (100)	4 (100)	0.999
Disease-free survival days <sup>1</sup>	52 (0-1798)	53 (36-1798)	35 (0-439)	0.221
Death (%)	7 (77.8)	4 (80)	3 (75)	0.866
Survival days <sup>1</sup>	402	402	596	0.806

<sup>1</sup>The numbers are median value. AFP: Alpha fetoprotein.

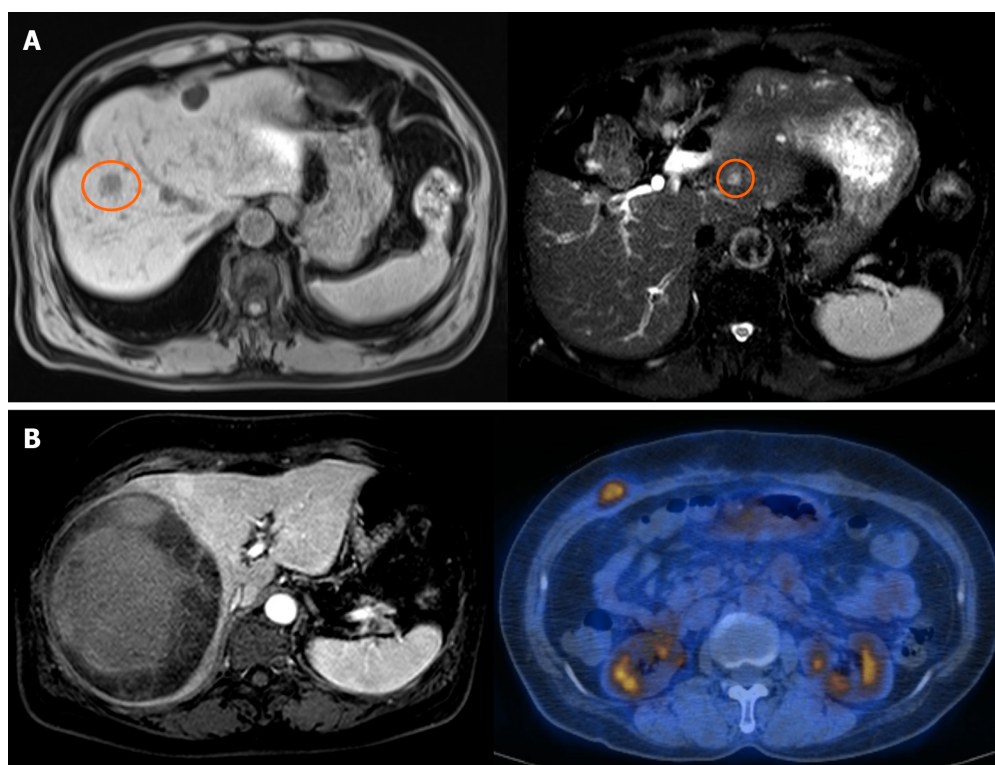
### Undifferentiated sarcoma group

Among the four patients with undifferentiated sarcoma, only one patient survived. Median survival duration was 20 mo, and median disease-free survival was 53 d. Case 6, a 53-year-old female, received Rt. trisectionectomy for a 25 cm undifferentiated sarcoma associated with partial cholangiocellular carcinoma. The tumor was partially ruptured, and a cytology test of ascites was positive for malignant cells with two regional lymph node metastases. The patient expired at post-operative 40 d before receiving chemotherapy. Case 9, a 60-year-old male, received Rt. lobectomy, Rt. nephrectomy, and diaphragm resection due to a 24 cm undifferentiated sarcoma, followed by a diagnosis of lung metastasis at post-operative 35 d. He received four cycles of AI regimen (doxorubicin/ifosfamide) and one cycle of docetaxel/gemcitabine chemotherapy until he expired at post-operative 10 mo.

Case 4, a 48-year-old female, received Rt. lobectomy due to a 13 cm undifferentiated sarcoma. The tumor had already penetrated to the liver capsule and had a high mitotic count (10/10 HPFs), and tumor emboli were in the Rt. portal vein. Multiple tumors recurred on the liver resection margin and the remnant liver at post-operative 49 d.



**Figure 1 Flow chart of selecting pure hepatic sarcoma.** 5 cases of angiosarcoma and 4 cases of undifferentiated sarcoma were included. HCC: Hepatocellular carcinoma; CCC: Cholangiocellular carcinoma.



**Figure 2 Images of a surviving patient (cases 7 and 8).** A: Case 7. Left: T1 magnetic resonance imaging (MRI) of pre-operative angiosarcoma on S8 (orange circle). Right: T2 MRI of recurrence on S2 five years after central hepatectomy; B: Case 8. Left: MRI of pre-operative sarcoma. An 11.5 cm circumscribed mass with hemorrhage on the Rt. lobe. Right: Positron emission tomography-computed tomography of the recurrent mass. Focal fluoro-deoxyglucose uptake at the Rt. anterior abdominal wall.

The patient received multiple series of chemotherapy, including five cycles of VIP regimen (etoposide/ifosfamide/cisplatin) followed by five cycles of AI regimen (doxorubicin/ifosfamide) and two cycles of docetaxel/gemcitabine. The patient survived 68 mo until she died due to tumor progression, lung metastasis, and liver abscess.

Case 8, a 60-year-old female who received Rt. lobectomy for an 11.5 cm undifferentiated sarcoma, is still alive after 30 mo (Figure 2B). The tumor had high cellularity, moderate pleomorphism, and tumor necrosis of more than 50% with a negative resection margin. After operation, the patient received six cycles of chemotherapy with

the VIP regimen (etoposide/ifosfamide/cisplatin), followed by RT to the pre-operatively diagnosed vertebral metastasis (1.5 cm tumor at T9). She had no cancer recurrence until abdominal metastasis was detected at post-operative 14 mo. The metastatic tumor was 2 cm, located between the abdominal investing fascia and the external oblique muscle. After a wide excision, the patient has been cancer-free for 30 mo.

### Cancer recurrence

All patients experienced recurrence of the primary cancer. Median disease-free survival was 52 d. Because one patient with angiosarcoma had tumor recurrence in the Left lobe at post-operative 4 years and 11 mo, the disease-free survival of angiosarcoma looks higher than that of undifferentiated sarcoma; however, this was not statistically significant (Figure 3). The age, sex, pathology type, and tumor markers showed no statistical influence on cancer recurrence (Table 3). Only the largest tumor size was associated with higher cancer recurrence, but only in univariate analysis (HR = 1.13,  $P = 0.49$ ) and not in multivariate analysis.

### Patient death

Among the total of nine patients, only two patients survived (one with angiosarcoma, one with undifferentiated sarcoma). The 5-year survival rate was 29.6% (Figure 4). There was no significant difference between survival of the angiosarcoma and undifferentiated sarcoma groups. Pathologic type, largest tumor size, number of tumors, and tumor markers did not influence patient death with univariate analysis (Table 4). Only adjuvant therapy had an effect on patient survival. The 5-year survival of patients who received adjuvant therapy was 44.4%, while all patients without adjuvant therapy expired within 1 year (Figure 5).

## DISCUSSION

In our study, all patients who received surgical treatment for primary hepatic sarcoma had tumor recurrence, and the 5-year survival rate was relatively low (29.6%). However, one patient with angiosarcoma is still alive after 11 years, and one with undifferentiated sarcoma is still alive after 30 mo.

With recent medical advances, survival outcomes after surgical resection of primary hepatic sarcoma are slightly increasing. One study with 22 patients who received primary surgical treatment showed a 5-year survival rate of 65.2%[5]. However, that study population included various sarcoma types, including rhabdomyosarcoma, leiomyosarcoma, and hemangiopericytoma, while the five cases of angiosarcoma had much poorer outcomes with only one patient surviving for six months. Another review article including 64 cases of hepatic angiosarcoma showed a median survival time of five months[6]. In this article, median survival of patients who received local excision alone or local excision combined with chemotherapy was 17 mo. In our study, the angiosarcoma group had a median survival of 13 mo, with a 5-year survival rate of 20%. One patient who received central hepatectomy and RFA for recurrence at the Lt. lateral section still lives after 11 years.

Hepatic undifferentiated sarcoma is also known to have poor outcomes[7]. Sometimes, it is misdiagnosed as other cystic tumor on pre-operative images and revealed as undifferentiated sarcoma on pathologic review after surgical resection[8,9]. However, it may have better outcomes compared to angiosarcoma when surgically resected. A recent review article including 271 patients who received partial hepatectomy showed a 5-year survival rate of 70%[10]. The 5-year survival rate for the undifferentiated sarcoma group in our study was 50%. One patient who still lives, received Right lobectomy for an 11.5 cm tumor, followed by chemotherapy and RT for vertebral metastasis. About 14 mo later, she underwent a local excision at the abdominal wall metastasis and has maintained a disease-free state for 15 mo. A 48-year-old female patient who received Rt. lobectomy followed by chemotherapy lived more than five years but expired at 5 years and 7 mo due to tumor cachexia.

Even though the outcome of surgical resection for primary hepatic sarcoma is not ideal, surgical resection is still considered to be a better treatment than chemotherapy alone. In a study with 30 primary hepatic sarcoma patients, those who received R0-surgical resection experienced much better outcomes than those who did not, except for patients with the specific pathologic type of epithelioid hemangioendothelioma[2]. Another study in which 6 patients received transcatheter arterial chemoembolization or transcatheter arterial embolization alone for hepatic angiosarcoma resulted in all

**Table 3 Risk factors for cancer recurrence**

	Univariate HR (95%CI)	P value	Multivariate HR (95%CI)	P value
Sex (male)	0.82 (0.20-3.31)	0.779		
Age	0.99 (0.96-1.03)	0.694		
Pathology (HAS/UDS)	0.50 (0.12-2.07)	0.339	0.63 (0.11-3.55)	0.602
Largest tumor size	1.13 (1.00-1.27)	0.049	1.12 (0.97-1.28)	0.115
Tumor number	1.28 (0.79-2.07)	0.311		
AFP	1.02 (0.77-1.33)	0.917		
CA 19-9	1.00 (1.00-1.00)	0.527		
Adjuvant therapy	0.24 (0.04-1.47)	0.121	0.25 (0.04-1.71)	0.157

HAS: Hepatic angiosarcoma; UDS: Undifferentiated sarcoma; AFP: Alpha fetoprotein; CI: Confidence interval; HR: Hazard ratio.

