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EDITORIAL

Metabolic-associated fatty liver disease: New nomenclature and approach with hot debate

Yasser Fouad

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Abstract

An international panel recently proposed an update to the terminology and diagnostic criteria for fatty liver disease. The experts proposed a change in the nomenclature from non-alcoholic fatty liver disease (NAFLD) to metabolic (dysfunction)-associated fatty liver disease (MAFLD). This single-letter change, we believe, heralds the dawn of a new era in clinical practice and in clinical and basic research as well. The new nomenclature with the easily applicable approach has stimulated the enthusiasm of the researchers worldwide, resulting in a large number of publications over the past two years. Several recent studies have provided tremendous evidence of the superiority of the MAFLD criteria over the NAFLD criteria. Many studies in different geographic areas of the world including the United States, Europe, and Asia on a large number of patients proved that the utility of MAFLD criteria was higher than that of the NAFLD criteria in different aspects of fatty liver diseases. Consequently, many societies, physician and nurse groups, health stakeholders, representatives of regulatory sciences, and others endorsed the new nomenclature. Here we highlight the endorsement of the new name by different societies and groups and the outcome of different studies on the new nomenclature in addition to a short discussion of the debate by some experts.

Key Words: Non-alcoholic fatty liver disease; Metabolic-associated fatty liver disease; Liver disease; Fibrosis; New nomenclature; Redefinition

Core Tip: An international panel recently proposed an update to the terminology and diagnostic criteria for fatty liver disease. The authors proposed a change in the nomenclature from non-alcoholic fatty liver disease (NAFLD) to metabolic (dysfunction)-associated fatty liver disease (MAFLD). Several studies have been published recently, and showed tremendous evidence of the superiority of MAFLD criteria over NAFLD criteria. Consequently, many societies, physician and nurse groups, health stakeholders, representatives of regulatory sciences, and others endorsed the new nomenclature.

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INTRODUCTION

The World Health Organization (WHO) has motivated scientists, doctors, and healthcare providers to use the appropriate medical terms and change the terms according to the patient's interest and the medical care provided. This call by the WHO was to overcome the stigmas and inaccuracies that may confer upon people, regions, and economies[1].

In the recent medical history, renaming of the diseases involved primary biliary cirrhosis, schizophrenia, epilepsy, autism, and others with ongoing trials to change the term ''noncommunicable diseases'' to a better positive name to gain more medical support by governments, societies, and stakeholders[2].

Since 1980 when the non-alcoholic fatty liver disease (NAFLD) was introduced[3], several trials have been carried out to rename the disease by different scientists and societies for different reasons. In 2019, Eslam *et al*[4] proposed changing the traditional NAFLD to metabolic dysfunction-associated liver disease (MAFLD). The single-letter change means a lot for researchers, physicians, and patients. The authors explained their vision of new nomenclature by linking the fatty liver to the metabolic syndrome which is the most common and most serious etiology of fatty liver diseases and under-evaluated when using the older nomenclature. Moreover, the new nomenclature gives the clinical community a chance to avoid the stigma of alcohol intake, avoid the negativity of NAFLD nomenclature, and overcomes trivialization[2]. The simplified criteria for diagnosis of MAFLD were put forward by consensus of an international panel of hepatologists in 2020[5]. These criteria pave the way for easy diagnosis of fatty liver diseases because of easy applicability. The consensus considered the diagnosis of MAFLD based on the presence of steatosis by imaging or histopathology in addition to the presence of diabetes mellitus or obesity/overweight or two out of seven metabolic dysfunction criteria (Figure 1). The new nomenclature and approach better clarify the role of metabolic dysfunctions in fatty liver disease and make the fatty liver closer to its pathophysiology.

The new nomenclature with the easily applicable approach stimulated the enthusiasm of researchers worldwide, resulting in a large number of publications over the last two years. Several studies have been published recently, showing tremendous evidence of the superiority of MAFLD criteria over NAFLD criteria. Many studies in different geographic areas of the world including the United States (US), Europe, and Asia on a large number of patients proved that the utility of MAFLD criteria was higher than that of the NAFLD criteria in different aspects of fatty liver diseases.

Among the many important findings, MAFLD criteria could better identify patients at risk of liver fibrosis than the NAFLD criteria in the American population[6]. High diagnostic ability of fatty liver index in the detection of steatosis was seen in patients with MAFLD[7]. Fibrosis-4 index and NAFLD fibrosis score could confidently be used to exclude advanced fibrosis in overweight, obese, and severely obese patients with MAFLD[8]. MAFLD is associated with a higher incidence of hepatocellular carcinoma[9]. MAFLD (not NAFLD) predicts extrahepatic malignancy[10]. MAFLD was better than NAFLD in identifying patients at high risk of renal diseases[11]. In a recent meta-analysis, MAFLD was associated with increased severity of COVID-19[12]. Renaming to MAFLD increases awareness of the disease among primary care providers and physicians in other specialties[13]. Change to MAFLD has a positive impact on clinical trials[14,15] MAFLD identifies the severity of the coexistence of fatty liver disease with other liver diseases[16,17].

Being convinced by the reasons for changing nomenclature, evidence of the superiority of the new name MAFLD, and the benefits of the new nomenc-lature, many international societies, patient groups, stakeholders, nurse groups, and representatives of pharmacist and regulatory sciences have endorsed the new nomenclature (Table 1). In an unprecedented manner, a unique gathering of more than a thousand international experts from more than 135 countries worldwide signed an agreement on a global multi-stakeholder endorsement of the MAFLD definition published recently.

Table 1 Metabolic-associated fatty liver disease endorsement by societies, groups, and stakeholders

Type of endorsement	Endorsed by	Ref.
Guidelines	APASL	[25]
Guidelines	Egyptian EMRG group	[26]
Consensus statement	Middle East and North Africa group	[27]
View point (perspectives)	International nurse and allied health groups	[28]
Position statement	ALEH	[29]
Position statement	The Chinese Society of Hepatology	[30]
Position statement	ISTP	[31]
Position statement	Arabic Association for the Study of Diabetes and Metabolism	[32]
Consensus statement	Malaysian Society of Gastroenterology and Hepatology	[33]
Viewpoint (perspectives)	International leaders in regulatory science and drug development	[34]
Position statement	International representatives of patient advocacy groups	[35]
Letter of endorsement	Global multi-stakeholder from more than 135 countries worldwide	[36]
Editorial of endorsement	Spanish Society of Gastroenterology	[37]

APASL: Asian Pacific Association for the Study of the Liver; EMRG: Egyptian MAFLD Research Group; ISTIP: The International Society of Tropical Pediatrics; ALEH: The Latin American Association for the Study of the Liver.

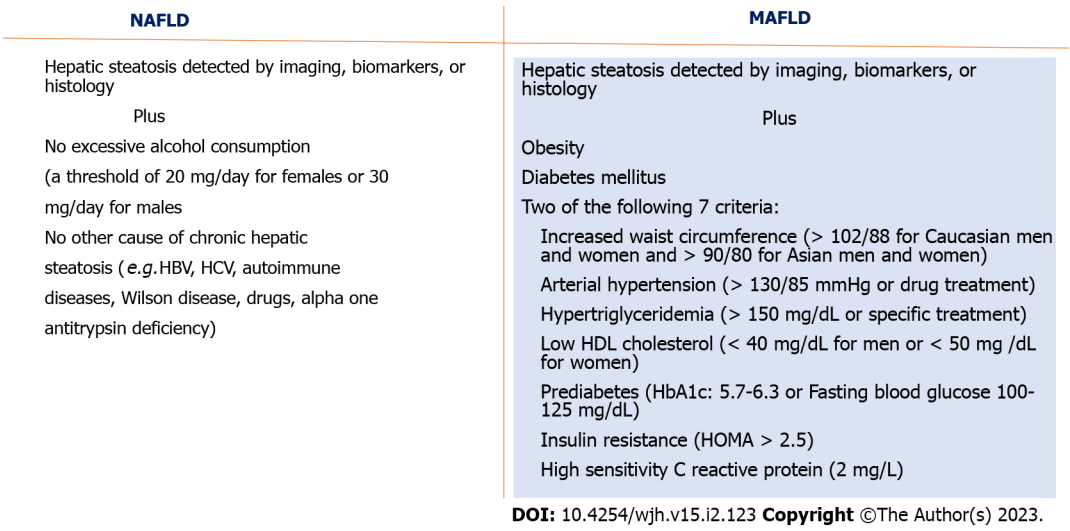

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Figure 1 Criteria for diagnosis of metabolic dysfunction-associated fatty liver disease and non-alcoholic fatty liver disease. NAFLD: Non-alcoholic fatty liver disease; MAFLD: Metabolic-associated fatty liver disease; HBV: Hepatitis B virus; HCV: Hepatitis B virus; HDL: High-density lipoprotein; HbA1c: Hemoglobin A1c; HOMA: Homeostasis model assessment.

Two major hepatology societies, The European Association for Study of the Liver and The American Association for the Study of Liver Diseases, have not endorsed the new name yet till writing this editorial. The debate from these societies focused mainly on the prematurity of change[18]. One of the main debates is about non-metabolic or lean NAFLD. Evidence proved that the non-metabolic NAFLD group seems to be comparable to subjects with no fatty liver in terms of cardiovascular-related mortality as well as all-cause mortality. Moreover, the non-metabolic NAFLD group seems to be at a very low risk of fibrosis (0.8%)[19]. Another concern was about pediatric NAFLD. In a recent study involving 1446 US adolescents aged 12–18 years from the National Health and Nutrition Examination Survey III, MAFLD criteria were met by most of these US adolescents with elastographic evidence of steatosis[20]. Additional debate was about clinical trials. In a recently published paper, a group of researchers declared that the new name and approach with positive inclusion criteria lead to easier recruitment of patients and are more likely to give positive results[21]. Being in the era of evidence-based medicine, we believe that the need for an evidence-based debate is mandatory. Once again, the MAFLD conceptual framework removes the concept that there is no alcohol involvement, links the liver disease

which is commonly seen in metabolic dysregulation with its systemic effects, and performs better in patient identification, risk stratification, disease awareness, and networking with metabolic disease physicians[22,23].

The important question in the current situation is why some experts do not change their attitude toward the new nomenclature despite the obvious conspicuous evidence. The answer is not clear although, pleasingly, since the very beginning, the weight of evidence appears to have led to the persuasion of an ever-increasing number of stakeholders on the increasing benefits. Another important issue is that experts who advocate against the redefinition despite the robust evidence should explain to the hepatology community how and why we discard the rapidly progressive growing body of new literature[24].

CONCLUSION

In summary, we have a redefinition of a very prevalent disease worldwide. The new nomenclature MAFLD is simple, with superior utility, and is supported by a tremendous amount of evidence. It is endorsed by many societies and full global adoption is a matter of time.

FOOTNOTES

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REVIEW

Current status and prospect of treatments for recurrent hepatocellular carcinoma

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Abstract

Owing to its heterogeneous and highly aggressive nature, hepatocellular carcinoma (HCC) has a high recurrence rate, which is a non-negligible problem despite the increasing number of available treatment options. Recent clinical trials have attempted to reduce the recurrence and develop innovative treatment options for patients with recurrent HCC. In the event of liver remnant recurrence, the currently available treatment options include repeat hepatectomy, salvage liver transplantation, tumor ablation, transcatheter arterial chemoembolization, stereotactic body radiotherapy, systemic therapies, and combination therapy. In this review, we summarize the strategies to reduce the recurrence of high-risk tumors and aggressive therapies for recurrent HCC. Additionally, we discuss methods to prevent HCC recurrence and prognostic models constructed based on predictors of recurrence to develop an appropriate surveillance program.

Key Words: Review; Recurrence; Hepatocellular carcinoma; Hepatectomy; Liver transplantation; Transcatheter arterial chemoembolization

Core Tip: The current rate of recurrence after initial hepatocellular carcinoma treatment remains unsatisfactory. Repeat hepatectomy and salvage liver transplantation are the preferred options for patients who meet the criteria. However, for patients whose clinical situation do not allow these treatments, non-surgical treatment can also provide survival benefits. Additionally, adjuvant treatment strategies to prevent recurrence and proper surveillance are effective tools to improve overall patient survival. This review summarizes the existing literature to help guide clinical decision-making and provide directions for further research.

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INTRODUCTION

Hepatocellular carcinoma (HCC), a heterogeneous disease with multiple etiologies, is the major subtype of primary malignancies of the liver, accounting for 70%-85% of primary liver cancers[1]. Globally, HCC is the third most common cause of cancer-related mortality, and its incidence is rising[2]. Treatment options for HCC have improved, but frequent recurrence after treatment is a major concern. International guidelines provide detailed treatment options for each stage of HCC, and depending on the patient's liver function and tumor burden, treatment options vary from radical treatment options, such as resection, transplantation, ablation, and combination therapy, to palliative treatment options, such as transcatheter arterial chemoembolization (TACE), systemic therapy, and supportive care. Although hepatectomy is the preferred option for patients with HCC who meet the criteria, 67.6% of patients develop tumor recurrence or metastasis after hepatectomy[3]. Moreover, few patients can undergo radical hepatectomy due to insufficient liver function reserve, vascular invasion, extrahepatic metastases, and the size and number of lesions[4]. With the continuous development and maturation of transplantation technology, liver transplantation has become the best long-term treatment for patients with early-stage HCC. However, liver transplantation also has limitations, including a 25% risk of recurrence even if the patient meets the strict Milan criteria and a lack of donor organs, which limit the use of transplantation[5]. Ablation is another way to treat patients with small HCC who are not candidates for surgery due to comorbidities, liver dysfunction, or tumor location. However, the risk of recurrence after ablative therapy is as high as 80%; therefore, this option is limited to patients who cannot undergo surgical resection but are suitable for liver-directed therapy[6]. The combination of TACE and ablation is one of the most widespread and efficacious combination therapies. The latest version of the Barcelona Clinic Liver Cancer (BCLC) guidelines suggests that the combination of TACE and ablation as a radical treatment solution for 3-5 cm masses has the advantage of reducing heat deposition and expanding the scope of ablation compared with a single treatment option[7]. Nevertheless, 76.4% of patients undergoing TACE with ablation develop recurrence, probably because of the presence of portal vein collateral circulation and high alpha-fetoprotein (AFP) levels[8]. Finally, palliative care options mostly play a role in improving the symptoms and quality of life of patients with advanced HCC that is incurable. Given the high risk of recurrence with radical treatment regimens, refining and optimizing treatment options for recurrent liver cancer are urgent issues.

In addition to differences in recurrence risk, various treatment modalities have varying patterns of recurrence, which affects the choice of treatment options for recurrent HCC. In general, hepatectomy is mostly associated with intrahepatic recurrence, with few extrahepatic metastases, most probably due to residual minuscule lesions. Lee *et al*[9] observed that tumor recurrence after resection was detected in the liver in 80.1% of patients and suggested that curative therapeutic results might be achieved through repeat hepatectomy or local ablation. In addition, HCC recurrence can be classified into early and late recurrence, depending on the time of recurrence after surgery. It is generally believed that early recurrence may be associated with tiny preoperative or intraoperative metastases and the continued growth of tiny postoperative residual lesions, mostly close to surgically resected lesions. Late recurrences are mostly new tumors arising from the malignant transformation of normal liver cells due to latent cancer-causing factors in the liver, such as frequent recurrent inflammation of the liver and cirrhotic fibrosis[10]. There is no consensus on the dividing line between early and late recurrences; however, a 2-year cutoff after resection has been widely used to distinguish between the two types of HCC recurrence[11]. Treatment options for recurrent HCC after resection vary according to the type of recurrence pattern and timing. The best treatment plan should be developed by fully integrating multiple treatments and following the principle of the maximum benefit to the recipient.