**Table 4 Risk factors for patient death**

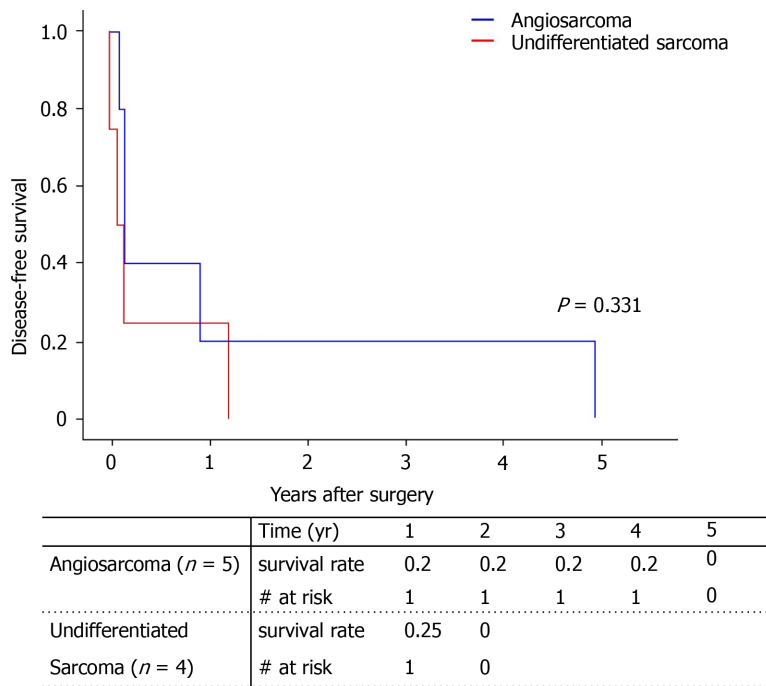
	Univariate HR (95%CI)	P value
Sex (male)	1.36 (0.30-6.24)	0.693
Age	1.00 (0.96-1.04)	0.922
Pathology (HAS/UDS)	0.99 (0.22-4.47)	0.989
Largest tumor size	1.08 (0.97-1.20)	0.179
Tumor number	1.28 (0.79-2.07)	0.311
AFP	0.99 (0.74-1.31)	0.922
CA 19-9	1.00 (0.99-1.01)	0.677
Adjuvant therapy	0.00 (0.03-2779)	0.002

HAS: Hepatic angiosarcoma; UDS: Undifferentiated sarcoma; AFP: Alpha fetoprotein; CI: Confidence interval; HR: Hazard ratio.

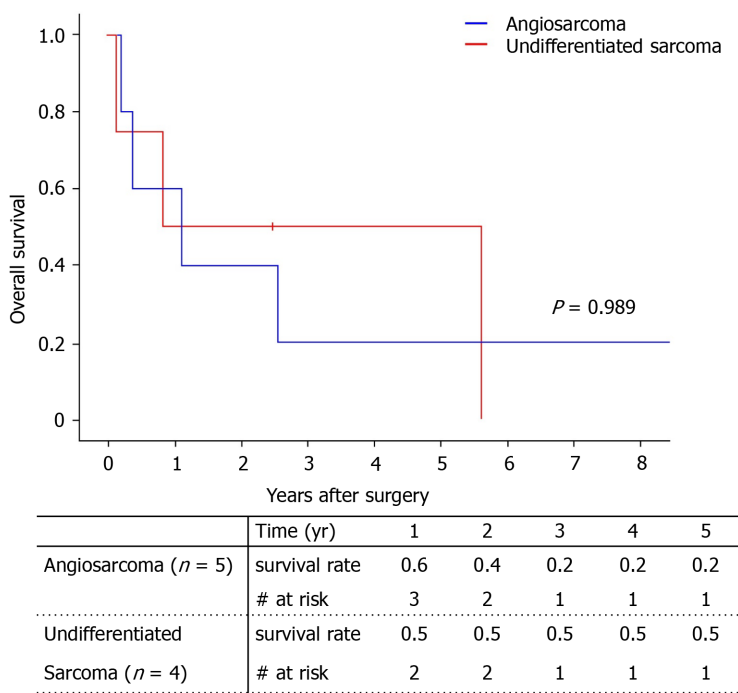
patients expiring within 1 year[11]. A recent study of 8 patients with R0-resected hepatic angiosarcoma showed median survival and disease-free survival of 59 and 11 mo which emphasizes the radical surgical resection is best approach for long-term survival[12].

Still, adjuvant chemotherapy after resection is recommended for hepatic sarcoma. In cases of angiosarcoma, one study showed that a combination of surgical treatment and adjuvant chemotherapy may be beneficial[13]. A review article with 64 cases of angiosarcoma suggested that surgery with chemotherapy is the optimal choice for survival[6]. May *et al*[14] studied five pediatric patients with hepatic undifferentiated sarcoma who underwent a uniform approach of local resection and vincristine, actinomycin-D, cyclophosphamide. All patients survived with median survival of 53 mo. Lenze *et al*[15] described 14 patients with undifferentiated sarcoma who remained alive for a median of 28.5 mo after receiving both surgical resection and adjuvant chemotherapy, which was a significantly better outcome compared to patients without adjuvant chemotherapy. However, the optimal regimen of chemotherapy for hepatic sarcoma has not yet been established. Kim *et al*[16] showed that palliative chemotherapy may be beneficial to survival even if the hepatic angiosarcoma is unresectable. Transarterial chemoembolization showed some effectiveness in acute intra-abdominal hemorrhage of hepatic angiosarcoma cases[17]. In our study, three patients who did not receive adjuvant therapy had poorer survival than patients who received adjuvant therapy. However, two of them could not receive chemotherapy due to poor general condition (angiosarcoma patients), and one patient expired before scheduled chemotherapy (undifferentiated sarcoma). There is a case report of immunotherapy about a patient with primary hepatic angiosarcoma with multiple liver metastasis treated by pazopanib plus procedural death factors-1 inhibitor and RAK cells showing stable disease after treatment[18]. Although this is only one case report, this study showed a hope of new era of treatment which may aid surgical





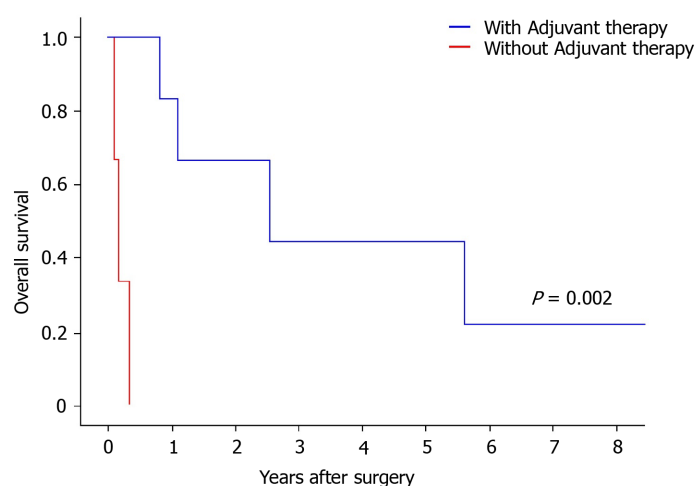
**Figure 3 Disease-free survival.** Median disease-free survival was 52 d. There was no statistical difference between angiosarcoma and undifferentiated sarcoma groups.



**Figure 4 Overall survival.** The 5-year survival rate was 29.6%. There was no significant difference between survival of the angiosarcoma and undifferentiated sarcoma groups.

resection of hepatic angiosarcoma.

In our study, two patients with hepatic angiosarcoma received living donor liver transplantation (LT). The 2-year-old girl had recurrence at 53 d and expired at 31 mo after LT, while the 60-year-old male had recurrence at 11 mo and expired at 14 mo. Selection of treatment between surgical resection and LT is an issue of concern. A study of 237 patients registered in the National Cancer Database of North America concluded that both hepatic resection and LT may lead to similar long-term survival with selected pathologic cases such as epithelioid hemangioendotheliomas[19]. However, this study also found that the prognosis of angiosarcoma was worse with



	Time (yr)	1	2	3	4	5
Received adjuvant therapy ( $n = 6$ )	survival rate	0.83	0.67	0.44	0.44	0.44
	# at risk	5	4	2	2	2
No adjuvant therapy ( $n = 4$ )	survival rate	0.25	0.25	0		
	# at risk	1	1	0		

**Figure 5 Overall survival depending on adjuvant therapies.** Patients who received adjuvant therapy showed higher overall survival rate.

both resection and transplantation. A review article of 64 angiosarcoma cases did not recommend LT for angiosarcoma due to the higher recurrence rate[6]. This result accords with the poor outcomes of LT seen in the hepatic angiosarcoma patients in our study. For hepatic undifferentiated sarcoma, liver transplantation cases are rare. Walther *et al*[20] studied 3 patients who received LT and remained in clinical remission for a mean of 35 mo. Wu *et al*[10] showed 14 patients who received LT for hepatic undifferentiated sarcoma with a 5-year survival rate of 78.9%. When weighing the benefits of LT against the risks for the liver donor or the shortage of deceased donor, LT in hepatic undifferentiated sarcoma is still controversial and needs further research.

Our study has limitations in that it is a retrospective study and has a small number of patients due to the rarity of the tumor. Furthermore, three patients did not receive adjuvant therapy, not due to clinical decision, but due to either poor patient condition or the patient expiring prior to receiving the adjuvant therapy.

## CONCLUSION

Primary hepatic sarcoma has poor outcomes even after surgical resection. However, surgical resection may have some benefit for extending long term life expectancy in some cases. Adjuvant therapy may support the outcomes. Liver transplantation for primary hepatic angiosarcoma also continues to have poor survival outcomes.

## ARTICLE HIGHLIGHTS

### Research background

Primary hepatic sarcoma is a malignant tumor which arises from hepatic mesenchymal tissue. It consists of angiosarcoma, undifferentiated (embryonal) sarcoma, leiomyosarcoma, and epithelioid hemangioendothelioma.

### Research motivation

Due to its rarity and various prognosis, the treatment plan of primary hepatic sarcoma is not established yet.

### Research objectives

We aim to analyze the tumor characteristics, treatment and prognosis of the primary

hepatic sarcoma cases which was surgically resected in a single center.

### Research methods

After exclusion of cases with open and closure, early loss to follow-up and sarcomatoid tumors, total nine cases of primary hepatic sarcoma were surgically resected from August 2001 to September 2016. The research data collection and analysis were achieved with retrospective approach. Baseline patient's characteristics, tumor characteristics and treatment modality with tumor recurrence and patient's survival were analyzed. The analysis was done separately according to tumor pathologic type.

### Research results

Among five angiosarcoma and four undifferentiated sarcoma patients, only two patients survived and all patients experienced tumor recurrences (5-year survival rate: 29.6%). Follow-up post-operative durations of survived angiosarcoma patient and undifferentiated sarcoma patient were 11 years and 30 mo, respectively. Adjuvant therapy had a positive role on survival gain ( $P = 0.002$ ). However, this study has a limitation of a retrospective approach and a small case number.

### Research conclusions

In spite of known poor prognosis, surgical resection of primary hepatic sarcoma may help extending the life expectancy of patient. Aggressive adjuvant treatment after resection may aid the better outcome.

### Research perspectives

Accumulation of primary hepatic sarcoma data followed by finding of specific prognostic factor should be researched. New era of adjuvant therapies, such as immunotherapy for primary hepatic sarcoma is also needed to be developed.

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# Endoscopic retrograde cholangiopancreatography drainage for palliation of malignant hilar biliary obstruction — stent-in-stent or side-by-side? A systematic review and meta-analysis

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## Abstract

### BACKGROUND

Biliary drainage, either by the stent-in-stent (SIS) or side-by-side (SBS) technique, is often required when treating a malignant hilar biliary obstruction (MHBO). Both methods differ from each other and have distinct advantages.

### AIM

To compare both techniques regarding their efficacy and safety in achieving drainage of MHBO.

### METHODS

A comprehensive search of multiple electronic databases (MEDLINE, Embase, LILACS, BIREME, Cochrane) was conducted and grey literature from their inception until December 2020 with no restrictions regarding the year of



Baracat R contributed revising the article, drafting the article, final approval; de Moura ETH contributed revising the article, drafting the article, final approval; Bernardo WM contributed analysis and interpretation of data, drafting the article, final approval; de Moura EGH contributed analysis and interpretation of data, drafting the article, revising the article, final approval.

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publication or language, since there was at least an abstract in English. The included studies compared SIS and SBS techniques through endoscopic retrograde cholangiopancreatography. Outcomes analyzed included technical and clinical success, early and late adverse events (AEs), stent patency, reintervention, and procedure-related mortality.

## RESULTS

Four cohort studies and one randomized controlled trial evaluating a total of 250 patients (127 in the SIS group and 123 in the SBS group) were included in this study. There were no statistically significant differences between the two groups concerning the evaluated outcomes, except for stent patency, which was higher in the SIS compared with the SBS technique [mean difference (d) = 33.31; 95% confidence interval: 9.73 to 56.90,  $I^2 = 45\%$ ,  $P = 0.006$ ].

## CONCLUSION

The SIS method showed superior stent patency when compared to SBS for achieving bilateral drainage in MHBO. Both techniques are equivalent in terms of technical success, clinical success, rates of both early and late AEs, reintervention, and procedure-related mortality.

**Key Words:** Endoscopic retrograde cholangiopancreatography; Biliary tract neoplasms; Biliary; Hilar; Stenting; Drainage

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**Core Tip:** Biliary drainage is often required when treating a malignant hilar biliary obstruction. There are two types of drainage: Stent-in-stent (SIS) and side-by-side (SBS) techniques. Both of them differ from each other and have distinct advantages. This study aimed to compare both techniques regarding their efficacy and safety. Our systematic review and meta-analysis demonstrated no statistically significant differences between the SIS and SBS techniques; except for stent patency which was superior in the SIS technique. The choice of palliation for drainage must be guided by both local expertise and resource availability.

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**DOI:** <https://dx.doi.org/10.4254/wjh.v13.i5.595>

## INTRODUCTION

Malignant hilar biliary obstruction (MHBO) is a late manifestation of certain types of cancer. This is diagnosed as unresectable in up to 80% of cases, and capable of causing potentially fatal complications, such as cholangitis and sepsis[1-6]. Thus, aimed at improving the quality of life and survival rate of patients, a discussion on the optimal method for palliation of drainage is very valuable[7-10].

The endoscopic biliary stent, introduced at the beginning of the 1980s, was a significant advance in the treatment of extrahepatic obstruction[11-13]. In biliary obstruction, self-expandable metal stents (SEMS) seem to provide prolonged patency of drainage when compared to plastic stents[3,4,14-17]. The endoscopic approach is preferred for drainage over the percutaneous and surgical approaches due to its more physiological nature, minimal invasiveness[3,4,6,18-20], low rate of adverse events (AEs), and shorter hospital stays[21]. One predictor of the effectiveness of biliary drainage is when the drained hepatic volume is above 50%. This often requires a bilateral decompression[15,22], which is associated with a lower chance of reintervention when compared to unilateral drainage in the palliation of drainage of

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MHBOs[23].

Bilateral drainage of the bile ducts can be performed *via* two methods: Stent-in-stent (SIS) or side-by-side (SBS)[15] placement of metal stents (Figure 1). In the SIS technique, one of the stents is positioned through the wire mesh of the other, configuring into a Y-shaped aspect. On the other hand, in the SBS method, both stents are placed side by side[22]. The SIS technique, in contrast to the SBS technique, does not require a dilated common bile duct, and thus allows the placement of higher caliber biliary stents[17], and presents a more physiological nature of drainage[3]. The SBS technique provides an easier procedural execution[3,15], and in the case of stent occlusion, reintervention is often more feasible[17].

In theory, there are advantages to both techniques, which casts doubt whether there is enough evidence to favor one method to the detriment of the other. Furthermore, few comparative studies have addressed the subject, making it still unclear which of the two methods is the optimal approach. To gather the best available data in the literature, we have designed this systematic review and meta-analysis on the subject. We aimed to compare the feasibility, safety, and efficacy of both the SIS and SBS techniques for palliative drainage in MHBO.

## MATERIALS AND METHODS

### Protocol and registration

This study was performed in conformity with the PRISMA[24] and it was registered in the International Prospective Register of Systematic Reviews under the file number CRD42020191262. The study was approved by the Ethics Committee of Hospital das Clínicas, Faculty of Medicine at The University of São Paulo.

### Eligibility criteria

The data search was made without limitations of publication date or language, since there was at least an abstract in English. We considered clinical trials or observational studies published either as full text or as an abstract with the necessary data, comparing SIS and SBS metal stent placement in patients with malignant hilar biliary strictures. The following outcomes were observed: Technical and clinical success, early AEs (occurring within the first month after the procedure), late AEs (occurring after 30 d), stent patency, reintervention, and procedural-related mortality.

The exclusion criteria were studies using non-human subjects and trials that evaluated percutaneous biliary access drainage.

### Information sources

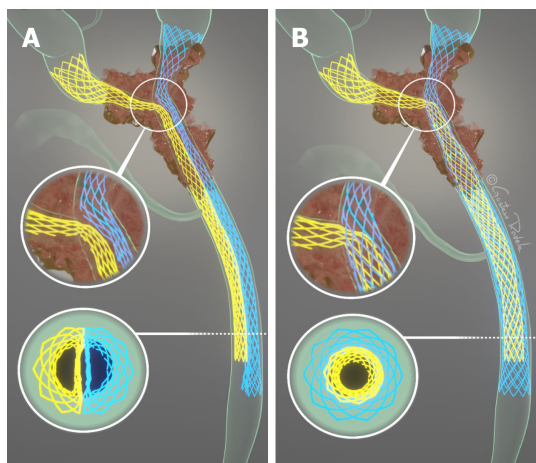
We identified the studies by searching electronic databases and scanning reference lists of the selected articles. This search strategy was applied in electronic databases [MEDLINE, Embase, Central Cochrane, LILACS (*via* BVS), BIREME, and Google Scholar] and grey literature from their inception until December 2020 (Figure 2).

### Search strategy and study selection

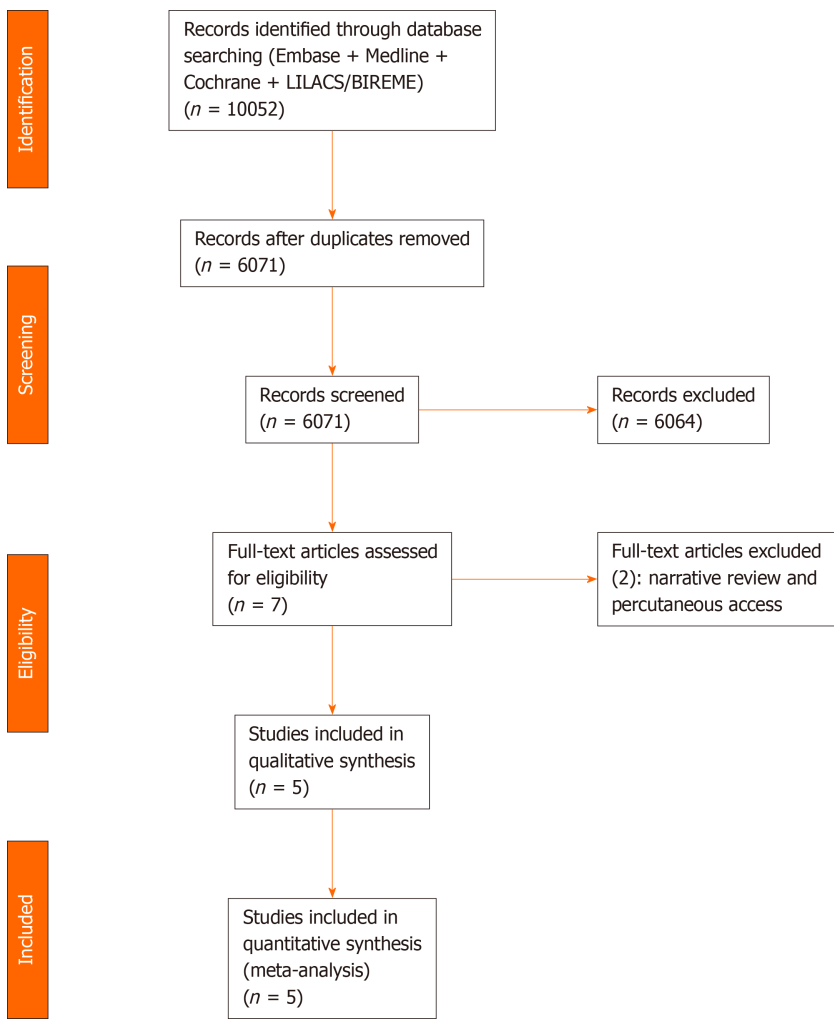
The following search strategy was used in all databases: [(Neoplasia OR Neoplasias OR Neoplasm OR Neoplasms OR Tumors OR Tumor OR Cancer OR Cancers OR Malignancy OR Malignancies) AND (Biliary Tract OR Biliary Tree OR Biliary System OR Bile Duct OR Bile Ducts)] OR [(Bile Duct Neoplasms OR Bile Duct Neoplasm OR Bile Duct Cancer OR Bile Duct Cancers OR Biliary Tract Neoplasm OR Biliary Tract Neoplasms OR Biliary Tract Cancer OR Biliary Tract Cancers) AND (Prostheses and Implants)] OR Prosthetic OR Implants OR Implant OR Prostheses OR Prosthesis OR Endoprosthesis OR Endoprostheses OR Stent OR Stents OR Stent-in-stent OR Side-by-Side.

### Data collection process and data items

Two researchers reviewed the title and abstract of each article after the removal of duplicated articles. Articles that were found to be relevant were selected for full-text review. The final decision on the selection of the studies was based on predetermined inclusion and exclusion criteria. Any disagreement on the selection of studies was resolved by consensus with a third experienced researcher. The target data of the selected studies were entered and organized in a Microsoft Excel spreadsheet by the same two reviewers who conducted the selection. The reviewers extracted from the articles the outcomes of interest and information concerning the population and study



**Figure 1** Two methods of bilateral drainage of the bile ducts. A: Side-by-side; B: Stent-in-stent.



**Figure 2** Flow diagram showing the article selection process.

characteristics. When the data of the published articles were insufficient, the corresponding authors were consulted by e-mail for further elucidation.

**Risk of bias in individual studies and quality of evidence**

The risk of bias in the cohort studies was assessed by the Risk of Bias in Non-randomized Studies-of Interventions (ROBINS I) Cochrane tool[25]. For randomized clinical trials, the risk of bias was defined by version 2 of the Cochrane Risk-of-Bias

tool for Randomized Trials (RoB2)[26].

The quality of evidence, expressed as high, moderate, low, and very low, was assessed utilizing the objective criteria from GRADE (Grading Recommendations Assessment, Development, and Evaluation) for each of the pre-specified results and outcomes using GRADEpro-Guideline Development Tool software (McMaster University, 2015; Evidence Prime, Inc., Ontario, Canada)[27].

### **Synthesis of results and data analysis**

For continuous variables, we used mean or median values[28] along with the standard deviation and the total number of patients. Regarding the outcomes expressed by categorical variables, the absolute number of events and the total number of patients was employed, with calculation of the regular and absolute risk differences for each group utilizing the Mantel-Haenszel test. The mean values of each continuous outcome were calculated, as well as the 95% confidence interval (CI). *P* values < 0.05 were considered statistically significant and the results were exposed through forest plots.

Heterogeneity was calculated using the Higgins method ( $I^2$ ). When heterogeneity < 50% was found, the fixed-effect model was used. In outcomes with high heterogeneity among studies ( $I^2 > 50\%$ ), sensitivity analysis employing funnel plots were conducted to identify publication bias (outliers). If the heterogeneity levels were still high even after outlier exclusion, we maintained the outlier and applied the random-effects model to express the results (true heterogeneity). If the heterogeneity levels were low after outlier exclusion, we applied the fixed-effects model.