Similarly, with the demarcation line being set at 2 years, HCC recurrence after liver transplantation can be divided into early and late recurrence. A higher original tumor burden and more aggressive

features may account for early recurrence in patients who undergo liver transplantation[12]. A high primary tumor burden predisposes to missed or undetectable extrahepatic metastases before transplantation, leading to the recurrence of HCC. Similarly, more aggressive tumors tend to trigger the engraftment and growth of circulating HCC cell clones in the target organ after transplantation[12].

Early recurrent HCC tends to involve multiple organs and has a poor prognosis; therefore, its treatment plan should be selected carefully[13]. In contrast, late recurrence appears to be the result of transplantation of a small number of latent advanced HCC cells, and patients tend to have more favorable tumor characteristics at this time; thus, TACE and local ablation may be capable of achieving positive outcomes[5]. Radiofrequency ablation (RFA) is one of the main applications of ablation therapy, which is typically performed for unresectable solitary tumors < 3 cm in diameter and has comparatively high safety and efficacy. However, RFA is prone to leaving residual tumor cells owing to incomplete ablation, thus causing local recurrence[4]. Heat dissipation effects and tumor size are the primary limiting factors for RFA, and combination therapy may be a solution[4].

Prevention and treatment of recurrent HCC have become an urgent issue. In this review, we evaluate the available evidence on the effectiveness of adjuvant therapy, summarize the treatment options for the recurrence of primary liver cancer after treatment, and describe appropriate monitoring protocols for predictors of liver cancer recurrence to ultimately identify the optimal management strategy for patients with recurrent liver cancer.

ADJUVANT THERAPY TO PREVENT RECURRENCE OF PRIMARY LIVER CANCER

Given the high recurrence rate after HCC treatment, adjuvant therapy has been proposed to reduce the risk of HCC recurrence and further improve the long-term survival of patients with liver cancer. Nonsurgical therapy, including antiviral therapy, TACE, systemic therapy, radiation therapy, and other strategies, may be performed preoperatively to improve liver function or postoperatively to improve patient survival outcomes.

Antiviral therapy

Previous studies have shown that high hepatitis B virus (HBV) levels, HBV e-antigen positivity, and HBV reactivation are strongly associated with a high risk of recurrence of HBV-related liver cancer after resection[14]. Similarly, a recent study showed that in HCC patients with viral infection who underwent living liver transplantation, HBV recurrence tended to cause HCC recurrence, and hepatitis D virus infection was considered an independent risk factor for HBV-HCC co-occurrence after transplantation [15]. This suggests that antiviral therapy plays an essential role in the prevention of postoperative recurrence of viral hepatitis-related HCC.

Currently, the primary antiviral treatments include nucleoside analogs (NAs), interferons, and direct antiviral agents (DAAs)[16]. NAs can significantly reduce the incidence of HBV-associated HCC by lowering the patient's HBV load. Several studies have confirmed the effectiveness of NAs in preventing liver cancer[17,18]. The Asian Pacific Association for the Study of the Liver guidelines on the management of HCC state that NAs can be utilized as secondary prevention for the development of HBV-associated HCC[19]. Interferons are broad-spectrum antiviral agents that act mainly through the action of cell surface receptors to produce antiviral proteins, thereby improving the body's immune regulation ability, inhibiting the replication of HBV, and enhancing antiviral ability[20]. Interferons conjugated to polyethylene glycol are particularly effective in preventing HBV-associated HCC[21]. A meta-analysis demonstrated that interferon therapy reduced recurrence in patients with hepatitis-associated HCC whose tumors did not exceed 3 cm in diameter[22]. Moreover, a randomized controlled trial published by Lo *et al*[23] in patients with HBV-related HCC showed that the 5-year survival rate was improved from 24% to 68% (P = 0.038) in patients receiving postoperative adjuvant interferon therapy, particularly in those with pTNM stage III/IVA tumors. DAAs effectively inhibit viral replication and are highly efficacious in the treatment of HCC caused by hepatitis C virus (HCV) infection. A systematic review that included 24 studies reported that patients treated with DAAs had an acceptable risk of recurrence, with a recurrence rate of 21.9% [95% confidence interval (CI): 16.2-28.3][24]. Combinations of multiple antiviral therapies are also a worthwhile adjuvant treatment option for patients with HBV-associated recurrent HCC, with combination strategies significantly improving the antiviral efficacy and long-term patient survival compared with monotherapy[25,26].

TACE

TACE is the standard of care for intermediate to advanced HCC and the primary method of bridging or step-down therapy before liver transplantation[27]. Many studies have shown that TACE as an adjuvant therapy has certain advantages in improving the prognosis of patients with HCC and preventing cancer recurrence. Liu *et al*[28] systematically analyzed the outcomes of 117 patients with HCC who underwent hepatectomy between 2010 and 2014 and received postoperative TACE and found that postoperative TACE improved the 1-year disease-free survival (DFS) compared with surgical resection only (64.5% *vs* 45.5%, P = 0.04). In addition, they recommended postoperative TACE for patients with tumors > 5 cm

with microvascular invasion or satellite nodules[28]. Other studies also support this view and concluded that postoperative TACE is a safe intervention to prevent tumor recurrence in patients with BCLC early- and intermediate-stage HCC with microvascular invasion[29-31]. However, preoperative TACE is controversial, and a meta-analysis of randomized controlled trials based in Asia showed that preoperative TACE did not improve the long-term prognosis of patients with resectable HCC, possibly because of the risk of tumor progression or deterioration of liver function in patients undergoing TACE [32].

Radiation therapy

Because HCC is a relatively radiation-sensitive tumor, radiation therapy is one of the commonly used treatments for liver cancer. A recent systematic review evaluating the impact of different postoperative treatments on patients with HCC with microvascular invasion after radical resection revealed that postoperative radiotherapy is more effective in reducing recurrence than postoperative TACE[33]. Yoon *et al*[34] shared the same view and concluded that the combination of TACE and radiotherapy is a promising treatment option to alleviate symptoms in patients with HCC and portal vein tumor thrombosis. A narrow-margin (< 1 cm) hepatectomy is prone to residual microscopic lesions that can spread through intrahepatic vessels and lead to recurrence due to detailed control issues during the procedure. However, a prospective randomized study found that adjuvant radiotherapy for central HCC after narrow-margin hepatectomy is technically feasible and relatively safe. Subgroup analysis showed that adjuvant radiotherapy significantly improved recurrence-free survival (RFS) in patients with HCC ≤ 5 cm in diameter, although there was no difference in overall survival (OS)[35]. An additional prospective phase 2 study concurred with this finding and suggested that intraoperative electron radiotherapy was more beneficial for survival in patients with microvascular infiltration after resection[36].

In 1999, Lau *et al*[37] first proposed that adjuvant therapy with intra-arterial administration of 1850 MBq of ^{131}I-labeled lipiodol after radical resection significantly reduced recurrence in patients with HCC and improved DFS and OS. However, Chung *et al*[38] found that administration of adjuvant intra-arterial ^{131}I-labeled lipiodol after resection showed negligible improvement in controlling HCC tumor recurrence and that patients were at risk for hypothyroidism and hepatic artery dissection during angiography. Conversely, several meta-analyses have positively evaluated the efficacy of adjuvant treatment with intra-arterial ^{131}I-lipiodol[39-41]. A systematic review including three case-control studies and two randomized controlled trials showed robust evidence that adjuvant ^{131}I-labeled lipiodol prolongs DFS and OS by up to 5 years after resection in patients with sound liver function and low microvascular invasion[40]. Therefore, more well-designed, randomized pilot studies are required to draw solid conclusions.

Adjuvant chemotherapy

Chemotherapy is the most widely administered cancer treatment. Generally speaking, chemotherapy is mostly utilized in the systemic treatment of primary liver cancer; however, with the development of modern technology, regional adjuvant chemotherapy also plays an important role in the prevention of liver cancer recurrence. However, chemotherapy has its limitations, as many drugs kill both cancer and healthy cells[42]. Therefore, chemotherapy is also utilized in combination with other therapies, such as surgery, radiotherapy, and immunotherapy, which have shown positive synergistic effects. As early as 1996, Yamamoto *et al*[43] systematically analyzed the efficacy of oral adjuvant chemotherapy in 67 patients with HCC who underwent radical resection between 1988 and 1990. They found that the OS and RFS were significantly higher in patients who received adjuvant oral 1-hexylcarbamoyl-5-fluorouracil than those who did not among patients with mild hepatic dysfunction, but no significant differences in survival were observed in patients with moderate hepatic dysfunction[43]. A subsequent randomized controlled trial had a different conclusion on the controversial question of whether adjuvant chemotherapy after resection can prevent recurrence of HCC. This trial showed similar relapse-free survival rates in the postoperative oral uracil-tegafur (UFT) and no adjuvant therapy groups and a significantly higher proportion of late recurrence in the UFT group than in the control group (74% *vs* 53%, $P = 0.02$)[44]. Interestingly, Ueda *et al*[45] discovered that adjuvant chemotherapy with UFT after TACE significantly prolonged the time to treatment failure in patients with advanced HCC, and no serious adverse events were observed with this regimen. This regimen may have adjuvant and anti-angiogenic functions in the treatment of advanced HCC. In addition, Nagano *et al*[46] found that adjuvant interferon-/5-fluorouracil could benefit patients with advanced HCC after palliative hepatectomy. Therefore, combining chemotherapy with another treatment may be a solution to the poor efficacy of adjuvant chemotherapy when applied alone. Similarly, adjuvant chemotherapy after liver transplantation can provide survival benefits. A systematic evaluation and meta-analysis showed that implementing adjuvant chemotherapy early after liver transplantation in patients with advanced HCC can significantly prolong patient survival and delay liver cancer recurrence[47].

Hepatic arterial infusion chemotherapy (HAIC) is a type of chemotherapy primarily administered to patients with advanced intrahepatic HCC, such as those with major portal vascular invasion and intrahepatic multinodular lesions with Child-Pugh class B liver function[48]. As patients with HCC with vascular invasion tend to have a poor prognosis after surgical resection, the postoperative adminis-

tration of HAIC has been increasingly emphasized by investigators. A retrospective study that included 73 patients with HCC with visible vascular invasion found that DFS was significantly higher in the hepatic resection with HAIC group than in the control group without HAIC (33.1% *vs* 11.8%, $P = 0.029$) after 5 years of follow-up; however, there was no significant difference in OS between the two groups [49]. Hsiao *et al*[50] had similar findings and suggested that patients with HCC with multiple small nodules in close proximity to each other or a single large tumor with several satellite nodules could achieve greater benefit when HAIC was performed as an adjuvant treatment after resection. Preoperative HAIC can also be a means of downstaging before resection in patients with advanced HCC. Lee *et al*[51] showed that the median survival time and response rate of patients with advanced HCC who underwent hepatectomy after preoperative HAIC were 14 ± 1.7 mo and 26.4%, respectively.

The drug combinations for HAIC are also being continuously explored by scholars in various countries. A Japanese HAIC study compared the outcomes of 476 patients with HCC who received HAIC (5-fluorouracil and cisplatin) with 1466 patients who did not receive active treatment and showed that the median survival time was longer in patients who received chemotherapy (14.0 mo) than in those who did not receive active treatment (5.2 mo, $P < 0.0001$)[52]. However, several cisplatin (DDP)-based HAIC regimens are dose limited by renal, neurological, and gastrointestinal toxicity, making it difficult to achieve the desired outcomes[53]. In contrast, with the publication of the EACH study[54], oxaliplatin is coming into the limelight as a systemic chemotherapeutic agent. The study explored whether infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) as palliative chemotherapy for patients with advanced HCC provides survival benefit and efficacy compared with doxorubicin, and found that this regimen may offer some benefits for Asian patients[54]. Subsequently, Chinese scholars modified and applied the FOLFOX regimen to HAIC and achieved impressive results. In the ASCO 2021 meeting, Li *et al*[55] first explored the efficacy of neoadjuvant HAIC (FOLFOX regimen) and compared it with that of direct surgery in patients with HCC with ultra-Milan standard BCLC stage A/B; they found that the objective response rate (ORR) in the neoadjuvant HAIC group reached 63.6%, and the disease-control rate reached 96.0%. Furthermore, the team found that this protocol was also effective in HCC patients with microvascular invasion. The study showed that patients who received one or two cycles of postoperative adjuvant arterial perfusion chemotherapy had significantly better OS and DFS compared to patients without any adjuvant therapy (97.7% *vs* 78.5%; 58.7% *vs* 38.6%; $P = 0.037$ and 0.023, respectively)[56]. Thus, HAIC based on the FOLFOX regimen is gaining more and more attention in the academic community for its high ORR and surgical conversion rate.

Systemic therapy

The liver tumor microenvironment has complex immune tolerance capabilities[57]. Immunotherapy can enhance the body's immune response, break the immune tolerance of the tumor microenvironment, and reactivate immune cells to recognize and kill tumor cells. Immunotherapies mainly include adoptive cell transfer-based therapies, tumor vaccines, and immune checkpoint inhibitors (ICIs)[58]. Adoptive cell transfer-based therapy involves isolating immunocompetent cells from the bodies of cancer patients. Through cytokine stimulation, *in vitro* culture, or tumor antigen loading, a large number of amplifications and functional identifications are performed *in vitro,* and then cells are injected back into the patient's body. These cells are now primed to enhance the patient's immune function and kill tumor cells. Cytokine-induced killer cells (CIKs) and genetically modified natural killer or T cells are the main immune cells used for this process in liver cancer[58]. A randomized trial published by Takayama *et al* [59] in 2000 first demonstrated the safety and efficacy of adoptive immunotherapy in reducing recurrence and improving patient survival after HCC resection. A study of patients with HCC undergoing curative therapy also showed that adjuvant injection of activated CIKs improved RFS and OS[60]. However, other studies have shown a limited effect of adoptive T cell therapy in solid tumors, possibly due to the poor persistence of adoptive T cells *in vivo*, their cytotoxicity, and other defects[61]. Tumor vaccines are immunotherapies in which the patient's tumor antigens are infused back into the patient in various forms to enhance immunogenicity, thereby activating the patient's immune system to attack tumor cells. This is the theoretical basis of tumor vaccine treatment for liver cancer[61]. Repáraz *et al*[62] indicated that tumor vaccines have significant potential in combination with ICIs for the prevention and treatment of HCC. A recent review that included 31 clinical trials worldwide held the same opinion and concluded that HBV-associated HCC may benefit more from tumor vaccines than HCV-associated HCC[63]. Currently, tumor vaccines for patients with HCC mainly include dendritic cell (DC) vaccines, AFP vaccines, and other vaccines. DC vaccines, a common tumor vaccine, can provide clinical benefits to patients with HCC by stimulating antitumor T cell responses without significantly increasing toxicity[64]. AFP vaccines are peptide-based tumor vaccines used in HCC and are characterized by low immunogenicity and tolerance to the host immune system[62]. Immune checkpoints play a protective role in the body's immune system by preventing excessive activation of T cells from damaging the body's tissues. Cytotoxic T lymphocyte-associated antigen-4 and programmed death 1 are the two main immune checkpoints considered in the treatment of HCC, and ICIs developed against these checkpoint molecules have been widely adopted clinically for liver cancer. Numerous studies have reported ICIs as an appropriate therapy option pre- and post-transplantation, but most of these studies were retrospective or case reports; therefore, ICIs should be administered to patients undergoing liver transplantation with caution[65]. Similarly, there is a shortage of randomized

controlled trials of ICIs after HCC resection or ablation, although several relevant trials are underway, testing drugs such as pembrolizumab (KEYNOTE-937, NCT03867084), nivolumab (CheckMate 9DX, NCT03383458), and atezolizumab plus bevacizumab (IMbrave050, NCT04102098), which are expected to yield promising results.