The data of interest extracted from the selected studies were meta-analyzed using RevMan software (Review Manager Software version 5.4 – Cochrane Collaboration Copyright© 2020).

## **RESULTS**

### **Study selection and study characteristics**

A total of 10052 articles were identified through our searches in the MEDLINE, Embase, LILACS, BIREME, and Central Cochrane databases. After the removal of duplicates, evaluation of the titles and abstracts, and text analysis, four retrospective cohort studies[29-32] and one randomized controlled trial (RCT)[33] were included in the meta-analysis (Figure 2). The characteristics of the included studies are summarized in Table 1.

Three[29,30,32] of the four retrospective studies presented a moderate overall risk of bias, assessed by the ROBINS-I tool, mainly due to confounding, the bias in the selection of participants, and bias in the selection of the reported results. The other included study[31] presented a serious risk of bias. The RCT study[33] presented a low risk of bias in our analysis (RoB2) (Tables 2 and 3). Detailed information concerning the risk of bias for each outcome is described in Table 4.

### **Technical success**

All four cohorts[29-32] (181 patients) and the RCT study[33] (69 patients) assessed technical success. The overall analysis showed no difference between both SIS and SBS [risk difference (RD) = 0.06; 95%CI: -0.00 to 0.13,  $I^2 = 0\%$ ,  $P = 0.06$ ] (Figure 3).

The overall certainty of the evidence was moderate for the cohorts and high for the RCT study, according to GRADE.

### **Clinical success**

Three studies evaluated clinical success, namely two cohorts[30,32] (116 patients) and the RCT study[33] (69 patients). This outcome was similar for both SIS and SBS techniques in the overall analysis (RD = 0.07; 95%CI: -0.05 to 0.18,  $I^2 = 56\%$ ,  $P = 0.26$ ) (Figure 4).

The overall certainty of the evidence was low for the cohort and high for the RCT study, according to GRADE.

### **Early AEs**

Three cohorts[30-32] (157 patients) and the RCT study[33] (69 patients) evaluated early complications. In the overall analysis, both SIS and SBS techniques performed similarly regarding this outcome (RD = -0.09; 95%CI: -0.19 to 0.01,  $I^2 = 2\%$ ,  $P = 0.07$ ) (Figure 5).

Table 1 Type of intervention and outcome of study

Ref.	Design	Year	Technical success		Clinical success		Rate of early adverse events		Rate of late adverse events		Stent patency		Reintervention		Procedure-related mortality	
			SIS	SBS	SIS	SBS	SIS	SBS	SIS	SBS	SIS	SBS	SIS	SBS	SIS	SBS
Lee <i>et al</i> [33]	RCT	2019	34/34	32/35	32/34	29/35	4/34	4/35	6/34	8/35	Median 253 d (28-420); SD 98; mean 253	Median 262 d (9-455); SD 111.5; mean 262	15/34	12/35	0/34	0/35
Naitoh <i>et al</i> [30]	Cohort	2012	24/24	25/28	24/24	24/28	1/24	3/28	2/24	8/28	Median 104 d (20-600); SD 145; mean 207	Median 155 d (15-881); SD 216.5; mean 155	NA	NA	0/24	0/28
Kim <i>et al</i> [31]	Cohort	2012	18/22	15/19	NA	NA	5/22	6/19	11/22	7/19	NA	NA	NA	NA	NA	NA
Law <i>et al</i> [29]	Cohort	2013	7/7	17/17	NA	NA	NA	NA	0/7	0/17	NA	NA	3/7	9/17	0/7	0/17
Ishigaki <i>et al</i> [32]	Cohort	2020	40/40	23/24	37/40	23/24	9/40	11/24	4/40	3/24	Median 169 d (108-445); SD 84.25; mean 169	Median 205 d (85-NA); SD 24.39; mean 123.75	NA	NA	NA	NA

SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; NA: Not available.

The overall certainty of the evidence was moderate for both cohorts and the RCT study, according to GRADE.

### Late AEs

Five studies[29-33] compared late complication rates, evaluating a total of 181 patients in the cohorts and 69 patients in the RCT. In the overall analysis, there was no significant difference between the two groups (RD = -0.04; 95%CI: -0.14 to 0.05,  $I^2 = 0\%$ ,  $P = 0.39$ ) (Figure 6).

The overall certainty of the evidence was moderate for both cohorts and the RCT study, according to GRADE.

### Stent patency

Three studies assessed stent patency: two cohorts[30,32] (116 patients) and the RCT[33] (69 patients). The overall analysis revealed increased stent patency when SIS was performed [mean deviation (MD) = 33.31; 95%CI: 9.73 to 56.90,  $I^2 = 45\%$ ,  $P = 0.006$ ] (Figure 7).

The overall certainty of the evidence was moderate for the cohort and high for the RCT study, according to GRADE.

### Reintervention

One cohort[29] compared reintervention rates, evaluating a total of 24 procedures—7 in the SIS group and 17 in the SBS group. We found no difference between the two groups in the overall analysis (RD = 0.05; 95%CI: -0.15 to 0.26,  $I^2 = 0\%$ ,  $P = 0.60$ ).



**Table 2 Risk of bias for ROBINS-I**

Ref.	D1	D2	D3	D4	D5	D6	D7	Overall
Naitoh <i>et al</i> [30] 2012	Moderate	Moderate	Low	Low	Low	Moderate	Moderate	Moderate
Kim <i>et al</i> [31] 2012	Serious	Serious	Low	Serious	Serious	Serious	Serious	Serious
Law <i>et al</i> [29] 2013	Moderate	Moderate	Low	Low	Moderate	Moderate	Serious	Moderate
Ishigaki <i>et al</i> [32] 2020	Moderate	Moderate	Low	Low	Low	Moderate	Moderate	Moderate

D: Domains; D1: Bias due to confounding; D2: Bias due to selection of participants; D3: Bias in classification of interventions; D4: Bias due to deviations from intended interventions; D5: Bias due to missing data; D6: Bias in measurement of outcomes; D7: Bias in selection of the reported result.

**Table 3 Risk of bias for RoB2**

Ref.	D1	D2	D3	D4	D5	Overall
Lee <i>et al</i> [33], 2019	Low	Low	Low	Low	Low	Low

D: Domains; D1: Bias due to randomization process; D2: Bias due to deviations from intended interventions; D3: Bias due to missing outcome data; D4: Bias due to measurement of the outcome; D5: Bias due to selection of the reported result.

(Figure 8).

The overall certainty of the evidence was low for the cohort and high for the RCT study, according to GRADE.

### Procedure-related mortality

Two cohorts[29,30] compared procedure-related mortality, evaluating a total of 76 procedures—31 in the SIS group and 45 in the SBS group. We found no difference between the two groups (RD = 0.00; 95%CI: -0.05 to 0.05,  $I^2 = 0\%$ ,  $P = 1.00$ ) (Figure 9).

The overall certainty of the evidence was moderate for the cohorts and high for the RCT study, according to GRADE.

## DISCUSSION

Despite being targeted by promising therapies in several clinical trials[34,35], bile duct tumors are often diagnosed as unresectable when they present with biliary obstruction. Therefore, internal drainage *via* the endoscopic deployment of stents has a pivotal role in this condition.

To the best of our knowledge, this is the first systematic review and meta-analysis comparing both the SIS and SBS techniques for the palliation of biliary drainage in MHBOs. This is a relevant topic for clinical practice, and many studies have non-comparatively evaluated these biliary drainage methods in the past. Despite presenting higher stent patency with the SIS method, we have found through our meta-analysis that there were no statistically significant differences concerning technical success, clinical success, early AEs, late AEs, reintervention, and procedure-related mortality.

For both groups, technical success was achieved in most cases, and we consider that the included studies were conducted at high-volume centers. The main challenge in the SBS method consists of the deployment of the second stent along with the first one. This is especially important since the distal end of both stents should ideally remain at the same level to facilitate an eventual reintervention. New devices have been developed, including systems with a thinner delivery system, which allows the simultaneous deployment of both prostheses. This system prevents the risk of a failed second placement and is associated with a shorter procedural time, as reported by Inoue *et al*[36]. Traditionally, the dilation on the wire mesh of the first stent before inserting the second one is necessary for the SIS technique. This prerequisite increases the difficulty and cost of the procedure. However, stents with larger cells have been developed, specifically for this usage, with high rates of technical success for the SIS method[37]. We consider that despite the fact that achieving bilateral biliary drainage

Table 4 Description of bias for each outcome (GRADE)

Certainty assessment							Summary of findings				
Participants (studies) follow up	Risk of bias	Inconsistency	Indirectness	Imprecision	Publication bias	Overall certainty of evidence	Study event rates (%)		Relative effect (95%CI)	Anticipated absolute effects	
							With SBS	With SIS		Risk with SBS	Risk difference with SIS
Early adverse events: Cohorts											
157 (3 observational studies)	Serious <sup>1</sup>	Not serious	Not serious	Not serious	None	Moderate	20/71 (28.2)	15/86 (17.4)	RR 0.54 (0.31 to 0.96)	282 per 1.000	130 fewer per 1.000 (from 194 fewer to 11 fewer)
Early adverse events: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Serious <sup>2</sup>	None	Moderate	4/35 (11.4)	4/34 (11.8)	RR 1.03 (0.28 to 3.79)	114 per 1.000	3 more per 1.000 (from 82 fewer to 319 more)
Late adverse events: Cohorts											
181 (4 observational studies)	Serious <sup>1</sup>	Not serious	Not serious	Not serious	None	Moderate	18/88 (20.5)	17/93 (18.3)	RR 0.82 (0.46 to 1.47)	205 per 1.000	37 fewer per 1.000 (from 110 fewer to 96 more)
Late adverse events: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Serious <sup>2</sup>	None	Moderate	8/35 (22.9)	6/34 (17.6)	RR 0.77 (0.30 to 1.99)	229 per 1.000	53 fewer per 1.000 (from 160 fewer to 226 more)
Procedural-related mortality: Cohorts											
76 (2 observational studies)	Serious <sup>1</sup>	Not serious	Not serious	Not serious	None	Moderate	0/45 (0.0)	0/31 (0.0)	Not pooled	Not pooled	Not pooled
Procedural-related mortality: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	0/35 (0.0)	0/34 (0.0)	RR 0.00 (-0.05 to 0.05)	0 per 1.000	- per 1.000 (from 0 fewer to 0 fewer)
Technical success: Cohorts											
181 (4 observational studies)	Serious <sup>1</sup>	Not serious	Not serious	Not serious	None	Moderate	80/88 (90.9)	89/93 (95.7)	RR 1.06 (0.97 to 1.16)	909 per 1.000	55 more per 1.000 (from 27 fewer to 145 more)
Technical success: RCT											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	32/35 (91.4)	34/34 (100.0)	RR 1.09 (0.97 to 1.22)	914 per 1.000	82 more per 1.000 (from 27 fewer to 201 more)
Clinical success: Cohort											
116 (2 observational studies)	Serious <sup>1</sup>	Serious <sup>3</sup>	Not serious	Not serious	None	Low	47/52 (90.4)	61/64 (95.3)	RR 1.05 (0.87 to 1.26)	904 per 1.000	45 more per 1.000 (from 118 fewer to 235 more)

<b>Clinical success: RCT</b>											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	29/35 (82.9)	32/34 (94.1)	RR 1.14 (0.96 to 1.35)	829 per 1.000	116 more per 1.000 (from 33 fewer to 290 more)
<b>Reintervention: Cohort</b>											
24 (1 observational study)	Serious <sup>1</sup>	Not serious	Not serious	Serious <sup>2</sup>	None	Low	9/17 (52.9)	3/7 (42.9)	RR 0.81 (0.31 to 2.13)	529 per 1.000	101 fewer per 1.000 (from 365 fewer to 598 more)
<b>Reintervention: RCT</b>											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	12/35 (34.3)	15/34 (44.1)	RR 1.29 (0.71 to 2.33)	343 per 1.000	99 more per 1.000 (from 99 fewer to 456 more)
<b>Stent patency: Cohort</b>											
116 (2 observational studies)	Serious <sup>1</sup>	Not serious	Not serious	Not serious	None	Moderate	52	64	-	The mean stent patency: Cohort was 0	MD 45.75 higher (18.92 higher to 72.58 higher)
<b>Stent patency: RCT</b>											
69 (1 RCT)	Not serious	Not serious	Not serious	Not serious	None	High	35	34	-	The mean stent patency: RCT was 0	MD 9 lower (58.49 lower to 40.49 higher)

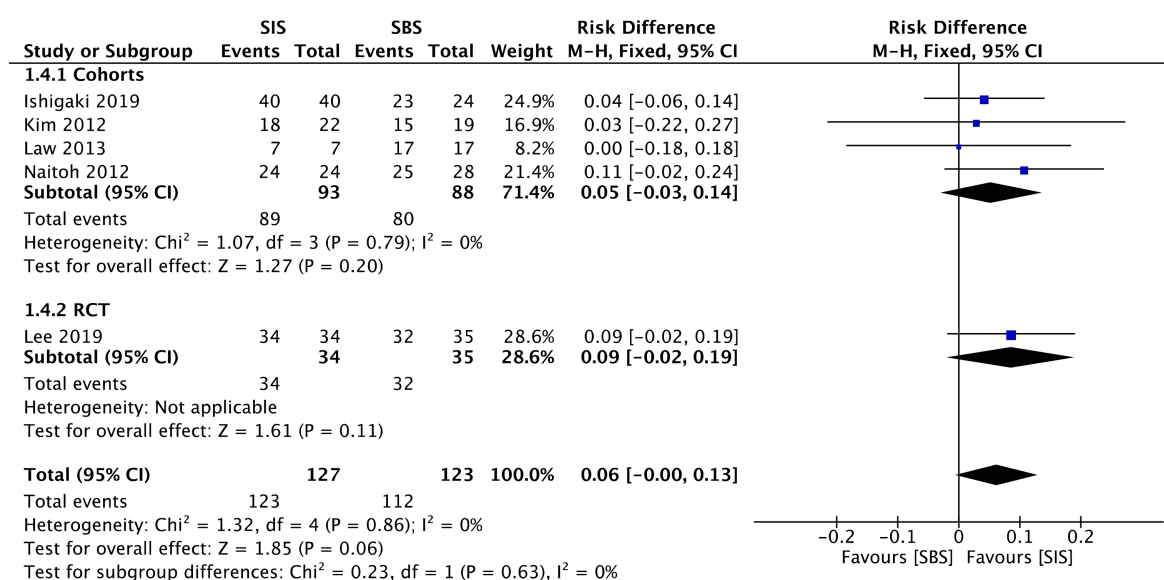
<sup>1</sup>There are risk of bias in selection of the reported result, according to ROBINS-I tool.<sup>2</sup>Wide confidence interval range.<sup>3</sup>High heterogeneity, calculated using the Higgins method (*I*<sup>2</sup>).

CI: Confidence interval; RR: Risk ratio; MD: Mean difference; RCT: Randomized controlled trial.

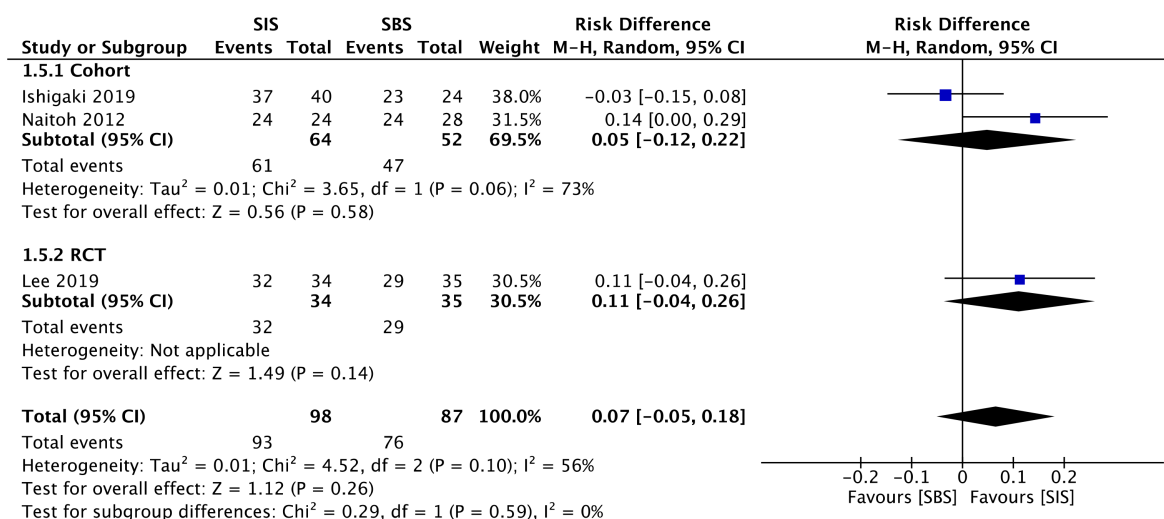
in the MHBOs is technically challenging, technical success rates were increased and equivalent between both SIS and SBS, probably due to the endoscopist's vast expertise and the availability of suitable material.

Clinical success was defined in the studies as a total bilirubin decrease in the first month to at least 50% or 75% of the pre-treatment value. Although there was no statistical difference between the groups, we have reservations regarding this outcome definition and we think this outcome should be evaluated very carefully. One reason for this could be that the studies that evaluated this outcome opted for a conservative definition, based on a little significant drop in bilirubin levels, and not on laboratory level standards. Also, they failed to assess other laboratory or clinical parameters.

The use of uncovered SEMS is preferred over fully covered SEMS (FCSEMS) for palliative drainage of malignant biliary obstructions[21], just as it was done in the assessed studies. This is due to the risk of obstruction in intrahepatic lateral branches and cystic and pancreatic ducts, abscess-related factors, cholecystitis, and acute pancreatitis (AP). Inoue *et al*[38] and Yoshida *et al*[39] reported the occurrence of hepatic abscesses (11.8% and 6.3% of cases, respectively) when using 6 mm FCSEMS. Although these results cannot be attributed to the stents, they allow us to consider such a hypothesis. In our study, the SIS and SBS techniques presented similar results



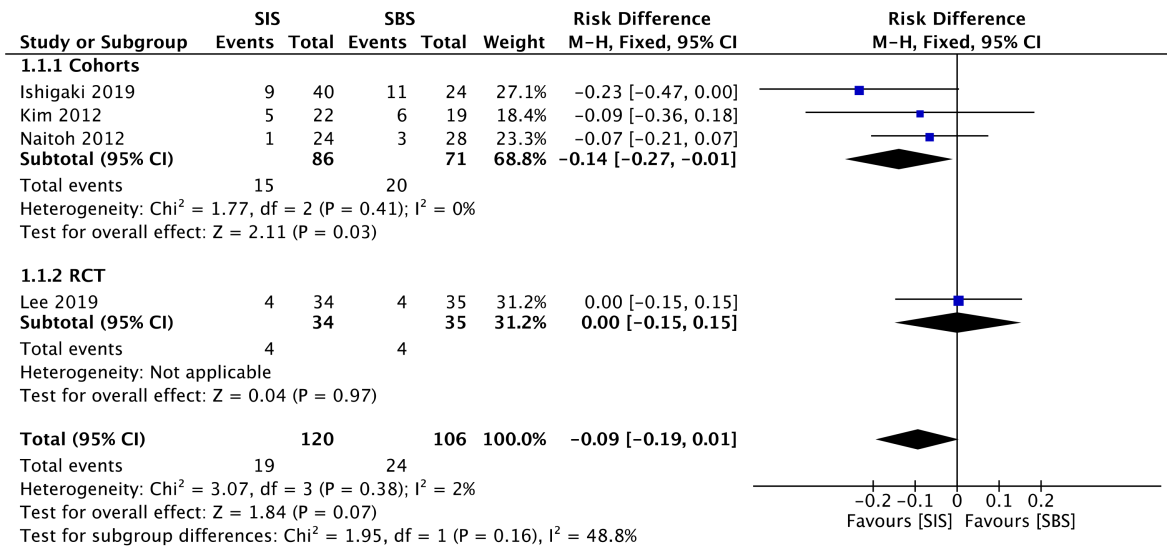
**Figure 3 Forest plot — studies reporting rate of technical success using a fixed-effects model.** CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.



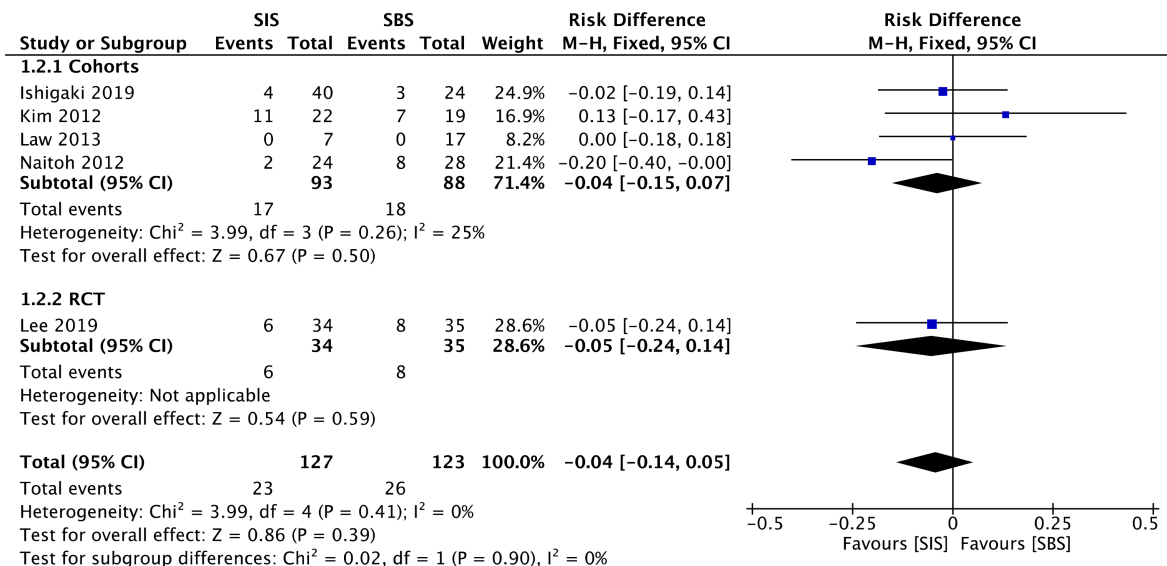
**Figure 4 Forest plot — studies reporting rate of clinical success using a random-effects model.** CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

regarding late complications, such as cholangitis, cholecystitis, and biloma formation. In the cohort meta-analysis, SBS resulted in higher early complication rates ( $RD = -0.14$ ; 95%CI: -0.27 to -0.01,  $I^2 = 0\%$ ,  $P = 0.03$ ), such as AP. Tarnasky *et al*[40] had already reported a higher risk of AP in patients referred to biliary stenting for hilar biliary stricture. Furthermore, stent deployment in SBS with the distal end of the stent across the papilla, instead of above the papilla, seems to raise the risk of AP[41]. Nevertheless, in the cohorts meta-analyzed in the present study both techniques were utilized, thus impeding the attribution of the aforementioned complication exclusively to that reason. However, a RCT and general analysis showed no statistically significant differences. These data suggest that both techniques are safe as part of a minimally invasive treatment, with no differences regarding the occlusion of intrahepatic, cystic, or pancreatic ducts. Even if it is not possible to arrive at this conclusion from only this meta-analysis, it seems to us that the stent type has more influence on the complication rates than the drainage technique itself. The safety of endoscopic treatment and each specific technique, is reinforced by the absence of procedural-related deaths in all the casuistry of this study.