Molecular targeted therapy is of epoch-making significance in the field of cancer treatment and is mainly based on the pathways involved in the pathogenesis of cancer. Molecular targeted therapeutics specifically cause the death of tumor cells. Sorafenib is an approved multi-target tyrosine kinase inhibitor for the treatment of patients with advanced and unresectable HCC[66]. Numerous retrospective studies have shown that adjuvant sorafenib treatment improves recurrence and prolongs survival, especially in patients at high risk of postoperative recurrence[67-69]. However, a phase 3, randomized, double-blind, placebo-controlled trial (STORM trial) evaluating the efficacy of adjuvant sorafenib after resection or ablation of HCC found no difference in the median RFS between the adjuvant sorafenib and placebo groups (33.3 mo *vs* 33.7 mo, $P = 0.26$)[70]. Sorafenib treatment in the perioperative period of liver transplantation is equally ineffective and strongly associated with a worse prognosis[71]. In contrast, lenvatinib has shown promising results as an adjuvant therapy for patients who have undergone liver transplantation. A retrospective case-control study showed that adjuvant lenvatinib can prolong DFS in patients with high-risk HBV-related HCC following liver transplantation [72]. Bevacizumab, an angiogenesis inhibitor, has shown poor results as adjuvant therapy in patients with HCC. Pinte *et al*[73] found that patients treated with adjuvant bevacizumab after TACE not only had no improvement in OS but also developed sepsis and vascular side effects. Consequently, for the prophylactic treatment of patients with HCC, adjuvant treatment strategies with molecular-targeted drugs should be carefully selected.

TREATMENTS FOR RECURRENT HCC

The treatment of recurrent liver cancer is mostly based on the diagnosis and treatment guidelines for primary liver cancer[74] combined with clinical experience. Multiple studies have shown that surgical resection, liver transplantation, and non-surgical treatment (such as ablation and TACE) for recurrent HCC can lead to survival benefits comparable to those of the first treatment[75-79]. However, most of these were small-sample studies at a single institution, the evidence is weak, and the results are difficult to generalize. Ideally, treatment strategies for recurrent HCC can be based on the same criteria as those for primary cancer; however, given the intratumoral heterogeneity and different clonal lineages between primary and recurrent HCC, it is still advisable to perform a comprehensive overview of the tumor before choosing the best treatment modality. In addition, patient characteristics (such as sex, age, and psychological state), conditions of the first operation (such as surgical area and main blood vessels severed during the first operation), and basic liver function status should also be comprehensively evaluated. A suggested flowchart to guide treatment decision-making in the setting of recurrent HCC is presented in Figure 1.

Repeat hepatectomy

Hepatectomy remains a safe and effective treatment for recurrent HCC. Reoperation in patients with HCC with good liver function significantly prolongs survival, especially in patients exhibiting recurrence within 2 years and those with a primary tumor burden exceeding the Milan criteria[80,81]. Yoh *et al*[3] observed similar findings; in their study, 128 patients who underwent repeat surgery had better liver function and a significantly longer time to recurrence than 548 patients who did not undergo reoperation (16.5 mo *vs* 11.4 mo; $P < 0.001$). Although repeat hepatectomy is most commonly performed for patients with intrahepatic metastases, surgical resection can also provide benefits to patients with recurrent extrahepatic lesions under conditions of limited isolation of metastases, preservation of liver function, and adequate control of the primary tumor[82]. Repeat hepatectomy is also a recommended treatment option for patients with recurrent HCC occurring more than 18 mo after the initial resection, and survival rates are significantly higher for patients with multiple distant metastases than for those with intrahepatic metastases[83]. Numerous retrospective studies have suggested that appropriately selected patients undergoing partial hepatectomy can achieve long-term survival after both initial hepatectomy and liver transplantation, with 5-year OS and RFS rates ranging from 22%-84% and from 10%-43%, respectively (Table 1). Third repeat hepatectomy is also a promising technique for recurrent tumors, and it has been reported that three or more repeat hepatectomies for recurrent HCC are reasonable and safe; however, they should be performed with caution because of the high recurrence rate, long operative duration, and high patient selectivity of resection[84,85]. For recurrent HCC after liver transplantation, patients who undergo repeat hepatectomy tend to have a worse prognosis and are more susceptible to deterioration in liver function. Therefore, an alternative, less invasive laparoscopic approach can be applied for repeat hepatectomy in these patients. Recurrent HCC was previously considered a contraindication to laparoscopic surgery; however, recent studies have shown that laparoscopic surgery for recurrent HCC is reliable, and there is no significant difference in tumor recurrence or survival after laparoscopic surgery compared with open surgery[86,87]. In contrast, the advantages of

Table 1 Overall survival and recurrence-free survival after re-resection for hepatocellular carcinoma recurrence

Ref.	Type	Year	*n*	1-, 3-, and 5-yr OS	1-, 3-, and 5-yr RFS
Huang *et al*[83]	Retrospective	1995-2010	82	71%/41%/22%	N/A
Itamoto *et al*[168]	Retrospective	1990-2004	84	88%/67%/50%	-/-/10%
Li *et al*[169]	Retrospective	1997-2015	103	92%/-/54%	N/A
Lu *et al*[81]	Retrospective	2004-2015	138	92%/82%/73%	N/A
Ho et al[103]	Retrospective	2001-2007	54	90%/-/72%	N/A
Sun *et al*[170]	Retrospective	1997-2003	57	70%/61%/31%	N/A
Wang *et al*[104]	Retrospective	2004-2010	128	98%/84%/64%	95%/72%/43%
Roayaie *et al*[171]	Retrospective	1994-2009	35	-/-/67%	-/55%/-
Faber *et al*[80]	Retrospective	1990-2009	27	96%/70%/42%	70%/46%/30%
Liu *et al*[87]	Retrospective	2008-2015	30	97%/85%/75%	79%/46%/30%
Sun *et al*[172]	Retrospective	2002-2014	43	98%/83%/56%	57%/32%/29%
Song *et al*[173]	Retrospective	1994-2012	39	89%/89%/84%	66%/49%/43%
Chan *et al*[93]	Retrospective	2001-2008	45	90%/57%/35%	41%/24%/24%

OS: Overall survival; RFS: Recurrence-free survival; HCC: Hepatocellular carcinoma; N/A: Not applicable.

laparoscopic liver resection include shorter operation time, less intraoperative bleeding, and faster recovery compared with traditional surgery; therefore, laparoscopic liver resection can be a safe alternative to open surgery.

Salvage liver transplantation

Salvage liver transplantation (SLT) is an appropriate treatment for recurrent HCC complicated by severe cirrhosis and liver decompensation. Available studies suggest that SLT in patients with recurrence after initial hepatectomy is a highly applicable strategy with long-term survival outcomes comparable to those of early liver transplantation[88-90]. SLT is a proven curative treatment technique for patients with recurrent HCC who meet the Milan criteria, with 5-year OS and RFS rates ranging from 42%-67% and from 32%-68%, respectively (Table 2). An intention-to-treat analysis of curative SLT in patients with cirrhosis and HCC by de Haas *et al*[89] showed that SLT had a favorable curative potential and that a model for end-stage liver disease score > 10 and the absence of TACE were predictors of successful SLT. In addition, Lim *et al*[90] compared the prognosis of 77 patients with HCC who underwent SLT with that of 314 patients with HCC who underwent a second surgery. They found that the 5-year intention-to-treat OS rates calculated from the time of the first hepatectomy were similar between the two groups (SLT, 72%; second surgery, 77%; $P = 0.57$), and the 5-year DFS rate after transplantation was much higher than that after a second hepatectomy (SLT, 72%; second surgery, 18%; $P < 0.001$)[90]. However, owing to organ shortages and cancer progression while on waiting lists, SLT can provide benefit to only a limited number of patients, making it far less widely used than repeat liver resection. Therefore, secondary resection of recurrent HCC may be considered a better therapeutic option than SLT in the current context of organ shortages. Nevertheless, given adequate organ reserves, SLT remains the preferred option for patients with cirrhosis after primary HCC resection or for those who undergo inoperable resection but meet the criteria for liver transplantation. It is worth pointing out that the existing international consensus suggests that SLT is not amenable for the treatment of HCC recurrence after transplantation[91].

Tumor ablation

Non-surgical treatment is typically proposed for recurrent HCC in the setting of inadequately preserved liver function or advanced tumor stage. Ablation therapy, such as RFA, has also been studied in the setting of recurrent HCC, with 5-year OS rates ranging from 9%-33% and 5-year RFS rates ranging from 32%-68% (Table 3). An updated meta-analysis showed that RFA is the preferred choice for recurrent HCC meeting the Milan criteria, with OS and DFS rates being similar to those of patients undergoing resection[92,93]. One study showed that 297 patients with isolated HCC ≤ 5 cm who underwent percutaneous ultrasonography-guided RFA following the recurrence of liver cancer had a similar OS to 263 patients who underwent initial RFA during the same period[94]. Similarly, Yang *et al*[95] concluded that RFA is generally effective and safe for the treatment of HCC recurrence after hepatectomy and that ablation is more effective in patients who relapsed 1 year after resection. RFA is also an advantageous

Table 2 Overall survival and recurrence-free survival after salvage transplantation for recurrent hepatocellular carcinoma

Ref.	Type	Years	*n*	1-, 3-, and 5-yr OS	1-, 3-, and 5-yr RFS
Guerrini *et al*[174]	Retrospective	2000-2011	28	-/-/42%	N/A
Chan *et al*[175]	Retrospective	2005-2017	776	96%/75%/67%	89%/68%/68%
Chan *et al*[176]	Retrospective	1993-2009	19	-/-/50%	68%/58%/58%
Bhangui *et al*[177]	Prospective	-	31	-/-/54%	-/-/48%
Shan *et al*[178]	Retrospective	2006-2015	45	65%/53%/42%	48%/32%/32%
Liu *et al*[179]	Retrospective	2001-2011	39	88%/78%/61%	14%/24%/33%
Hu *et al*[180]	Retrospective	1999-2009	888	73%/52%/46%	N/A
Wang *et al*[181]	Prospective	2001-2013	74	88%/79%/62%	87%/74%/67%

OS: Overall survival; RFS: Recurrence-free survival; HCC: Hepatocellular carcinoma; N/A: Not applicable.

Table 3 Overall survival and recurrence-free survival after ablation therapy for recurrent hepatocellular carcinoma

Ref.	Type	Years	*n*	1-, 3-, and 5-yr OS	1-, 3-, and 5-yr RFS
Sun *et al*[172]	Retrospective	2002-2014	57	98%/77%/53%	61%/27%/17%
Ho *et al*[103]	Retrospective	2001-2007	54	-/-/83%	N/A
Liang *et al*[182]	Retrospective	1999-2007	66	77%/49%/40%	N/A
Song *et al*[173]	Retrospective	1994-2012	178	99%/83% 71%	70%/41%/30%
Zhang *et al*[183]	Retrospective	2007-2014	50	100%/64%/64%	N/A
Feng *et al*[184]	Retrospective	2006-2016	199	91%/69%/56%	57%/28%/15%
Chan *et al*[93]	Retrospective	2001-2008	45	84%/43%/29%	32%/12%/9%
Koh *et al*[185]	Retrospective	2002-2011	42	-/-/24%	N/A
Chen *et al*[186]	Retrospective	2009-2015	57	78%/41%/37%	70%/38%/33%
Lu *et al*[81]	Retrospective	2004-2015	194	94%/75%/62%	N/A
Wang *et al*[104]	Retrospective	2004-2010	162	97%/73%/37%	90%/54%/27%

OS: Overall survival; RFS: Recurrence-free survival; HCC: Hepatocellular carcinoma; N/A: Not applicable.

alternative to prolong patient survival when surgical resection is contraindicated or technically infeasible[96]. Microwave ablation (MWA) is another commonly used modality for tumor ablation. Compared with RFA, MWA can reduce the time required for ablation by 60% and is more effective in eradicating tumors 3-5 cm in size[97]. As both RFA and repeat hepatectomy are indicated for HCC tumors with similar characteristics, a randomized controlled trial compared repeat hepatectomy and RFA for recurrent HCC. After a randomized 1:1 assignment of 217 patients with the same tumor characteristics to repeat hepatectomy or percutaneous RFA, the study found no statistically significant difference in survival outcomes between the two treatment strategies for patients with early-stage recurrent HCC. However, subgroup analysis found that repeat hepatectomy may be correlated with better local disease control and long-term survival in patients with tumor diameters > 3 cm or AFP levels > 200 ng/mL. In addition, because of cirrhosis, multifocal lesions, and vascular invasion, repetitive hepatectomy for recurrent HCC is limited, and only 15%-30% of patients are eligible[98]. Ablation therapy has the advantages of less trauma, less impact on liver function, and fewer complications than surgical treatment. Therefore, RFA remains a potential treatment option for patients with recurrent HCC who are unsuitable for repeat resection or salvage transplantation. However, salvage ablation is usually only appropriate for small recurrences that are detected early.