The outcome of stent patency, evaluated as moderate and high levels of evidence for the cohort and the RCT, respectively, showed a  $MD = 33.31$ , favoring SIS, with a



**Figure 5 Forest plot — studies reporting rate of early adverse events using a fixed-effects model.** CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

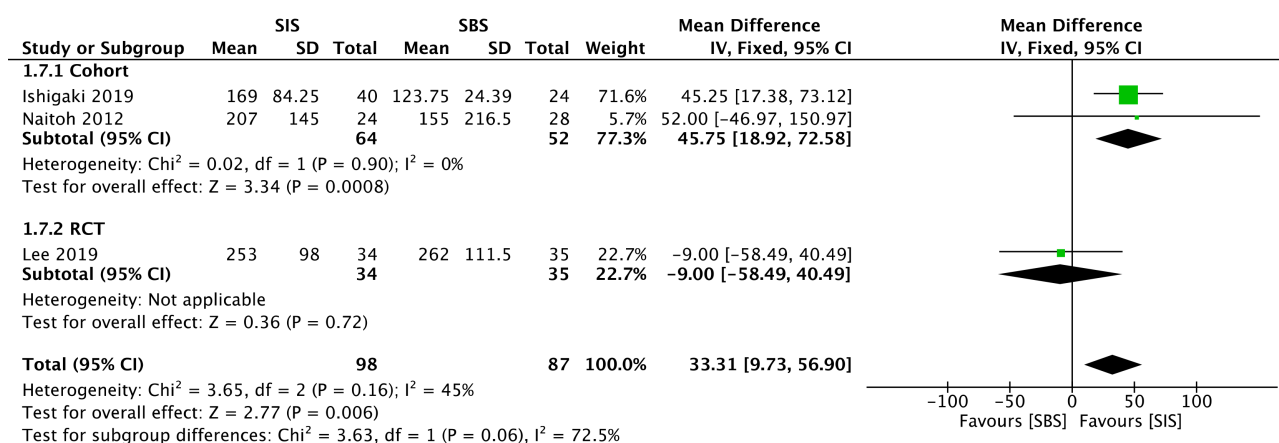


**Figure 6 Forest plot — studies reporting rate of late adverse events using a fixed-effects model.** CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

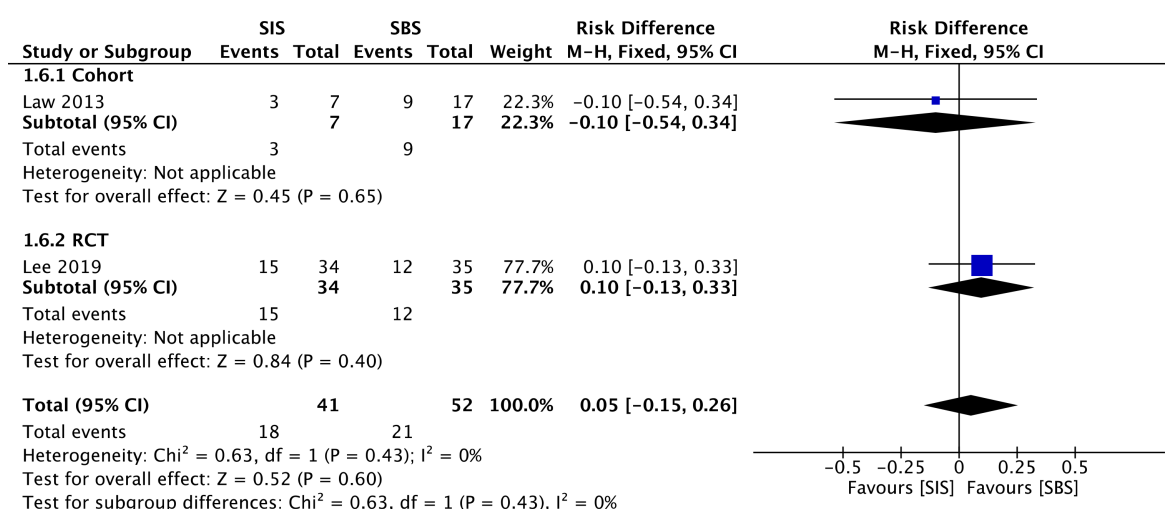
95%CI: 9.73 to 56.90. Although the reason behind such a difference is unclear, we believe that the SIS technique may allow greater stent expandability, and consequently larger internal caliber, in comparison with the SBS technique. Nevertheless, this result should be analyzed very carefully since some studies do not specify the exact caliber of the employed stent, and one of them disclosed the use of calibers slightly larger in the SIS technique. The use of SEMS in the studies is a positive factor regarding stent patency, corroborating the findings of the specific study that showed higher patency with these types of stents when compared with the plastic stents (131 d *vs* 47 d)[42]. Our study found no difference regarding the reintervention rate. The main cause of post-procedural obstruction was tumor progression (ingrowth or overgrowth) provoking cholestasis and cholangitis, and thus requiring reintervention. The reintervention approach usually adopted in these cases is the placement of an inner metallic stent, after the cleansing of ductal debris with a balloon extraction and/or cholangioscopy. Radiofrequency ablation can also be considered, but related studies are still scarce[21].

Our study has some limitations. There is only one RCT in the literature comparing both analyzed techniques. Besides the RCT, only 4 comparative retrospective observa-





**Figure 7 Forest plot — studies reporting the number of days of stent patency using a fixed-effects model.** CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial.



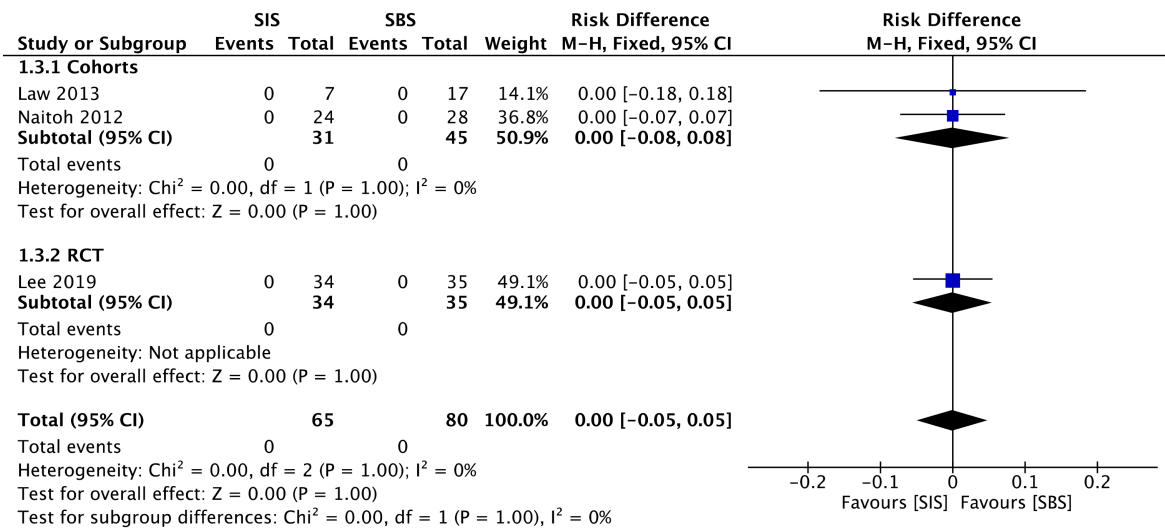
**Figure 8 Forest plot — studies reporting the rates of reintervention using a fixed-effects model.** CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

tional studies are available in the literature. Furthermore, the number of patients in the included studies is small, perhaps because this disease does not have a high prevalence. Although the study by Kim *et al*[31] is available only as an abstract, it possessed all the necessary data for this analysis. Moreover, the prostheses used in different studies were from different manufacturers, with no information on diameter measurements for comparison. Given such limitations, new RCTs may have a valuable role in new systematic reviews, thus improving the quality of evidence.

Despite the aforementioned limitations and to the best of our knowledge, our study is the first systematic review with a meta-analysis on this topic. We firmly believe this has significant clinical applicability given the increasing demand for bile duct drainage in the palliation of malignant hilar tumors.

## CONCLUSION

There is no significant difference between the SIS or SBS techniques in terms of early and late complication rates, technical success, clinical success, reintervention, and procedural-related mortality. The SIS technique was superior in terms of stent patency when compared to the SBS technique, which may guide decision-making regarding the best therapeutic modality for each patient.



**Figure 9 Forest plot — studies reporting the rates of procedural-related mortality using a fixed-effects model.** CI: Confidence interval; SIS: Stent-in-stent; SBS: Side-by-side; RCT: Randomized controlled trial; M-H: Mantel-Haenszel test.

## ARTICLE HIGHLIGHTS

### Research background

Patients with malignant hilar biliary obstruction (MHBO) benefit from bilateral palliative endoscopic drainage. However, there is no consensus on which is the optimal technique for placing a metal stent: Stent-in-stent (SIS) or side-by-side (SBS).

### Research motivation

Many patients undergo palliative endoscopic retrograde cholangiopancreatography (ERCP) drainage, due to the advanced stage of the disease at the time of diagnosis, unresectable in most cases. However, choosing the best management for drainage can be a real technical challenge. Therefore, we aimed to compare both drainage techniques in an attempt to identify the optimal approach.

### Research objectives

To perform a systematic review and meta-analysis of available studies that compare SIS and SBS deployment in patients with MHBO undergoing ERCP drainage.

### Research methods

The systematic review and meta-analysis followed the PRISMA Guidelines. Electronic searches were performed in MEDLINE, Embase, Cochrane, LILACS, and BIREME databases, and the grey literature. Comparative cohorts and randomized controlled trials (RCTs) were included. Studied outcomes were technical and clinical success, early and late adverse events (AEs), stent patency, reintervention, and procedure-related mortality.

### Research results

Four comparative cohorts and one RCT were included in the final analysis with a total of 250 patients, of whom 127 belonged to the SIS group and 123 to the SBS group. Stent patency was significantly higher in the SIS group. Procedure-related mortality was similar in both groups, and no significant differences were found in the rates of technical success, clinical success, early AEs, late AEs, and reintervention.

### Research conclusions

There was no difference between the groups concerning technical and clinical success, early and late AEs, reintervention, and procedure-related mortality. However, there was longer stent patency in patients undergoing the SIS technique. This result suggests that SIS may be the preferred technique for bilateral palliative metal stent deployment in patients with inoperable MHBO.