TACE

Most recurrent HCC cases are not amenable to curative treatment techniques, including repeat resection, transplantation, and ablation. Therefore, TACE is the most common treatment modality for recurrent HCC after primary resection. TACE exerts a combined antitumor effect by embolizing tumor vessels and increasing local drug concentrations[99-101]. Although numerous studies have shown that

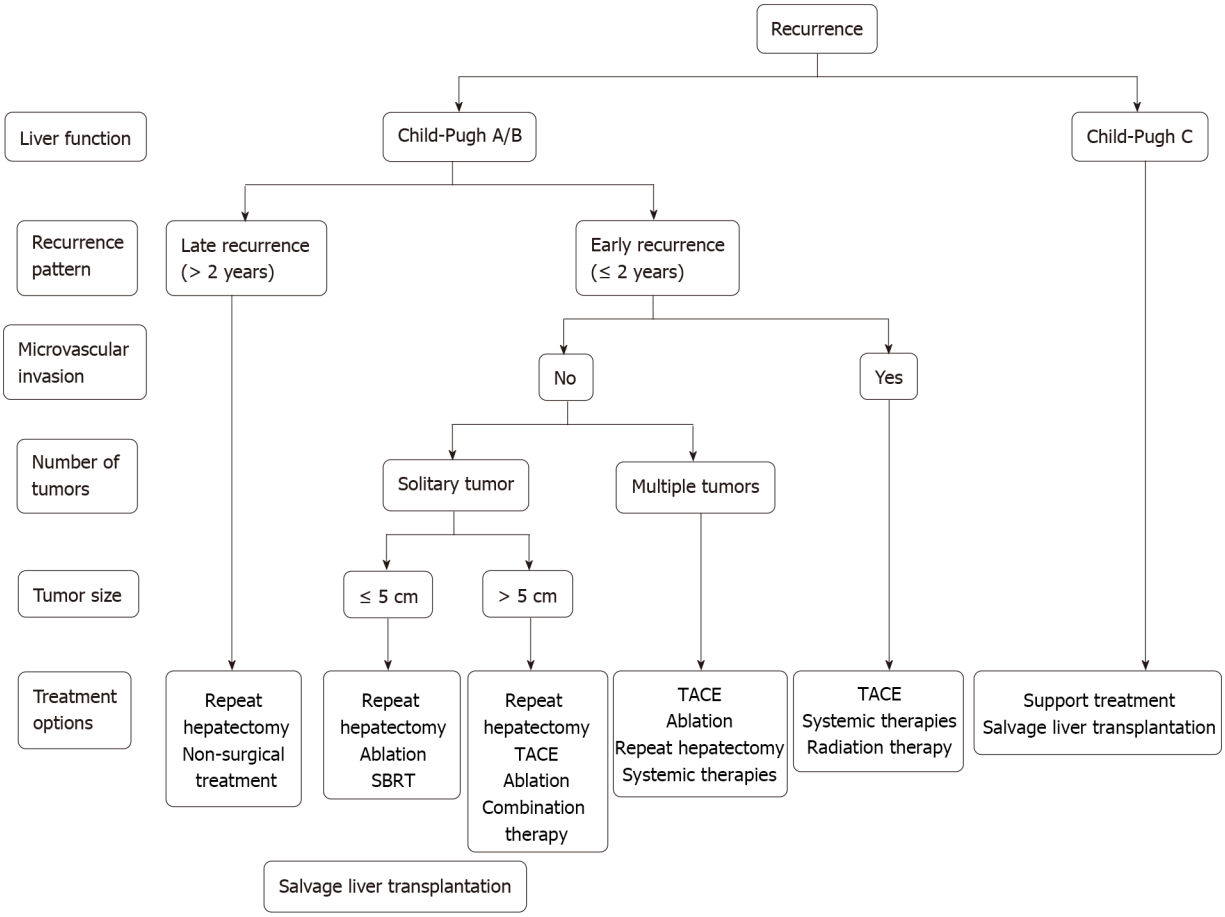

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Figure 1 Suggested flowchart of recurrent hepatocellular carcinoma management. SBRT: Stereotactic body radiotherapy; TACE: Transcatheter arterial chemoembolization.

TACE is inferior to repeat hepatectomy and SLT[102-104], according to a prospective cohort study, TACE is more appropriate for patients with multifocal disease and early (≤ 1 year) recurrence than other treatment techniques, such as repetitive hepatectomy and RFA[105]. Similarly, it has been proposed that TACE is a more effective treatment for prolonging patient survival in patients with BCLC stage 0 or A recurrent HCC with microvascular invasion, especially those who developed recurrence < 1 year after surgical resection[106]. Furthermore, two randomized controlled trials demonstrated that TACE is the only transarterial embolization modality that offers a survival advantage over best supportive care for patients with HCC who cannot receive curative treatment techniques[107,108].

Selective internal radiotherapy with yttrium-90 is also an available solution for patients with intermediate-to-advanced HCC with portal vein thrombosis as a safe alternative to TACE[109]. However, there are no experimental data on the application of yttrium-90 in the treatment of recurrent HCC. Both regimens can be used for the treatment of recurrent tumors after liver transplantation in patients with multiple lesions[110], but there are few relevant studies, and more robust evidence is needed to demonstrate the safety and efficacy of this regimen.

Stereotactic body radiotherapy

Stereotactic body radiotherapy (SBRT) is an emerging treatment option for HCC, where it is mainly performed for the local control of small HCCs. A matched-pair study demonstrated that 36 patients receiving SBRT had better OS than 138 patients with relapsed HCC who received other treatments or no treatment (2-year OS, 72.6% *vs* 42.1%; $P = 0.013$)[111]. A review evaluating the efficacy and prognosis of five different strategies for the treatment of recurrent intrahepatic HCC indicated that SBRT was superior to TACE in terms of OS and DFS but less effective than curative treatment techniques. In contrast, the prognostic efficacy of SBRT was better than that of ablation and TACE among patients with tumors > 3 cm and second only to repeat hepatectomy[102]. In addition, a small, single-center, retrospective study evaluating six patients with recurrent intrahepatic HCC after liver transplantation treated with SBRT found no local progression or death in patients at a median follow-up of 15.5 mo, which may imply that SBRT is safe for use in this setting[112]. Notably, a study by Eriguchi *et al*[112] suggested that repeated stereotactic radiotherapy is feasible for the treatment of HCC. The 3-year OS rate of patients with HCC treated with SBRT at least twice between 2012 and 2019 was 62.8% after the

second course of treatment. However, there are few prospective studies on the application of SBRT for recurrent HCC.

Systemic therapies

In recent years, systemic therapies, such as molecular targeted drug therapy and immunotherapy, have become a major focus in the treatment of intermediate and advanced liver cancer. Multiple studies have revealed that sorafenib, a representative molecular targeted therapy, prolongs the survival of patients with recurrent HCC after liver transplantation[113-116]. A case-control study showed that 15 patients with HCC treated with sorafenib had a better prognosis than 24 patients who relapsed after liver transplantation on supportive care (median survival for relapse: 21.3 mo *vs* 11.8 mo, $P = 0.0009$)[115]. Martin *et al*[116] also demonstrated a similar safety profile for sorafenib in patients with HCC who developed recurrence after resection. Regorafenib, another molecular targeted therapy, has gained attention as an option for the treatment of recurrent HCC after liver transplantation. In sorafenib-resistant patients who develop disease progression, the application of regorafenib for recurrent tumors after liver transplantation is safe and significantly prolongs patient OS compared with supportive therapy (13.1 mo *vs* 5.5 mo; $P < 0.01$)[117]. Regorafenib and lenvatinib are currently approved for the treatment of recurrent HCC in Japan[118]. However, many patients have *de novo* or acquired resistance to monotherapy; therefore, drug combinations are gradually gaining recognition among investigators. Immunotherapy, such as ICIs, has also proven to be advantageous in the treatment of recurrent HCC when combined with tyrosine kinase inhibitors. One study suggested that the combination of lenvatinib plus pembrolizumab for patients with postoperative refractory recurrent metastatic HCC resulted in partial remission and an OS of up to 60 mo after surgery[119]. Similarly, the combination of mammalian target of rapamycin target inhibitors and sorafenib is safe and effective in patients with post-transplant relapsed HCC[120]. Nevertheless, studies on systemic therapy for the treatment of recurrent HCC after resection are still insufficient, and more data are needed to confirm the therapeutic value of this strategy in the relevant populations.

Combination therapy

A combination of nonsurgical treatments for recurrent HCC is being tested in multiple studies, with the combination of TACE and ablation being the most promising. Heat dissipation may be the reason for the poor ablation effect of RFA. Applying both RFA and TACE can block the blood supply to the tumor, expand the tumor ablation margin to destroy satellite lesions, and minimize the heat loss caused by the heat sink effect, whereas the effect of chemotherapeutic anticancer agents on cancer cells is enhanced by the heat therapy effect[121]. Song *et al*[122] analyzed the outcomes of 96 patients with recurrent HCC ≤ 5 cm treated with a combination regimen of TACE-RFA and found that TACE-RFA as a first-line local therapy led to better DFS than TACE alone. This was also confirmed by a prospective randomized trial in which sequential TACE-RFA was more effective than RFA alone in patients with recurrent HCC ≤ 5 cm in diameter[123]. Furthermore, the combined TACE-RFA regimen was superior in prolonging patient survival compared with sorafenib alone for advanced recurrent HCC. This study revealed that the median OS (14.0 mo *vs* 9.0 mo; $P < 0.001$) and time to progression (7.0 mo *vs* 4.0 mo; $P < 0.001$) were significantly longer in the TACE-RFA combination group than in the sorafenib group[124]. In addition to the TACE-RFA combination, the combination of sorafenib and TACE is effective in patients with recurrent intermediate-stage HCC and microvascular invasion, and this treatment strategy yields a longer survival time than TACE alone[125]. Similarly, TACE combined with camrelizumab was reported to have an acceptable safety profile, although its efficacy was comparable to that of TACE alone[126]. Hence, TACE combined with systemic therapy has outstanding potential for recurrent liver cancer, but the variety of combination therapies is relatively small. Larger prospective clinical studies are needed to optimize the treatment sequence and identify the appropriate combination therapy regimens. The strategy of ablation combined with systemic therapy for the treatment of recurrent HCC is currently being studied in different institutions, including in phase III clinical trials (ClinicalTrials.gov numbers: NCT05444478, NCT05277675, and NCT04663035).

PREDICTORS AND SURVEILLANCE OF RECURRENT HCC

The previous sections have highlighted the high risk of recurrence of liver cancer and the limitations of available treatments. For example, surgical resection is the most effective treatment. However, owing to the low sensitivity and specificity of resection caused by the technical level and unclear diagnosis, it is likely that some patients with early recurrence of HCC will be unable to undergo the optimal treatment [127]. Therefore, predicting and monitoring for recurrence of HCC after the initial treatment is key to prolonging survival and avoiding harm to the life and health of patients due to tumor progression. Although there are some treatment measures to prevent the recurrence of HCC, these preventive treatments are not targeted, which can easily lead to overtreatment and increase patients' economic burden and decrease quality of life. Therefore, more accurate indicators are needed to supplement the stratification of prognostic and the risks of postoperative metastasis and tumor recurrence in patients

with HCC. The use of molecular biological methods to study and identify effective molecular markers is one of the key means to assist clinical diagnosis, guide clinical intervention, and provide early warning of cancer.

Recurrence and metastasis are the main reasons for the poor prognosis of HCC. However, there is no sensitive and specific method for predicting early recurrence and metastasis of HCC. Several molecular markers or their combinations have been published or reported for the diagnosis or prediction of HCC; however, there is still a lack of molecular markers or combinations that can be used to predict HCC recurrence and metastasis.

Influencers and predictors of recurrent HCC

Pathological factors: Owing to the high malignancy of HCC cells, the rapid growth of cancerous tissue, and the rich blood supply to the liver, cancer cells can easily invade the blood vessels of the liver and metastasize to other parts of the liver hematogenously. Therefore, many pathological factors associated with primary tumor characteristics and the underlying liver are intimately related to the recurrence of HCC, including the size and number of tumors, tumor capsule, portal vein tumor thrombus, stage and differentiation of the tumor, and degree of cirrhosis[128,129]. The size and number of tumors are important factors affecting recurrence after surgery. Some people regard the integrity of the tumor capsule as an indicator of tumor invasiveness; however, the capsule of liver cancer is actually a pseudocapsule (usually constructed from connective fibrous tissue) formed by squeezing the surrounding normal liver tissue during tumor growth[130-132]. Cancerous infiltrates are often found in the liver tissue outside the intact capsule, and there is little evidence of a clear relationship between capsule integrity and postoperative recurrence. However, the existence of an intact capsule has certain significance in the determination of the surgical margin during radical resection[132]. For patients with a tumor diameter > 3 cm and incomplete imaging of the tumor capsule, a wide resection margin is preferred[132]. The presence of intrahepatic portal vein tumor thrombus is another important factor associated with the postoperative recurrence of liver cancer, and intrahepatic metastasis is easily formed in patients with intrahepatic portal vein tumor thrombus[128]. It is generally believed that the stage and classification of the tumor are strongly correlated with prognosis: The lower the differentiation of a malignant tumor, the more invasive it is[128]. Therefore, primary liver cancers with poor differentiation are prone to early metastasis, resulting in incomplete resection and postoperative recurrence. Cirrhosis may affect recurrence, because it limits the size of the resection margin, thereby reducing the rate of radical resection. In addition, spleen stiffness measurements directly related to the degree of liver disease and portal hypertension, as assessed using transient elastography, appear to be the only predictors of late recurrence of HCC[129]. Finally, factors related to surgery are also strongly associated with the recurrence of HCC, including tumor margins[133], intraoperative bleeding and blood transfusion[134], and intraoperative compression of the tumor[135]. Tumor margin is the most important factor in the criteria for radical resection of liver cancer. A larger resection margin is associated with a lower detection rate of tumor thrombus and a lower recurrence rate after surgery [133]. Intraoperative bleeding and blood transfusion reflect the degree of surgical trauma, and the magnitude of intraoperative estimated blood loss is related to the biological characteristics of the tumor and the extent of surgery[134]. Moreover, estimated blood loss during HCC resection can affect the postoperative course of hepatitis and the recovery of immune function. Intraoperative compression of the tumor may cause shedding of cancer tissue or tumor cells, resulting in intrahepatic metastasis or distant dissemination and becoming an important source of postoperative recurrence[135].

Serum biomarkers: Serum AFP and albumin levels were the earliest serological markers used to assist in the diagnosis of HCC. Serum AFP ≥ 400 ng/mL is highly suggestive of HCC if pregnancy, chronic or active liver disease, gonad embryonic-derived tumors, and other gastrointestinal tumors can be ruled out. AFP L3 can be used as a prognostic indicator of HCC recurrence. Additionally, in patients with chronic HBV infection and those at a high risk of cirrhosis, AFP L3 can be an early indicator of HCC. After radical resection of HCC, a lack of obvious decrease in AFP L3 indicates the presence of metastasis or residual carcinoma[136]. In addition, given the high false-negative rate of AFP in the detection of early or small HCC, prothrombin induced by vitamin K deficiency or antagonist-II (PIVKA-II) can be used as a complement to AFP. As early as 1984, Liebman *et al*[137] found abnormally elevated levels of des-γ-carboxy prothrombin in patients with primary HCC and proposed its use for the laboratory diagnosis of HCC. Many have since studied this serum marker further and compared it with the traditional diagnostic marker AFP. Feng *et al*[138] evaluated the diagnostic efficacy of AFP and PIVKA-II when used separately and in combination in patients with primary and recurrent HCC and observed that the combination of both markers dramatically improved the diagnostic efficiency compared to either marker alone. Conversely, a recent retrospective cohort study indicated that preoperative PIVKA-II positivity, but not preoperative AFP positivity, was an independent risk factor for early recurrence of HCC[139]. This suggests that PIVKA-II is equally effective as a serum diagnostic biomarker and can be considered an alternative to AFP.

Inflammatory markers: C-reactive protein (CRP), which is synthesized by hepatocytes and regulated by interleukin-1 (IL-1) and IL-6, has important clinical value as a marker of acute and chronic inflam-

mation. Several recent studies have found that CRP is an independent risk factor for tumor recurrence in patients with HCC who exceed the Milan criteria after liver transplantation[140,141]. Similarly, elevated postoperative serum CRP may be a prognostic indicator for patients with HCC after elective hepatectomy[142]. Further, the peripheral blood neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) are correlated with the prognosis of malignant tumors[143,144]. Halazun *et al* [143] followed up 150 patients who underwent liver transplantation for HCC and found that the tumor recurrence rate of 13 patients with an NLR ≥ 5 was 62%, and the 5-year OS and DFS rates after surgery were significantly lower than those of patients with an NLR < 5. Further, multivariate analysis showed that a high NLR was a risk factor affecting the DFS rate of recipients (hazard ratio = 19.98; P = 0.005). Another study of 865 patients who underwent liver transplantation for HCC found that the risk of HCC recurrence increased 1.89 times for each logarithmic unit increase in the NLR[144]. A meta-analysis conducted by Lai *et al*[145] revealed that an elevated PLR was associated with an increased risk of HCC recurrence after liver transplantation (odds ratio = 3.33; 95%CI: 1.78-6.25; P < 0.001). Although these studies show the predictive potential of these inflammatory markers, there is heterogeneity and poor reproducibility. In addition, the cutoff values of inflammatory markers vary greatly between studies; therefore, the optimal cutoff requires further study, and it is difficult to use these as biomarkers widely in clinical practice.

Immunohistochemical indicators: Patients with liver cancer often have a history of HBV or HCV infection, liver cirrhosis, and other backgrounds, and the resulting inflammatory response often leads to large numbers of lymphocytes in or around the lesion. The ratio of CD4/CD8+ T cells in the tumor is associated with recurrence after liver transplantation, and more CD4+ T cell infiltration reduces the risk of recurrence after liver transplantation[146]. Further, tumor or peripheral blood regulatory T (Treg) cells are associated with tumor invasion, and Treg cells reduce the antitumor effect of effector T cells, which promotes tumor immune escape[147,148]. The imbalance between regulatory and cytotoxic T cells in HCC is also expected to be an effective prognostic factor. Clinical studies have found that Treg cells are significantly higher in HCC tissues than in non-cancerous liver tissues, suggesting that Treg cell infiltration in HCC can inhibit antitumor immunity and high Treg cell infiltration in HCC is a predictor of poor prognosis[149].

Genetic biomarkers: In the process of tumor invasion and metastasis, tumor cells need to break through the barriers of the extracellular matrix and basement membrane. Matrix metalloproteinase (MMP)-9 can degrade the extracellular matrix; therefore, tumors with high expression of MMP-9 have stronger invasion and metastasis abilities. Most patients with high MMP-9 expression in liver cancer tissues and plasma have portal vein tumor thrombus or intrahepatic metastasis[150]. The level of serum vascular endothelial growth factor (VEGF) in patients with liver cancer is significantly higher than that in patients with benign liver disease and healthy individuals. High VEGF is closely related to portal vein tumor tether, tumor size, and TNM stage[151]. VEGF plays an important role in the invasion and metastasis of liver cancer, and preoperative examination of serum VEGF levels is of great significance in predicting the invasion and metastasis of liver cancer[152,153]. *AFP* mRNA in circulating blood can be used to detect the presence of circulating cancer cells[154,155]. Reverse transcription-polymerase chain reaction indicated the presence of *AFP* mRNA in the peripheral blood of 59.7% of patients with liver cancer[154]. Therefore, the presence of disseminated HCC cells in the blood circulation can be detected before treatment is initiated. The positive rate of *AFP* mRNA in the peripheral blood is significantly correlated with the clinical stage and postoperative recurrence of liver cancer, and 57% of patients with postoperative recurrence have *AFP* mRNA in the peripheral blood[156]. Therefore, *AFP* mRNA expression in the systemic circulation can be used to assess the risk of recurrence and metastasis.

Surveillance of recurrent HCC

The establishment of prognostic models based on predictors is important in the field of HCC recurrence prevention and monitoring. Hwang *et al*[156] integrated three variables (tumor size > 5 cm, high AFP, and high des-γ-carboxy prothrombin) by direct multiplication and constructed the ADV score as a comprehensive proxy for predicting prognosis after isolated HCC resection. This score had a sensitivity of 73.9% and specificity of 66.7%[157]. This team then performed preoperative evaluation and postoperative follow-up of 526 patients with isolated HCC ≥ 8 cm treated by hepatectomy, which led to the development of a comprehensive, predictive surrogate marker that is equally valid in patients with very large HCC. The PPM prediction model constructed in that study is based on four factors, including AFP ≥ 100 ng/mL, hypermetabolic 2-18F-fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) findings, microvascular invasion, and satellite nodules, and had C-indexes of 0.66 for tumor recurrence and 0.69 for patient survival. In contrast, in the new version of the PPM prediction model constructed based on two previously studied factors, ADV7 log and FDG-PET, the C-indexes for tumor recurrence and patient survival were 0.64 and 0.70, respectively[158].

The construction of a reliable risk score for recurrence of HCC after liver transplantation could vastly improve surveillance strategies and help identify patients who may benefit from adjuvant therapy. The RETREAT score constructed by Mehta *et al*[157] is effective in predicting recurrence after transplantation in patients with HCC who meet the Milan criteria. The score includes three main factors:

Microvascular invasion, post-transplant AFP, and the sum of the maximum diameter and number of surviving tumors. Compared with the Milan criteria, the RETREAT score improved the prediction of HCC recurrence at 1 (0.40, $P = 0.001$) and 5 (0.31, $P < 0.001$) years after liver transplant[159]. Based on the RETREAT score, Costentin *et al*[158] recently proposed a novel composite prediction tool, the R3-AFP score, to optimize the prediction of HCC recurrence after liver transplantation. In addition to the factors included in RETREAT, the model also incorporated pre-transplant AFP and pathological variables, which led to the classification of patients into four risk groups, with a 5-year survival rate of 77.2% for patients in the very low-risk group[160].

In addition to these traditional Cox proportional hazard prediction models based on linearity assumption, the construction of prediction models by machine learning algorithms has become an important method for predicting tumor recurrence. Given the complex, multidimensional, nonlinear relationships between clinical data, machine learning models outperform traditional regression models in predicting HCC progression[161]. The XGBoost model based on clinical data is effective in predicting the risk of early recurrence in patients after MWA, with an area under the curve of 0.75 (95%CI: 0.72-0.78)[162]. Moreover, incorporating magnetic resonance imaging (MRI) data in a machine learning model for recurrent HCC after transplantation can effectively improve the predictive performance of the model compared to incorporating clinical parameters alone[163]. Therefore, appropriate monitoring protocols can be developed to maximize the prevention of recurrence and prolong patient survival after HCC resection.

The main methods currently used for the clinical monitoring of HCC recurrence are serum AFP monitoring, regular abdominal ultrasonography, and computed tomography (CT). In addition, MRI has strong soft tissue resolution and can reflect the changes in blood flow and enhancement at the lesion site and has been widely used in clinical practice to monitor the recurrence of liver cancer. Gadoxetic acid (Gd-EOB-DTPA) is a relatively safe and well-tolerated liver-specific contrast agent that adequately combines the properties of conventional extracellular contrast agents and hepatocyte-specific magnetic resonance contrast agents with the higher soft tissue resolution of MRI. Therefore, EOB-MRI has better detection and diagnostic efficacy for HCC than CT[164]. The apparent diffusion coefficient (ADC) in magnetic resonance diffusion-weighted imaging (DWI) can quantify the overall diffusion of a lesion. Chuang *et al*[165] revealed that tumor recurrence after liver transplantation could be effectively predicted by analyzing the correlation between tumor recurrence, explant pathologic findings, and the ADC. Furthermore, several lines of evidence suggest that Gd-EOB-DTPA and DWI are more advantageous in detecting small liver lesions than CT[166,167]. Therefore, clinicians may be able to select more appropriate monitoring methods based on the combination of imaging and prognostic models to identify patients at high risk of recurrence and determine the optimal treatment.

CONCLUSION

In conclusion, since HCC has varied recurrence patterns and timing, the choice of treatment option after treatment for primary HCC varies. Repeat hepatectomy is the treatment of choice for recurrent HCC; laparoscopic surgery techniques are becoming increasingly sophisticated and offer a novel, safe, and effective surgical option for hepatectomy in patients with recurrent disease. However, the clinical application of repeat hepatectomy is limited due to the small number of eligible patients. Liver transplantation is preferable for patients with recurrent HCC complicated by severe cirrhosis and hepatic decompensation, and it has a better RFS than repeated hepatectomy; however, a shortage of organ donors and long wait times are two major factors that limit the utilization of SLT. In patients with recurrent HCC who are not candidates for resection or transplantation, nonsurgical treatment options are worth considering. Whether HCC recurs after resection or transplantation, ablative therapy, especially RFA, has become another treatment alternative advocated by many researchers, owing to its minimally invasive nature and convenient advantages. However, salvage ablation is recommended only for patients with early recurrence of tumors ≤ 3 cm in diameter. Although TACE does not provide the same survival benefit as repeat hepatectomy and SLT for recurrent HCC, it should be considered in patients with early recurrence with microvascular invasion or multiple lesions. Similarly, SBRT can provide good disease control and a modest survival benefit in patients with small HCC who relapse after operative treatment. Systemic therapy, including molecular targeted therapy and immunotherapy, is also gaining attention as an emerging therapeutic strategy for clinical application in recurrent liver cancer. Systemic therapy can provide benefit to patients with advanced recurrent HCC either as a single agent or in combination with other therapies. Combination therapy is a promising way to optimize therapeutic efficacy by combining different treatment options to reduce complications and prolong survival, and this may be a key research direction for the future. The flexible combination of systemic therapies and other complementary therapies may offer a breakthrough in the clinical efficacy of HCC treatment. Finally, despite the promising results of most of these studies, future prospective randomized controlled studies are still needed to provide more rigorous clinical evidence to develop and optimize treatment options for recurrent HCC.

FOOTNOTES

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REVIEW

Bioengineering liver tissue by repopulation of decellularised scaffolds

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Abstract

Liver transplantation is the only curative therapy for end stage liver disease, but is limited by the organ shortage, and is associated with the adverse consequences of immunosuppression. Repopulation of decellularised whole organ scaffolds with appropriate cells of recipient origin offers a theoretically attractive solution, allowing reliable and timely organ sourcing without the need for immunosuppression. Decellularisation methodologies vary widely but seek to address the conflicting objectives of removing the cellular component of tissues whilst keeping the 3D structure of the extra-cellular matrix intact, as well as retaining the instructive cell fate determining biochemicals contained therein. Liver scaffold recellularisation has progressed from small rodent *in vitro* studies to large animal *in vivo* perfusion models, using a wide range of cell types including primary cells, cell lines, foetal stem cells, and induced pluripotent stem cells. Within these models, a limited but measurable degree of physiologically significant hepatocyte function has been reported with demonstrable ammonia metabolism *in vivo*. Biliary repopulation and function have been restricted by challenges relating to the culture and propagations of cholangiocytes, though advances in organoid culture may help address this. Hepatic vasculature repopulation has enabled sustainable blood perfusion *in vivo*, but with cell types that would limit clinical applications, and which have not been shown to have the specific functions of liver sinusoidal endothelial cells. Minority cell groups such as Kupffer cells and stellate cells have not been repopulated. Bioengineering by repopulation of decellularised scaffolds has significantly progressed, but there remain significant experimental challenges to be addressed before therapeutic applications may be envisaged.

Key Words: Regenerative; Bioengineering; Scaffolds; Liver; Decellularisation;

Recellularisation

Core Tip: Given the limited resource of livers for transplantation, repopulation of decellularised scaffolds with recipient cells offers a theoretically attractive organ source without the need for immunosuppression. Bioengineered livers have progressed from small rodent to large animal blood perfusion models. Although some hepatocyte function has been achieved, challenges remain in cholangiocyte repopulation, reconstitution of the vasculature, and other minority cell groups. The cell types used in experimental models to date have yielded advances but may need to be altered if the currently distant prospect of clinical application is to be envisaged.

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INTRODUCTION

Chronic liver disease is a major health concern, with 1.5 billion individuals affected worldwide, and associated with an annual global mortality of 2 million people[1]. In the United Kingdom, liver disease is the third commonest cause of premature death[2], and is associated with societal and health care costs measured in the billions of pounds per annum[3]. In the United states, 44000 people die of chronic liver disease each year[4], with an estimated annual hospitalisation costs demonstrating an increasing trend and measured at 18 billion dollars per year in 2016[5], to which must be added similar magnitude financial costs of pre-hospital healthcare and social care burden[3].

Although vaccination programs and antiviral therapy may result in decreasing prevalence in chronic liver disease of viral aetiology, the consequences of alcohol and hepatic steatosis has resulted in a gradually increasing incidence of chronic liver disease[6-8]. Despite the enormous scope for prevention of progression to chronic liver disease through vaccination, antiviral therapy, and lifestyle interventions, the only treatment for end-stage liver disease remains liver transplantation. However, due to the shortage of available organs, 10% of patients die whilst on the waiting list for an organ[9], and many more are never considered for transplantation because of the need to optimise graft usage. Moreover, transplanted patients face the short and long-term side effects of immunosuppression.

These challenges have motivated the investigation of bioengineering liver tissue with a view to delivering bioengineered organs for transplantation. Despite progress in the generation of biogels and 3D bioprinting, reproducing the immensely complex 3D microarchitecture of liver parenchyma remains a major challenge. By decellularizing tissues with surfactant detergents, it is possible to remove the cellular component of tissues, leaving behind the 3D extracellular matrix (ECM) providing not only a scaffold but also cell fate instructions to appropriate repopulating cells. In the context of liver transplantation, many deceased organs are discarded because of inadequate cellular function[10]. As an aspirational objective, such organs could be decellularised, and repopulated with cells of recipient origin with a view to bioengineering immunologically syngeneic organs. The theoretical benefits would include timely generation of organs, transplanted in an elective manner, without the need for immunosuppression.

This review describes current progress in the field of bioengineering liver tissue from decellularised matrix and repopulating cells. To orientate the reader, the review sections will deal with the following areas: Section 2 (ECM structure and role in cell fate) provides a summary of the structure and function of the extracellular matrix, describing its paramount influence in cell fate and bioengineering, as well as an account of the evolution of synthetic and ECM substrate components to enhance tissue culture; Section 3 (General concepts in decellularisation and non-hepatic applications) provides an account of decellularization of tissues in general and non-hepatic applications, as a background context in which to consider liver decellularisation and repopulation; Section 4 (Scaffold sterilisation) discusses scaffold sterilisation; Section 5 (Liver decellularisation and recellularisation) provides an account of decellularisation and repopulation of liver tissue with subsections dealing with the variety of cellular components of liver parenchyma; Section 6 (Recellularisation of extra hepatic blood vessels) discusses the recellularisation of extra-hepatic blood vessels; Section 7 (Immunogenicity of decellularised scaffolds) provides an account of scaffold immunogenicity; and Section 8 (Conclusion) concludes the review with a discussion of the remaining challenges in the field.