### Research perspectives

Palliative biliary drainage is an increasingly performed procedure, but without consensus on the optimal technique, SIS or SBS. There is a small number of comparative studies in the literature. Future RCTs will have an important role in elucidating the most optimal drainage technique.

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## Acquired hepatocerebral degeneration in a metastatic neuroendocrine tumor long-term survivor — an update on neuroendocrine neoplasm's treatment: A case report

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### Abstract

#### BACKGROUND

Metastatic small bowel low-grade neuroendocrine tumors (NETs) have a good prognosis. Surgery is the only curative treatment; however, this may induce advanced liver disease, particularly in long-term survivor patients. Acquired hepatocerebral degeneration or Parkinsonism in cirrhosis is characterized by rapidly progressive extrapyramidal symptoms in patients with advanced liver disease.

#### CASE SUMMARY

A 70-year-old man presented to the emergency department with diminished

the manuscript; All authors read and approved the final manuscript.

#### Informed consent statement:

Informed written consent was obtained from the patient for publication of this report and accompanying images.

#### Conflict-of-interest statement:

Mirallas O, Saoudi N and Gómez-Puerto D declare that they have no competing interests funding related to this work.

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consciousness and disorientation, and was diagnosed with hepatic encephalopathy. The patient was diagnosed in 1993 with a metastatic small bowel NET, for which he twice underwent hepatic surgery, with metastatic resection in 1993 and a right hepatectomy in 2002 to remove two hepatic metastases. In 2003, the patient started first-line chemotherapy and in 2004 started the first of three consecutive biological treatments, followed by radio-molecular therapy, achieving stable disease for 14 years. Disease progression was identified and he underwent an endoscopic retrograde cholangiopancreatography. However, in 2019 advanced liver disease was identified. We diagnosed the development of acquired hepatocerebral degeneration, an unusual long-term side effect after multiple hepatic procedures.

## CONCLUSION

The importance of regular and ongoing surveillance in long-term NET survivors who undergo hepatic procedures should be integrated into the therapeutic management plan, as some of these negative outcomes could be prevented.

**Key Words:** Neuroendocrine tumors; Hepatocerebral degeneration; Parkinsonism; Somatostatin analogues; Everolimus; Hepatic metastases; Peptide radionuclide receptor therapy; Encephalopathy; Paramagnetic deposits; Case report

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**Core Tip:** To the best of our knowledge, this is the first case report of acquired hepatocerebral degeneration in a metastatic small bowel neuroendocrine tumor long-term survivor, an uncommon irreversible extrapyramidal neurodegenerative condition encountered in patients with cirrhotic chronic liver disease, and resulting in widespread cerebral, basal ganglia, and cerebellar damage.

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## INTRODUCTION

Neuroendocrine neoplasms (NENs) are a rare group of cancers accounting for about 0.05% of all newly diagnosed malignancies and 0.5% of all gastrointestinal and lung malignancies[1-3]. Nonetheless, the incidence rate increased 6.4-fold from 1973 to 2012[2,4]. NENs are a heterogeneous group of malignancies with a slightly higher female preponderance, and are most commonly found in the gastrointestinal tract and lungs[5].

The neuroendocrine system encompasses not only the endocrine glands but is also scattered throughout the exocrine parenchyma, the so-called diffuse endocrine system[6,7]. Histologically, NENs are clustered into two main groups. On one hand, neuroendocrine tumors (NETs) are typically well-differentiated tumors characterized by uniform nuclei with dense granules, histologically described as “salt and pepper.” By contrast, neuroendocrine carcinomas have a poorly defined phenotype with a high mitotic index, and up to 40% do not express neuroendocrine markers[6,7]. Diagnosis confirmation must always be accompanied by a biopsy of the primary tumor or metastases. The 2017 World Health Organization classification takes into account the grade of differentiation and the Ki-67 mitotic proliferation index, distinguishing four groups; G1, G2 and G3 NETs and neuroendocrine carcinomas. Ki-67 grading is an important prognostic factor, and is therefore a mandatory biomarker in pathological reporting[8-10].

Liver metastases represent another crucial prognostic factor. Surgery of metastases

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is the only treatment that offers a cure[11]. For unresectable lesions, there are multiple treatment options such as somatostatin analogues (SSA), interferon  $\alpha$ , local liver therapies, chemotherapy, peptide-receptor radionuclide therapy, angiogenesis inhibitors, and mammalian rapamycin inhibitors. SSA have both anti-secretory and antiproliferative effects, improving progression-free survival in both the PROMID trial (octreotide LAR *vs* placebo) and the CLARINET (lanreotide *vs* placebo) trial[12,13]. The NETTER-1 trial reported prolongation of progression-free survival (PFS) after treatment with  $^{177}\text{Lu}$ -Dotatate compared to treatment with octreotide in patients with a well-differentiated midgut-NET[14]. Notably in the case of gastrointestinal NETs, the certainty of evidence is highest for the combination of SSA plus  $^{177}\text{Lu}$ -dotatate[15].

Nonetheless, the downside of these options is that many of these treatments can result in injury of healthy liver parenchyma and development of sinusoid liver fibrosis, and consequently induce portal hypertension with progression to advanced liver disease. The main complications of chronic liver disease are hepatocellular carcinoma and portal hypertension[16,17]. According to a study published in 2013 by Tryc *et al*[17], about 4% of cirrhotic patients develop progressive hypokinetic-rigid syndrome, which is not present in hepatic encephalopathy, recently referred to in the literature as “cirrhosis-related-Parkinsonism” or “acquired hepatocerebral degeneration (AHD).” The most commonly reported symptoms of patients with AHD are bradykinesia, cerebellar symptoms, tremor, and rigidity[16,18,19].

It has been hypothesized that AHD originates from increased manganese deposits in the basal ganglia, particularly in the globus pallidum, damaging the presynaptic dopamine transporters and post-synaptic dopamine receptors in cirrhotic patients[17,20,21]. Treatment with levodopa can be effective when D2 receptors are available[17,22]. The study by Rose *et al*[20] analyzing postmortem human brain tissue, demonstrated an increase in manganese deposits in several brain structures of cirrhotic patients. The two main causes of increased manganese deposits that the authors found to be statistically significant resulted both from portocaval-shunt and liver dysfunction[20]. This manuscript is the first case in the literature to report AHD in a metastatic gastrointestinal NET long-term survivor.

## CASE PRESENTATION

### Chief complaints

A 70-year-old male patient presented in January 2019 to the emergency department with diminished consciousness and disorientation, without any other relevant symptoms.

### History of present illness

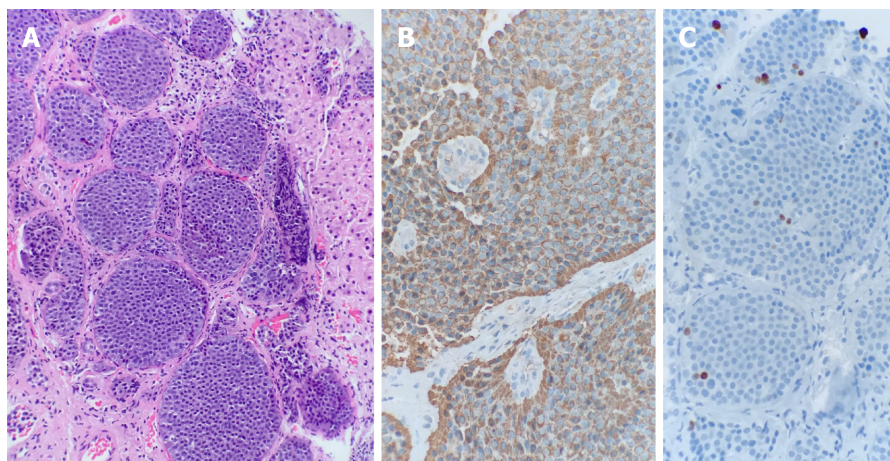
Neurological symptoms were first reported in May 2017 and asymmetric Parkinson's disease diagnosed in June 2018 for which he received levodopa.

### History of past illness

The patient had a medical history of high blood pressure, which was treated with diuretics. The patient denied use of potentially hepatotoxic drugs. He also had diabetes mellitus type 2 treated with metformin, without any other cardiovascular risk factors. He underwent a gastrectomy and Billroth II reconstruction in March 1993 for a gastric ulcer.

His oncological history started in 1993 when he was diagnosed with a metastatic midgut NET confirmed by a hepatic biopsy, in which the anatomic pathology reported a well-differentiated tumor with a Ki-67 expression of 1.26%, graded as a G1 tumor (Figure 1), and without hepatic enzyme alterations. He subsequently underwent two hepatic procedures: a single metastasis resection from the right hepatic lobe in July 1993 and a right hepatectomy was performed in 2002 to remove two hepatic metastases. There were no changes to laboratory data or computed tomography (CT) scans after both procedures.

In November 2003, the patient started first-line chemotherapy with streptozotocin and doxorubicin after a new hepatic lesion appeared. In 2004, the patient showed hepatic progression and began treatment with the biological agent octreotide. He achieved stable disease lasting until 2010, when a CT scan showed a new hepatic lesion in the surgical bed, three sub-centimeter hepatic lesions, and a new adenopathy in the hepatic hilum. The treatment was discontinued. Then the patient participated in the RAMSETE clinical trial evaluating the efficacy of everolimus at 10 mg daily in non-functioning extrapancreatic NETs, and was randomized to the active treatment arm.



**Figure 1 Anatomic pathology report of the tumor biopsy.** A: Hematoxylin and eosin staining showing neoplastic neuroendocrine cells with a typical insular pattern infiltrating hepatic parenchyma; B: Chromogranin A positivity with granular/dot-like cytoplasmic staining; C: Ki-67 staining shows a proliferation rate of 1.26% in the hot-spot, and the neoplasia was graded as G1.

The lesions showed tumor shrinkage with a total reduction of 23%, corresponding to stable disease per RECIST v1.1. The only side effects were low platelet counts and grade 1 pneumonitis (CTCAE v5.0). After 5 years with stable disease, a CT scan showed progressive disease in July 2015, and he changed to a third biological treatment, lanreotide at 120 mg monthly, again achieving stable disease as the best response.

In June 2017, he presented to the emergency department with cholangitis due to extrinsic compression of the bile duct from hepatic lesions, and a choledochal stent was inserted by endoscopic retrograde cholangiopancreatography. The CT scan showed progressive disease in the liver. A somatostatin receptor scintigraphy revealed liver, hepatic hilum and peritoneal uptake, and he started peptide receptor radionuclide therapy (PRRT) with  $^{177}\text{Lu}$ -Dotatate for three sessions and 30 mg octreotide LAR monthly, achieving partial response as the best response. One year later in July 2018, the patient returned to the emergency room with a new episode of cholangitis. Hepatic magnetic resonance imaging (MRI) showed extensive progression in the surgical bed, invading the biliary stent and causing partial obstruction. A new endoscopic retrograde cholangiopancreatography was performed to unblock the bile duct and antibiotic treatment was administered. At discharge, the patient continued on octreotide LAR, and had an ongoing best response of stable disease at the time of presentation to the emergency department in January 2019.

It should be noted that in May 2017, the patient began to complain of memory loss and distal tremor, but it was not until June 2018 that he was diagnosed with asymmetric Parkinson's disease by a neurologist. At this time, the CT scan showed heterogeneous liver with signs of portal hypertension. The patient began treatment with levodopa for Parkinson's disease, without a significant clinical response.

### **Personal and family history**

There was no relevant family history.

### **Physical examination**

On physical examination, he presented with flapping, jaundice, facial amimia, and cogwheel rigidity in both arms. There were no signs of Kayser-Fleischer rings. All other neurological examinations were normal.