ECM STRUCTURE AND ROLE IN CELL FATE

Introduction

Whilst a full account of the role of ECM in cell biology is beyond the scope of this review, its fundamental role in influencing cell behaviour requires emphasis in the context of the use of decellularised ECM scaffolds. This section describes the structure of the ECM and provides an overview of ECM cell interactions as well as the evolution in the use of ECM based substrates to enhance tissue culture.

ECM content and structure

Although the structure of ECM varies immensely between tissues in terms of proportion and layout of its constituents, common components can be identified and include Glycosaminoglycans, water, 4 major classes of extracellular proteins (the collagens, elastin, proteoglycans, and glycoproteins), and numerous growth factors as well as other bioactive cell behaviour influencing species.

Glycosaminoglycans such as chondroitin sulphate, heparan sulphate and hyaluronic acid[11] are long, negatively charged macromolecules consisting of linear repeats of uronic and amino disaccharide units. In isolation or when combined with proteins to form proteoglycans[12], Glycosaminoglycans bind water, which is critical for imparting compressive resistance to tissue.

Collagens imparts tissue tensile strength and structural integrity. They consist of 3 alpha chains, the various combinations of which make up the 28 known collagen types. In broad structure, Fibrillar collagen is assembled in triple helical structures which combine to form fibrils of varying size and thickness. Non fibrillar collagen does not form fibrils but rather a mesh like network, such as that in in basement membrane by collagen type 4[13].

Elastin complements collagen's tensile strength properties to provide elasticity[14].

Glycoproteins[15,16] are peptide units covalently bound to carbohydrate groups, but not in a linear or repeating pattern, as in proteoglycans. The glycoproteins are described as connecting molecules, in that they carry binding sites to multiple other molecules including other ECM molecules, secreted growth factors, and extra-cellular membrane receptors on cells including cell adhesion molecules. The principal glycoproteins are fibronectin and laminin. Cell attachment to glycoproteins is mediated through distinct peptide domains[17] such as the Arg GlyAsp (RGD) and Arg GluAsp Val (REDV) sequences in fibronectin[18,19], as well as Val-Al -Pro-Gly domain in elastin[20], which binds integrins on cell surface. Binding motifs may be overtly apparent or may be revealed after unfolding of ECM proteins by fibroblasts, or following the action of ECM degrading enzymes, thus introducing further complexity in the interplay between the ECM and multiple cell types in the control of cell behaviour[21]. Laminin is composed of alpha, beta and gamma heterotrimeric chains arranged in cross or Y shapes[12]. It is found in basement membrane and connects ECM components, with different forms and modifications resulting in specific controls on cell behaviour[22].

ECM cell interactions

The ECM is much more than simply a 3D scaffold which houses resident cells. It is also a source of critical biochemical and physical signalling which influences fundamental processes of cell survival, organization and differentiation[23].

ECM in development, cell migration, stem cell niche, and adult tissue fate: The importance of the ECM in cellular organisation is apparent from its synthesis and secretion in the very earliest stages of development, exemplified by the assembly of laminin and collagen 4 in mouse embryos as early as the blastocyst stage[24]. Moreover, major developmental defects are caused by ECM proteins[25], with ECM mutations resulting in wide ranging anomalies affecting body shape[26], as well as development of neural tube[27], and muscle[28].

In addition to broad control of development, the ECM acts as a regulator of the extent and direction of cell migration. Thus, laminin chain knockout results in uncontrolled and undirected neural crest cell migration[29], whilst fibronectin mutations result in impaired migration of cardiac precursor cells[30].

The ECM controls stem cell fate not only in development, but also in adult tissues where it plays a major role in the definition of the stem cell niche, keeping stem cell in a quiescent state until appropriate circumstances trigger a requirement for their proliferation[31].

The ECM also influences the behaviour of differentiated cells in adult tissues. Following cues from multitude factors (physical, chemical, oxygen partial pressure, and numerous others which together define physiological niche)[32], the ECM is altered and remodelled by resident cells in adult tissues. ECM remodelling is much more than a reconfiguration of local 3D scaffold shape: the ECM is a reservoir of multiple biologically active species which impact on cell behaviour, and which are recruited and released upon remodelling. Thus, although resident cells produce and deposit their local ECM, they are also influenced by it in a process referred to as 'dynamic reciprocity' or 'bidirectional crosstalk' between cells and their environment[33,34].

ECM – cell biochemical and biophysical signalling: The ECM interacts with cells *via* multiple receptors in the cell membrane including integrins, discoidin domain receptors, syndecans, CD44, and receptor

for hyaluronic acid. Of these, the most studied are the integrins. Distinct alpha and beta subunits combine to make 24 different known integrins, which act specifically on defined cell types in a contextual manner to determine cell growth and survival, promote invasion and migration, and direct cell differentiation and stem cell fate[35]. Mechanistically, the importance of binding motifs is emphasised by experiments demonstrating that blocking the integrin binding site of fibronectin (the RGD motif) by competitive inhibition with RGD peptides resulted in major embryonic symmetry anomalies[36].

In addition to direct communication with cells *via* cell membrane receptors, the ECM influences cell fate by acting as a reservoir of growth factors, morphogens and enzymes, which may be released as active forms in defined circumstances, in a manner that has been most studied in relation to many growth factor families including the transforming growth factor beta, platelet derived growth factor, fibroblast growth factor and insulin like growth factor superfamilies of growth factors[14]. These growth factor signals are added to and complemented by those of other bioactive species including, matrix cryptic peptides[37], matrix bound vesicles containing bioactive molecules (RNA, lipids, proteins)[38], with wide-ranging roles including impacts on cell differentiation[31] chemotaxis[39], mitogenesis[40], angiogenesis[41,42], and wound healing[43].

The ECM signals to cells and influences cell fate in biophysical ways as well as *via* biochemical mechanisms. For example, by determining cell shape with microprinted fibronectin islands, McBeath *et al*[44] showed that mesenchymal stem cells would differentiate to adipocytes if they assumed a rounded shape, and to chondrocytes if allowed to assume a spread shape.

The ECM also influences cell proliferation[45] and cell fate *via* its stiffness and elasticity. Thus, mesenchymal stem cells differentiation may be directed towards either neurological, muscle or bone phenotypes by varying the elasticity of the underlying substrate to mimic the corresponding tissue types[46], *via* mechanisms involving mechano-sensitive ion channels, and Yes-Associated Protein and Transcriptional Coactivator With PDZ-Binding Motif[14].

The evolution of complex substrates for cell culture

The powerful influence of the ECM in the control of cell fate has motivated the use of alternatives to 2D plastic cell culture with a variety of complex substrates to minimise the loss of functional specificity that is otherwise frequently observed. Thus, there has been a gradual evolution in the use of materials to mimic the ECM *in vitro*, culminating in the recent development of decellularised scaffolds, representing to date the most accurate version of native ECM.

Substrates for enhanced cell culture include synthetic or naturally occurring chemicals. The synthetic substrates are man-made polymers such as polycaprolactone, polyethylene glycol (PEG) and polyglycolic acid[23], or hydrogels composed of hydrophilic polymers such as polyacrylic acid, polyethylene glycol and polyvinyl alcohol[47]. These have the advantages of reliability, consistency, reproducibility, low variability, but tend to produce host inflammatory responses[48] and fundamentally lack the complexity of native ECM. The naturally occurring substrates are components of ECM, either in single form or in combinations of varying complexity.

Synthetic substrates may be bioengineered to include biological entities in a number of ways: (1) By crosslinking cell adhesion peptides (for example, the RGD domain of fibronectin or VPVGV domain of elastin) to synthetic polymers like PEG to promote cell interaction[49]; (2) By incorporation of specific growth factors to favour desired cell behaviour, for example neuronal[50], bone[51], and vascular[52] differentiation, with the option of positioning of boundary forming signals[53], or temporal control by determining the mechanism of release of the bioactive species[54]; and (3) by incorporating enriched ECM components into poly-ethylene-glycol hydrogels[55].

Hydrogels are hydrated polymers or materials with ≥ 30% (v/w) water content that maintain their structural integrity through crosslinks between their constituents[56], which can be synthetic polymers, or from ECM components in single form[57] or multi component form[58]. Cell derived hydrogels such as Matrigel, or hydrogels generated from specific decellularised tissues are more complex and have been used for organoid culture[59], as 2D substrates, or cell medium additives.

Whether synthetic, naturally occurring or combined, the deposition of substrate components on a given surface has evolved to high level of precision, achieving resolutions of fractions of micrometres, with micro-patterning techniques such as photolithography[60], elastomeric stamping[61], nanofiber lithography[62], electrospinning[63], and 3D bioprinting using 'bio ink' (ECM derived from specific tissue in hydrogel and colloid form)[56].

Nevertheless, despite the wide range of available substrates, be they synthetic, naturally occurring or combined, the complexity of specific tissue microarchitecture combined with the multitude of growth factors within the ECM, means reproducing ECM by the techniques mentioned above remains elusive. Hence the concept of decellularisation, whereby the cells of a specific tissue are removed, thereby leaving behind a native cell free ECM scaffold, theoretically maintaining both 3D micro-architecture and the ECM associated biological signalling.

GENERAL CONCEPTS IN DECELLULARISATION AND NON-HEPATIC APPLICATIONS

Introduction

The objective of whole organ decellularisation has 2 components: (1) To completely remove the cellular component, whilst (2) leaving the 3D microarchitecture and vital growth factor content of the ECM intact, such that repopulating cells have an environment which favours regeneration of the native tissue.

The first objective is necessary as there is evidence that residual cellular debris is not only toxic to repopulating cells, but also triggers inflammatory and destructive responses *in vivo*[64-67] (discussed in section 7 "Immunogenicity of decellularised scaffolds") rather than the desired regenerative events. The second objective is necessary to retain the vital physical and biochemical ECM properties by which it influences cell fate.

Herein lies a fundamental difficulty in decellularisation techniques, in that whilst both necessary, these 2 objectives are in conflict, as the stringent conditions required to clear toxic debris of decellularisation will also inflict some damage to the ECM. Small molecules like growth factors will be particularly susceptible to being washed away[68], but even large macromolecules, though less vulnerable because of size and cross linking, may also be damaged[69].

The following section outlines the techniques used for decellularisation and discusses their merits and disadvantages.

Decellularisation techniques

A multitude of decellularisation techniques have been developed using physical, chemical, and enzymatic, methods either singly or in combination, and adapted to suit the differing requirements of the native tissue being treated.

Physical methods: (1) Sonication. Sonication utilises an ultrasound emitting device to transfer acoustic energy in a solvent containing tissues to be decellularised[70]. Cell membranes are disrupted by the sonication waves, and resultant debris requires removal by other methods[71-74]. Sonication process may significantly increase temperature of the solvent and tissues, risking denaturisation, and therefore may need to be combined with a cooling mechanism[70]. Sonication is typically used with detergents to decellularise dense tissues such as tendons, ligaments[75], and cartilage[71], although has also been used in kidney decellularisation[76]; (2) Freeze-Thaw. Freeze-thaw achieves cell lysis through rapid thermal change, though debris requires additional methods for clearance[70]. The technique has been used in combination with detergents to reduce to the quantities of chemical reagents for decellularisation[77,78]. The formation of ice crystals may be detrimental to the ECM, leading some researchers to advocate the use of cryoprotectants to mitigate the detrimental effects without affecting cell lysis[79]; and (3) Immersion and agitation. The decellularisation effects of chemical reagents may be enhanced by agitation in instances where decellularisation is achieved by immersion in chemical reagents[70]. The length of immersion, and intensity of agitation depend on the tissue[80], and this approach is usually only appropriate for epidermal tissues and smaller organs, such as small intestine submucosa[81], trachea[82], other cartilaginous tissues[83,84], and thyroid gland[85].

Chemical methods: (1) Detergents. Detergents have been used extensively to decellularise large vascular organs by vascular perfusion[70]. Ionic detergents like sodium dodecyl sulphate (SDS) and sodium deoxycholate solubilise cell membranes and denature proteins[86-88]. Non-ionic detergents, of which Triton X-100 is the most frequently and successfully used, disrupt lipid-lipid, lipid-protein, and DNA-protein interactions[89,90]. Detergents are frequently combined in decellularisation techniques, with variations in concentration and perfusion time, and require washing steps to remove residual traces after decellularisation[91,92]; (2) Hypertonic and hypotonic solutions. Hypertonic saline causes dissociation of DNA protein interactions[93], which, combined with cell shrinkage and swelling, causes cell lysis[94]. Debris clearance further steps to achieve full decellularisation[95]; (3) Acids and bases. Bases, such as ammonium hydroxide, have been used as an adjunct to detergent based decellularisation techniques to enable clearance of DNA which, in alkali solution, denatures to low viscosity single stranded nucleic acid, facilitating its removal by perfusion[96,97]. Acids such as peracetic acid have been used predominantly for sterilisation of scaffolds (see section 4 "Scaffold sterilization"). However, both bases and acids have significant detrimental effects on the ECM, by damaging collagen and other structural proteins, as well as by denaturing key growth factors[81,98]; (4) Alcohols. Alcohols diffuse into cells and cause cell lysis by a dehydrating mechanism, and thus have been used as decellularising agents[99,100], but also as sterilising agents either alone or in combination with acids; (5) Chelating agents[101-104]. Chelating agents such ethylenediaminetetraacetic acid and ethylene glycol tetra acetic acid bind metallic ions that are essential for protein interaction[105,106], resulting in the disconnection of intercellular integral proteins and disruption of cellular adhesion in the ECM. Full decellularisation requires additional agents such as detergents[107]; and (6) Enzymatic treatments[108-111]. A variety of enzymes have been utilised for tissue decellularisation, with trypsin and nucleases being the most frequently used. Trypsin is a serine protease that hydrolyses proteins involved in cellular attachment [112], thus dissociating cells from the ECM[113]. Nucleases (DNases and RNases) cleave phosphodiester bonds between nucleotides in nucleic acids and have been used to improve the removal of remaining

nucleic acid debris in conjunction with other decellularisation agents[114,115].

Implications of decellularisation technique heterogeneity: The above summary of techniques provides an insight into the enormous heterogeneity of approaches. In addition to the variety of methods above, many protocols use varying combinations of 2 or more methodologies. Furthermore, techniques vary in other factors including temperature of decellularising process, flow rates of perfusion agents. Such considerations may be quite subtle, yet critically important: for example, one study of tracheal decellularisation involving repeated cycles of decellularising agent reported that the number of cycles critically altered the integrity of the scaffold between cycles 18 and 22[116].

This technique heterogeneity reflects the differing requirements of different tissues. Tissues with obviously different macroscopic structures require different methodology: for example, perfusing a decellularising agent *via* the circulation in vascular tissue such as liver may be effective, but unlikely to be so in tough avascular structure of tendon. In this regard, a tissue classification of laminate, amorphous, composite, whole organ suggested by Keane[117]. Moreover, the matrisome (the protein content profile of the ECM) is subtly tissue dependant[118] such that even tissues of similar consistency may behave differently. For example, similar tissues such as tendon and ligament may behave quite differently despite exposure to same decellularising agent[119,120]. Furthermore, even within a defined tissue type, individual variation with factors including age and sex may affect matrisome content[121-124].