### **Laboratory examinations**

Blood tests showed low platelet count, low albumin and evidence of cholestasis, with no other relevant alterations. Urine sediment and blood culture were negative, ruling out an infectious cause. The patient presented with Child-Pugh B and MELD 8 at admission. Ceruloplasmin was within normal ranges. There were no findings of hypovitaminosis, dyselectrolytemia, hypothyroidism, hepatitis virus serologies, or autoimmunity tests.



### Imaging examinations

A head CT scan performed with intravenous contrast material revealed no evidence of intracranial hemorrhage, mass, or acute territorial infarct. However, an abdominal CT scan and brain MRI shed light on our case. The CT scan showed signs of portal hypertension, describing splenomegaly and splenic dilatation with collateral circulation, as well as an intrahepatic portosystemic shunt without biliary tract obstruction (Figure 2A and B). Surprisingly, brain MRI showed symmetric basal ganglia hyperintensity in a T1 alteration and asymmetric extension to cerebral peduncles, compatible with deposits of paramagnetic substances (Figure 3A and B) related to the intrahepatic shunt described in the previous CT scan. A video-electroencephalography was performed and showed neuronal dysfunction of metabolic-toxic origin. To complete our analysis, the patient underwent a gastroduodenoscopy, which showed grade 3 esophageal varices and the liver MRI revealed multiple irregular metastases (Figure 4).

### FINAL DIAGNOSIS

The patient was diagnosed with hepatic encephalopathy and AHD secondary to advanced liver disease, most likely induced by a combination of previous hepatic resections, targeted therapies, and radionuclide treatment.

### TREATMENT

We started treatment with both oral and rectal laxatives, banding, and beta blockers at increasing dosage, improving the hepatic encephalopathy symptoms without developing side effects, thus restoring the patient to his basal state.

### OUTCOME AND FOLLOW-UP

The patient was discharged after 2 wk of treatment with only a remaining rigidity of the superior left extremity, a sign of non-reversible neuronal damage due to AHD. We did not administer any further oncological treatment, and best supportive care was maintained. Levodopa treatment was stopped. The patient was alive 6 mo after discharge.

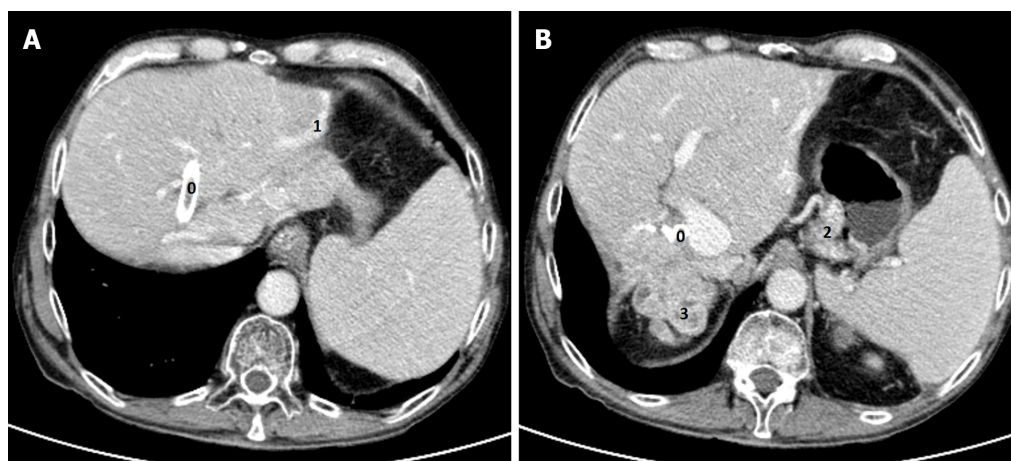
### DISCUSSION

NENs are rare and heterogeneous tumors with the particularity of secreting hormones, adding a further layer of complexity to their clinical management. On the other hand, this also gave the treating physician the opportunity to target these tumor cells with multiple approaches. We must consider carefully the treatment modalities available, since our choices will impact our patients' future. In this particular case, the patient developed an AHD after receiving multiple treatments for his metastatic midgut NET.

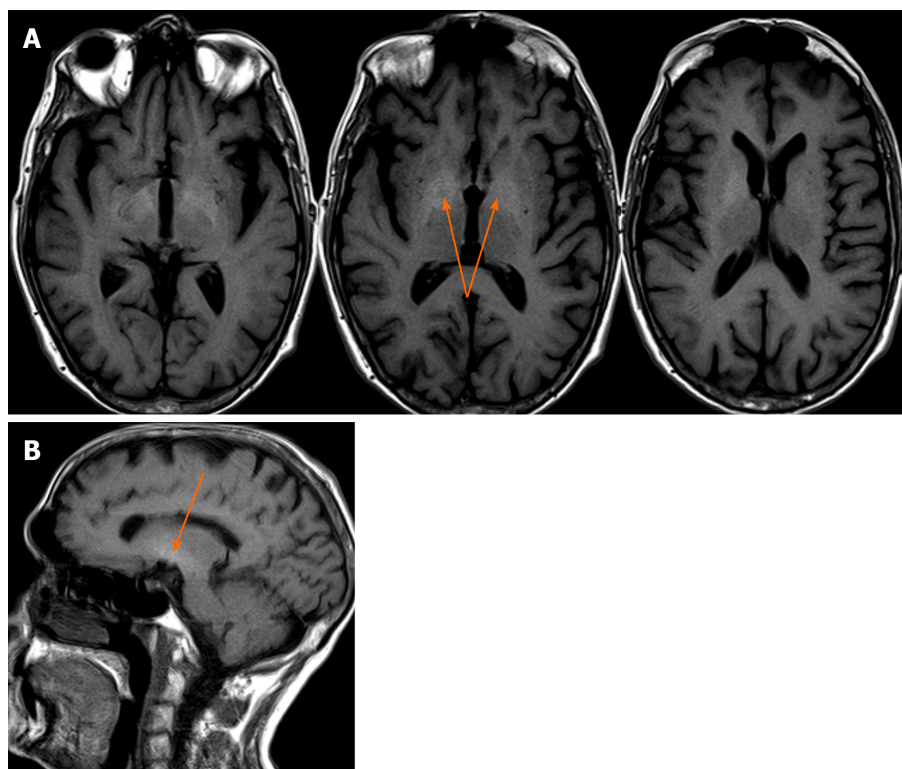
In particular, the patient underwent two hepatic surgeries and multiple hepatotoxic treatments, notably receiving four targeted therapies, three doses of PRRT, and lastly a somatostatin agonist. According to the NETTER-1 study, PRRT is superior to octreotide, but an important part of our treatment approach is to individualize the therapy according to the type of tumor, patient and treatments received previously. While PRRT treatment is a local radiotherapy that is highly selective for tumor tissue, it also affects the healthy surrounding hepatic parenchyma. Our patient had little healthy hepatic parenchyma left, and therefore had a greater susceptibility to local "ablative" therapies.

The patient experienced advanced liver disease after PRRT treatment, which likely acted as a trigger for AHD in a patient with unhealthy liver tissue, as we could identify in the CT scans and from consecutive liver laboratory tests before, during and after PRRT (Figure 5). The patient was not a candidate for closing the portosystemic shunt due to technical difficulties, and the patient's severe portal hypertension (Child B cirrhosis) and a high bleeding risk, so his only options were preventive medical treatment. Thus, the AHD symptoms of our patient persisted, since the cause was not





**Figure 2 Contrast-enhanced computed tomography (portal phase).** A: Biliary prosthesis (0), and abnormal vascular hepatic vein in the most marginal aspect of the left liver lobe related to a portosystemic shunt (1); B: Enlarged vein in the gastrohepatic ligament associated with small gastric mural varicose veins (2). Metastatic lesions at the hepatic hilum (3).

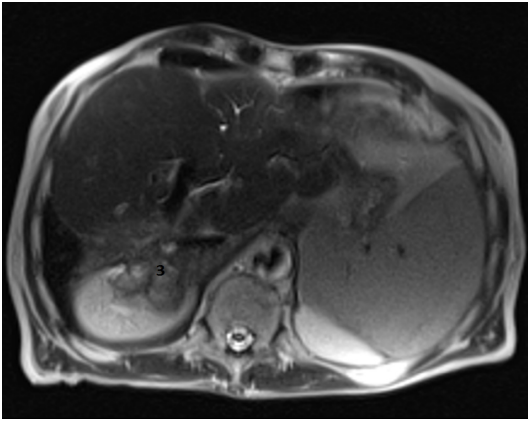


**Figure 3 Brain magnetic resonance imaging.** A: Axial; B: Sagittal projection showing T1-weighted imaging, hyperintense signal (arrow) within a lentiform nucleus extending into the midbrain.

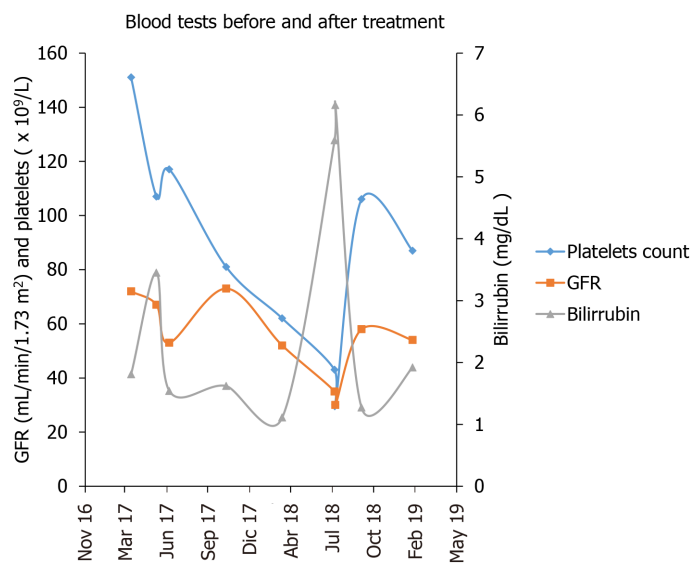
treated. On the other hand, the hepatic encephalopathy responded excellently, within days, to ammonia-lowering agents. The Parkinson's disease was initially thought to be primary, but with the extensive paramagnetic deposits in the basal ganglia and the poor response to levodopa, it would be more reasonable to consider it a Parkinsonism secondary to the portosystemic shunts and hepatic cirrhosis. As stated previously, the presence of manganese in the basal ganglia of cirrhotic patients is diagnostic of AHD and can result in irreversible neuronal damage.

## CONCLUSION

Herein, we report the first case described in the literature of AHD in a metastatic NET.



**Figure 4 Liver magnetic resonance imaging.** Axial T2-weighted imaging HASTE magnetic resonance imaging. Multiple irregular right liver metastatic lesions (3).



**Figure 5 Blood tests before and after treatment.** Laboratory test parameters showing platelet count ( $\times 10^9/L$ ), glomerular filtration rate (GFR) (in mL/min/1.73 m<sup>2</sup>) and total bilirubin (mg/dL) before and after peptide-receptor radionuclide therapy treatment.

Patients with NETs are typically long-time survivors, and we currently have multiple treatment modalities to choose from. Selection should be based on maximizing survival and reducing both the potential immediate and long-term side effects. The negative outcomes relating to hepatic injury in long-term NET survivors resemble those of patients with advanced liver disease. As such, regular monitoring and surveillance for potential complications in long-term cancer survivors should be recommended to rule out negative outcomes that may appear following treatment.

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