Though not intended to be comprehensive given the scope of this review, Table 1 provides examples of the breadth of tissues in which decellularisation has been studied, and range of decellularisation protocols. This reflects the fact that the field is at an empirical stage where methodology is in an assessment phase with multiple criteria to be considered. The optimal method of decellularisation may be difficult to determine and define particularly in the context of tissues with multiple cell types, as the optimum decellularising method for one cell type may not coincide with requirements for others. Attempts at decellularisation must perhaps be seen as producing an inevitably imperfect result, which may be corrected and refashioned by repopulating cells *in vitro* and in the host.

This high level of heterogeneity in tissue samples and technique raises the question of how to assess success in decellularisation. In this regard, Crapo *et al*[125] has suggested that successful decellularisation should be determined on the basis of producing ECM which (1) Does not contain more than 50 ng of DNA per mg dry weight; (2) with residual DNA fragments no longer than 200 bp; and (3) with no visible nuclear components, based on observations of *in vivo* adverse effects of these biochemicals[64,67,126]. The area of research is rapidly changing, and thus it is anticipated that new criteria of scaffold quality are likely to arise, as suggested by other authors[127-129]. Ultimately, the success of decellularisation is surely defined by the matrix to accept repopulating cells and whether those cells survive and collectively allow physiologically significant neo-organ function. These aspects will be discussed in detail as they pertain to liver function in section 5 (Liver decellularisation and recellularisation).

SCAFFOLD STERILISATION

Introduction

In vitro culture of mammalian cells provides ideal conditions for survival of cells of interest, but also for unwanted micro-organisms. Moreover, the potential for culture infection in decellularised scaffold experiments is higher than in standard cell culture given the non-sterile tissue of origin. Thus, not only for the success of *in vitro* scaffold repopulation, but also in terms of safety in the context of scaffold *in vivo* reimplantation, there is a need to eradicate microorganisms from decellularised scaffolds.

The ideal requirements for decontaminating agents would be (1) The ability to remove all microorganisms and spores; (2) to be removeable or non-toxic to repopulating cells or potential host; and (3) to leave the scaffold ECM unaltered. Thus, the end product could be tested in terms of its sterility, toxicity, and preserved biological properties.

In addition, there is a distinction to be made between sterilisation (killing or removing all microorganisms, including bacterial spores and disinfection (killing or removing all pathogenic microorganisms but not bacterial spores). Most protocols use disinfection techniques, but these may be deemed insufficient in the clinical context, should current experimental methodology progress to that stage. The section below provides an account of techniques used to remove micro-organisms from decellularised scaffolds prior to cell repopulation, as well as a summary of the studies that have compared the efficacy of these techniques.

Sterilisation and disinfection techniques

Irradiation: Irradiation using Gamma rays or electron beam act by inflicting direct damage to DNA and proteins, and by generation of oxidative species and free radicals. The advantages of irradiation are its delivery at room temperature, with no residual chemical toxicity, but with disadvantage of matrix denaturation with increasing dose[130]. To date, the main applications have been in bone and tendon [131] decellularisation.

Table 1 Examples of non-liver decellularisation protocols

Organ	Species	Decellularisation technique	Recellularization	Significant outcome	Ref.
Heart					
	Rat	SDS + Triton X-100	Neonatal cardio-myocytes	(1) Maintained eight constructs for up to 28 d by coronary perfusion in a bioreactor that simulated cardiac physiology; (2) Macroscopic contractions were observed by day 4; and (3) By day 8, under physiological load and electrical stimulation, constructs could generate pump function in a modified working heart preparation.	Ott *et al*[235]
	Pig	Freeze and Thaw + hypotonic solution + trypsin/EDTA/NaN3 + Triton X-100/EDTA/NaN3 + deoxycholic acid	Chicken embryonic cardio-myocytes	Cardiac extracellular matrix supported the formation of organized chicken cardiomyocyte sarcomere structure *in vitro*.	Wainwright *et al*[236]
	Rat	SDS *vs* POETE	Not performed	SDS decreased DNA and GAG and enriched the collagen content 10-fold.	Bruyneel *et al*[237]
	Pig	SDS *vs* Triton X-100 *vs* CHAPS *vs* OGP	Not performed	3% SDS as a detergent showed optimal decellularization.	Ferng *et al* [238]
	Rat	SDS + Triton X-100	Induced cardiac progenitor cells	(1) Optical mapping of recellularised scaffolds shows field-stimulated calcium transients that propagate across islands; and (2) Bipolar local stimulation demonstrated cell-cell coupling within scaffolds.	Alexanian *et al*[239]
Kidney					
	Rat	Saline + SNP + Triton X-100, DNAse + SDS	Murine pluripotent embryonic stem cells	(1) Primitive precursor cells populated and proliferated within the glomerular, vascular, and tubular structures; and (2) Cells lost their embryonic appearance and expressed immunohistochemical markers for differentiation.	Ross *et al* [240]
	Monkey	1% SDS *vs* 1% Triton X-100	Not performed	SDS at 48C to be most effective in preserving the native architecture.	Nakayama *et al*[241]
	Pig	0.5% SDS *vs* 0.25% SDS *vs* 1% Triton X-100 with 0.1% ammonium hydroxide	Not performed	0.5% SDS was the most effective detergent.	Sullivan *et al* [242]
	Pig	SDS	Not performed	(1) Kidney decellularized scaffolds implanted in Yorkshire pigs easily re-perfused, sustained blood pressure; (2) Scaffolds maintained renal ultrastructure; and (3) However, presence of inflammatory cells in the pericapsular region and complete thrombosis of the vascular tree were evident.	Orlando *et al* [243]
	Rat, pig, and human	SDS	HUVECs + Rat Neonatal kidney cells	(1) The resulting grafts produced rudimentary urine *in vitro* when perfused *via* their intrinsic vascular bed; and (2) Transplanted orthotopic grafts in rats, perfused by the recipient's circulation, produced urine *via* the ureteral conduit *in vivo*.	Song *et al* [244]
	Pig	Sonication + SDS + Triton X-100	Not performed	(1) Significant decrease in decellularization time with sonication; and (2) Sonicator power proved to have significant effect on the microarchitecture integrity of the scaffold.	Manalastas *et al*[76]
Lung					
	Rat	Heparinized PBS + SDS + Triton X-100	HUVECs	Orthotopic Transplantation of grafts with 6 h of perfusion *in vivo*.	Ott *et al*[245]
	Rat	PBS + SNP + CHAPS + EDTA + Benzonase	Rat neonatal lung epithelial + lung vascular endothelial cells	(1) *In vitro*, the mechanical characteristics of the engineered lungs were like those of native lung tissue; and (2) *In vivo* gas exchange for short time intervals (45 to 120 min).	Petersen *et al* [246]
	Mice	Triton X-100 + SDS + DNase	Embryonic stem cells	Demonstrated growth of foetal alveolar type II cells.	Price *et al* [247]
	Rat	Heparinized PBS + SDS + Triton X-100	HUVECs + rat foetal lung cells	Orthotopic transplantation of grafts with 7 d of perfusion *in vivo*.	Song *et al* [248]
Trachea					
	Rabbit	Freeze/thaw + Sonication + SDS	Not performed	(1) Orthoptic transplantation od decellularized scaffolds into segmental tracheal defects in rabbits; (2) Respiratory	Hung *et al* [249]

				epithelium regeneration on the inner surface; and (3) Cartilaginous tubular structures could not maintain structural integrity.	
	Pig	Freeze and Thaw + Agitation/immersion + SDS	Not performed	Successful decellularization.	Guimaraes *et al*[82]
	Rabbits	Sonication + 1 % SDS	Not performed	Orthotopic transplantation of partially decellularized trachea with no immunosuppression treatment resulted in 2 mo of survival in two rabbits and one long-term survival (2 years) in one rabbit.	Dang *et al*[71]
Nerve					
	Human	Triton X-100 + SDS + EDTA + sonication	Not performed	Detergent and sonication more effective than detergent only.	Suss *et al*[74]
Small intestinal submucosa					
	Pig	SDS/Triton X-100/DNase *vs* Agitation and immersion	Not performed	SDS/Triton X-100 combination for decellularization proved superior.	Syed *et al*[81]
Thyroid					
	Rabbit	SDS + immersion/agitation	HTFC	The scaffolds exhibited good cytocompatibility, supported HTFCs growth, and proliferation.	Weng *et al*[85]

HTFC: Human thyroid follicular cells; SDS: Sodium dodecyl sulphate; HUVECs: Human umbilical vein endothelial cells.

Ethylene oxide: Ethylene oxide is a toxic organic compound which reacts with sulfhydryl, amino and carboxyl groups in proteins and nucleic acid molecules[132]. It is a gas at room temp and very permeable so penetrates tissues well, but is very adsorbent to decellularised ECM so difficult to clear, and may form toxic species with water such as ethylene glycol[133].

Peracetic acid: Peracetic acid is produced by the reaction of hydrogen peroxide and acetic acid, with antimicrobial activity resulting from the peroxide group (O-O) oxidation of sulfhydryl groups in proteins[134], and with activity against viral particles when combined with ethanol[135]. Although its advantages are that its decomposition molecules (acetic acid, water, and oxygen), are non-toxic and water soluble, it does result in chemical alteration of ECM[136]. There have been wide ranging applications including many examples in liver, with some favourable outcomes in comparative studies (Table 2).

Hydrogen peroxide and hydrogen peroxide low-temperature plasma: Hydrogen peroxide is a powerful oxidant which reacts with cell membranes and causes the denaturation of nucleic acids and proteins[137]. The plasma form of Hydrogen peroxide, generated by magnetic excitation of gas at low temperature, contains many charged and reactive species which also denature proteins and nucleic acids and cell membranes. Despite the advantage that the end decomposition products (water and oxygen) are non-toxic, the highly reactive original species do result in chemical alteration of proteins [138].

Alcohol: Alcohol disinfects by denaturing proteins. Although it does not eradicate spores, it has been found to be relatively sparing of ECM structure, allowing its use in a wide range of decellularised tissues tissues[131,139]. In the case of liver decellularisation, its use has been mostly in relation to processed ECM[140-142], such as ECM based hydrogels.

Ultra-violet light: Ultraviolet light in the 200–300 nm wavelength range is associated with the strongest disinfection properties, produced by direct DNA damage and generation of ozone as a reactive species. Its advantages are the relative ease of delivery, and the absence of toxic residue, but its limitations are its superficial penetration only, reflected in its use restricted to thin dimension tissues such as small intestine[136], or in case of liver, used for slices of tissue[143].

Antibiotics: Antibiotics use has been reported[131] for treatment of decellularised ECM, including liver [144,145], but their limitations are the restricted spectrum of activity and inability to eradicate spores.

Conclusions

Thus, there are numerous microorganism eradication options, and, based on the properties of sterilisation methods and suitability for specific tissue types, some authors[131] have suggested guidelines to recommend particular methods of sterilisation. In practice, whether these theoretical recommendations deliver the desired microbiological outcome is uncertain, and therefore experimental comparisons of methods seems indicated.

Table 2 Summary of studies comparing different sterilization techniques used for decellularised scaffolds

Species	Organ	Sterilization technique	Outcome	Ref.
Sheep	Liver	Compared 6 different sterilization methods: (1) Freeze drying; (2) Ethylene oxide gas; (3) Gamma irradiation; (4) Gamma irradiation + Peracetic acid; (5) Gamma irradiation + Ethylene oxide gas; and (6) Gamma irradiation + Freeze drying	(1) Peracetic acid or ethylene oxide + gamma irradiation was associated with the best outcome; and (2) Freeze drying and Gamma irradiation completely sterilized the liver, but also reduced the mechanical properties.	Kajbafzadeh *et al*[96]
Porcine	Liver	Compared 3 different sterilization methods: (1) Peracetic acid; (2) Ethanol; and (3) Slightly acidic electrolyzed water	(1) Ethanol caused a significant loss in collagen content; (2) The retained glycosaminoglycan content decreased in all treatments; and (3) Peracetic acid and slightly acidic electrolyzed water treatments achieved the highest efficiency of sterilization.	Hussein *et al*[148]
Mouse	Lung	Compared 2 different sterilization methods: (1) Gamma irradiation; and (2) Peracetic acid	(1) Irradiation produced significant structural distortion; and (2) Peracetic acid had less effect on the resulting architecture.	Bonenfant *et al*[149]
Porcine	TMJ Fibro-cartilage disc	Compared 3 different sterilization methods: (1) Peracetic acid; (2) Gamma irradiation; and (3) Ethylene oxide.	(1) Gamma irradiation and Ethylene Oxide caused structural damage leading to inferior cell adhesions; and (2) Peracetic Acid caused minimal structural damage but also induced chemical modifications leading to better cell attachments.	Matuska *et al*[146]
Porcine	Kidney	Compared 4 different sterilization methods: (1) 70% Ethanol; (2 0.2% Peracetic acid in 1 M NaCl; (3) 0.2% Peracetic acid in 4% Ethanol; and (4) Gamma irradiation	(1) All four methods were successful in decontamination; (2) Gamma-irradiation was very damaging to collagen fibres and glycosaminoglycans, leading to less proliferation of human renal cortical tubular epithelium cells; and (3) 0.2% peracetic acid in 1 M NaCl was found to be the best method as it completely decontaminated the renal tissue and demonstrated to have preserved essential components of the ECM.	Poornejad *et al*[139]
Porcine	Liver	Compared 2 different sterilization methods: (1) Hydrochloric acid; and (2) acetic acid.	(1) ECM treated with Acetic acid showed higher initial attachment and albumin and urea production in HepG2/C3A cell cultures compared to Hydrochloric acid; and (2) Acetic acid preserved bioactive moieties compared to Hydrochloric acid.	Coronado *et al*[97]
Rabbit	Kidney	Compared 4 different sterilization methods: (1) Antibiotics (Penicillin G, Amphotericin B and Gentamicin; (2) Peracetic acid (0.5 %, 1% and 1.5 %); (3) Gamma irradiation 5 KG; and (4) 3 UV-irradiation 20-480 nm	(1) UV-irradiation is not able to sterile; (2) Gamma irradiation resulted in reduced mechanical strength and altered microstructure; and (3) 0.5 % Peracetic acid was the most efficient method to completely decontaminate rabbit decellularized kidney while preserving the mechanical properties and main components of the matrix.	Moradi *et al*[147]

ECM: Extracellular matrix.

In this regard, only a few comparative studies have been carried out for different organ systems including liver and are summarised in Table 2. Drawing confident conclusions from these studies is difficult because of heterogeneity in the range of techniques used, range of tissues examined, in different animal species.

However, from the studies where comparisons were made, there appears to be some degree of consistency favouring the use of peracetic acid, in achieving sterility with minimal ECM damage in sheep liver[96], porcine kidney[139], porcine temporo-mandibular joint disc[146], rabbit kidney[147], porcine liver[97,148], and mouse lung[149].

LIVER DECELLULARISATION AND RECELLULARISATION

Introduction

Since the first report of successful decellularisation and repopulation of liver tissue carried out in rat liver by Uygun *et al*[86], there have been significant developments with further reports in other models, and evolution in many aspects including the challenge of sizing up technology for larger species livers, investigation of optimal decellularisation method, progress in the variety, delivery, and functional assessment of repopulating cells, culminating in recent reports providing the first evidence of physiologically significant function in large animal bioengineered organs. This section provides an account of areas of advance, highlighting studies which have contributed incremental progress in the field, and for which additional information is given in Table 3.

Liver decellularisation

Similar to the situation in the non-hepatic context, numerous protocols for liver decellularisation have been reported[150,151], varying in nature of decellularising agents, technique, and time required

Table 3 Liver decellularisation recellularisation studies

Species	Decellularisation method	Recellularisation cell type and route	Outcome	Ref.
Female Lewis rats	SDS + Triton X-100	(1) Primary rat hepatocytes *via* the Portal vein; and (2) Rat cardiac microvascular endothelial cells *via* portal vein	(1) Demonstrated Successful decellularization/Recellularization with cell viability and function; (2) Demonstrated the feasibility of transplanting these recellularised liver grafts *in vivo* with minimal ischemic damage; and (3) The recellularised graft supports liver-specific function including albumin secretion, urea synthesis and cytochrome P450 expression at comparable levels to normal liver *in vitro*.	Uygun *et al*[86]
Fisher 344 rats	Triton X-100 + SDS	Rat liver progenitor cell line WB344 through the inferior vena cava	(1) Perfusion with 0.1% SDS for 1 hour completely cleared all DNA; and (2) Supplementation of all perfusion solutions with antibiotics/antimycotics prevented microbial growth, and the IDL could be stored at 4°C for several weeks.	Shupe *et al* [156]
Male Sprague Dawley rats	Trypsin + EGTA + Triton X-100	Primary mice hepatocytes *via*: (1) Direct parenchymal injection; (2) Continuous perfusion *via* the portal vein; and (3) Multistep infusion *via* the portal vein	Systematic comparison of three different reseeding methods showed that a multistep strategy provides the greatest seeding efficiency and the presence of functional hepatocytes.	Soto-Gutierrez *et al*[164]
Male Lewis rats	SDS + Triton X-100	Primary rat hepatocytes *via* the portal vein (from spheroid culture)	(1) Layer-by-layer heparin deposition was used to avoid thrombosis, followed by repopulation of hepatocytes, and successfully implanted as a TEL into the portal system; (2) Treatment of extended hepatectomized rats with a TEL improved liver function and prolonged survival; mean lifespan was extended from 16 to 72 h; and (3) At 72 h post operation, the TEL sustained functional and viable hepatocytes.	Bao *et al* [174]
Ferret	Distilled water + Triton X-100 + ammonium hydroxide	Human foetal liver cells + human umbilical vein endothelial cells co-infusion *via* the portal vein	Demonstrated delivery of cells to different compartments of the liver tissue *via* different pathways EC delivered through the vena cava selectively seeded larger and smaller blood vessels up to the pericentral area of the liver lobule and cells seeded through the portal vein reached predominantly the periportal area of the liver lobule.	Baptista *et al*[90]
Adult male Sprague–Dawley rats	SDS or Triton X-100 + sodium hydroxide	Primary rat hepatocytes *via* the portal vein	Decellularised scaffolds constructed by perfusion of Triton X-100 were of superior quality and can provide a more effective and ideal scaffold for tissue engineering and regenerative medicine.	Ren *et al* [161]
Porcine	SDS + DNase	Porcine hepatocytes *via* the portal VEIN	Demonstrated a protocol to decellularise rapidly a full-size porcine liver with small detergent volumes within 24 h.	Bühler *et al* [153]
Human	Distilled water + SDS + Triton X-100	Human cell lines hepatic stellate cells (LX2), hepatocellular carcinoma (Sk-Hep-1) and hepatoblastoma (HepG2) *via* suspension	Decellularised human liver cubic scaffolds were repopulated for up to 21 d using human cell lines with excellent viability, motility and proliferation and remodelling of the extracellular matrix.	Mazza *et al* [154]
Piglet	Triton X-100 + ammonium hydroxide	Murine endothelial cells (MS1) with combination of static and perfusion techniques (*via* the portal vein)	(1) Developed an effective method for re-establishing the vascular network within decellularised liver scaffolds by conjugating anti-endothelial cell antibodies to maximize coverage of the vessel walls with endothelial cells; (2) This procedure resulted in uniform endothelial attachment throughout the liver vasculature extending to the capillary bed of the liver scaffold and greatly reduced platelet adhesion upon blood perfusion *in vitro*; and (3) The reendothelialized livers, when transplanted to recipient pigs, were able to withstand physiological blood flow and maintained for up to 24 h	Ko *et al*[89]
Porcine	SDS + Triton X-100	Rat primary hepatocytes and human umbilical vein endothelial cells (cells cultured in scaffolds, but not in a perfusion circuit)	(1) The heparinized scaffolds showed improved anticoagulation and cytocompatibility compared to the control scaffold both *in vitro* and *in vivo* test; and (2) The layer-by-layer technique showed that heparinisation did not interfere with hepatocyte or endothelial cell repopulation.	Bao *et al* [176]
Porcine	SDS	Human EA.hy926 endothelial cells and HepG2 hepatic carcinoma cells *via* the portal vein	(1) The study demonstrated, exposing scaffold to heparin-gelatin mixture improved endothelial cell ability to migrate and cover vessel discs, perhaps by exploiting gelatin's multiple integrin binding sites which facilitate endothelial cell binding; and (2) Scaffolds repopulated with Hep G2 hepatocytes and endothelial cells after heparin gelatin coating showed improved *ex vivo* blood perfusion, in comparison to uncoated scaffolds.	Hussein *et al*[87]
Male Lewis rats	Trypsin + EGTA + Triton X-100	Primary rat hepatocytes *via* the bile duct and the portal vein	The study results suggest that biliary tree cell-seeding approach is promising, and that liver progenitor cells represent a good cell source candidate.	Ogiso *et al* [173]
Male Lewis rats	Trypsin + EGTA +	(1) Primary rat hepatocytes	(1) Hepatocytes co-seeded with LSECs retained their function	Kojima *et*

	Triton X-100	*via* the Bile duct; and (2) LSECs *via* the portal vein	compared with those seeded alone; (2) LSECs maintained hepatic function, and supported hepatocyte viability under blood perfusion in the engineered liver graft owing to their antithrombogenicity; and (3) Successfully achieved continuous blood flow into the vascularized liver graft by extracorporeal perfusion for at least 8 hours	*al*[172]
Female Lewis rats	SDS + Triton X-100	Human EA.hy926 endothelial cells *via* the portal vein	(1) Coupled the cell-binding domain REDV to the vasculature of decellularised rat livers; and (2) REDV coupling increased cell attachment, spreading and proliferation of endothelial cells within the scaffold resulting in uniform endothelial lining of the vasculature, and a reduction in platelet adhesion and activation	Devalliere *et al*[88]
Female Lewis rat	SDS	(1) Rat cholangiocytes *via* the common bile duct; and (2) Rat hepatocytes *via* the portal vein	(1) Demonstrated for the first time, whole liver grafts co-populated with hepatocytes and cholangiocyte; (2) Cholangiocytes formed duct-like structures, with the viable hepatocyte mass residing in the parenchymal space, in an arrangement highly comparable to the native tissue; and (3) Both albumin and urea assay results confirmed hepatocyte functionality and the gene expression analysis of cholangiocytes in recellularised liver grafts indicated viability and sustained gene expression of functional proteins.	Chen *et al*[177]
Adult Sprague–Dawley rats	Triton X-100 + NH4OH	Rat sinusoidal endothelial cells were perfused *via* the Portal vein in either RPMI media or in 5% gelatin hydrogel solution	(1) Used immortalized endothelial cells to repopulate decellularised rat liver scaffolds; (2) Gelatin hydrogels-based perfusion significantly increased the number of cells that were retained in the scaffolds; and (3) The Doppler ultrasound detected active blood flows within the re-endothelialised liver scaffolds 8 d post heterotopic transplantation.	Meng *et al*[190]
Male Lewis rats	Trypsin/EGTA solution + Triton X-100/EGTA	Human induced pluripotent stem cells derived hepatocyte-like cells *via* bile duct	(1) The first study to generate a recellularised liver model with human hepatic function using human induced pluripotent stem cells; and (2) This result suggested that the BD was an appropriate recellularization pathway regardless of the hepatocyte type.	Minami *et al*[250]
Porcine	SDS + Triton X-100	Human umbilical vein endothelial cells *via* the superior vena cava followed by *via* the portal vein	Decellularised whole porcine livers revascularized with human umbilical endothelial cells and implanted heterotopically into immunosuppressed pigs whose spleen has been removed sustained perfusion for up to 20 d.	Shaheen *et al*[191]
Porcine	Triton X-100 + SDS	(1) Human umbilical vein endothelial cells *via* the vena cava and the portal vein; and (2) Porcine hepatocytes *via* the bile duct	(1) Co-seeded primary porcine hepatocytes after human umbilical vein endothelial cell reendothelialization; and (2) Repopulated scaffolds were implanted heterotopically in a pig model and produced improved biochemical function in an acute liver failure model.	Anderson *et al*[175]
Female Sprague-Dawley rats	SDS + DNase	Human umbilical vein endothelial cells *via* the Portal vein	(1) Used aptamers (short, single-stranded DNA or RNA molecules that selectively bind to specific targets) with CD31 specificity; and (2) Aptamer coated scaffolds showed higher endothelial cell coverage, enabled perfusion with blood for 2 h with reduced platelet adhesion ex vivo, and restored liver function in a hepatic fibrosis rat model.	Kim *et al*[192]

TEL: Tissue-engineered liver; SDS: Sodium dodecyl sulphate; LSECs: Liver sinusoidal endothelial cells; REDV: Arg GluAsp Val.

ranging from hours[86,152] to days[153], to weeks[154] (rat, pig, human respectively) correlating with organ size. Perfusion of decellularising agents *via* the vasculature is the only means of reaching whole parenchymal space in a large organ such as the liver and has been used in all such studies.

The vessels available for infusion of decellularising agents are the portal vein, hepatic artery, and hepatic veins. Of these options, perfusion *via* the portal vein has been used most frequently although some authors report infusion *via* the hepatic veins *via* the inferior vena cava[155,156], the hepatic artery [157], and the hepatic artery and portal vein in combination[158]. Determining whether infusion route is an important factor in decellularisation quality is difficult as almost all studies report one particular technique, presumably arrived at empirically. Two studies suggest pulse flow *via* the hepatic artery provided better quality decellularisation, though whether this improved recellularisation potential with repopulating cells was not assessed[159,160].

Choice of detergent for decellularisation is equally varied though protocols using SDS and/or triton X-100 are the most frequently used, with SDS more effective at removing cellular debris, but at the expense of greater detriment to ECM structure. There are few comparative studies, with the exception of those of Ren *et al*[161], Wu *et al*[162], and Kajbafzadeh *et al*[96], showing lesser matrix degradation (with better structural protein, growth factor and glycosaminoglycan retention) and better repopulating cell function with triton X-100 in rat, porcine and sheep liver decellularisation models respectively.

In addition to biochemical content, mechanical structure of ECM important in contributing to signals which influence cell function[46]. In studies comparing protocols in sheep liver, Triton X-100 and SDS resulted in scaffolds with similar tensile strength, but Triton X-100 based protocols resulted in better retention of elasticity[96,163].

Disinfection and sterilisation of scaffold

Diverse methods have been used to eradicate micro-organisms from decellularised liver scaffolds. Once again there are few studies directly comparing the available methods, but those that exist provide some consensus in favour of perfusion with peracetic acid with reports in sheep[96] and porcine[97,148] liver models, suggesting that peracetic acid was optimal in the dual objective of achieving sterility and maintenance of matrix structure, albeit with protocols varying in concentration and time of exposure.

Characterisation of decellularised scaffold

As a result of the many decellularisation and sterilisation techniques, arises a need for some means of assessing the resultant scaffold to enable comparisons of scaffold quality not only for comparative research but also in view of future clinical applications. Ultimately, although the most meaningful quality criterion is how successfully a scaffold accommodates repopulating cells to generate a neo-organ with useful function, this high-level objective has proved difficult to achieve, resulting in the use of intermediary scaffold assessment methods. It is likely that as research advances, new criteria will emerge, with those which best predict end function becoming dominant.

DNA content: Some of the earliest scaffold quality criteria were put forward by Crapo *et al*[125], who suggested that successful decellularisation should be determined on the basis of producing ECM which (1) Does not contain more than 50 ng of DNA per mg dry weight; (2) with residual DNA fragments no longer than 200 bp; and (3) with no visible nuclear components[125], based on observations of *in vivo* adverse effects of these biochemicals[64,67,126]. In addition to gel electrophoretic methods to determine DNA fragment length, light microscopy with hematoxylin and eosin stain and DAPI stain have been used to demonstrate absence of residual DNA and supplemented by electron microscopy to visualise cell free matrix microarchitecture[86].

Protein and complex polysaccharide content: In contrast to nucleic acids which must be removed, there is a need to preserve structural proteins, growth factors and other complex molecules in the matrix. Many studies report qualitative and quantitative measures of the structural proteins collagen, laminin, elastin, fibronectin as well as glycosaminoglycans[86,158,161], whilst others quantify pre and post decellularisation content for known ECM associated growth factors including hepatocyte growth factor [161], basic fibroblast growth factor[164], vascular endothelial cell growth factor (VEGF) and insulin-like growth factor 1[165], and many others described by Park *et al*[166].

Non-destructive scaffold assessment: The above methods of scaffold assessment require physical sampling and destruction of the decellularised scaffold, preventing its subsequent use for recellularisation. Thus, pursuing the need to establish methods of scaffold assessment that leave the scaffold intact for further experimentation, Geerts *et al*[167] describe non-destructive methods of scaffold assessment by computerised tomography and biochemical analysis of decellularisation effluent perfusate.

Vascular tree structural integrity: The vasculature has a particular importance in the intended aim of recellularisation as parenchymal cell populations are critically dependant on a reliable blood supply. Thus many authors report preservation of ECM scaffold which define vessels as demonstrated by injection of coloured Dextran[90], radio-opaque dye[165], and corrosion casts[168].

Liver scaffold recellularisation

Repopulating cell heterogeneity: With recellularisation of scaffolds comes the choice of repopulating cells. Many different cell types have been investigated including cell lines, induced pluripotent stem cells (IPSCs), mesenchymal stem cells, foetal stem cells, primary adult cells, and their propagated form after culture in organoids -all with associated advantages and shortcomings.

Immortalised cell lines are useful experimental work tools in that they offer a homogeneous population with a stable phenotype, which can be easily propagated in large numbers. However, there is little or no scope for a role beyond experimentation and into clinical applications given the risk of unchecked proliferation and malignant transformation. IPSCs[166] are also very powerful experimental tools with all the advantages of cell lines, and the added benefits of phenotypic versatility, but are similarly limited in clinical applications because of malignant transformation concerns. Mesenchymal stem cells offer a potentially clinically relevant cell type in terms of sourcing, propagation and safety, with possible beneficial immune modulation effects[169], but are probably limited in their range of differentiation end points[170]. Hepatic foetal cells[90] offer advantages of propagation and differentiation plasticity, but have little clinical application potential because of ethical, availability, and immuno-allogeneicity issues. Primary cells[164] offer the advantages of stable, mature phenotype without concerns for malignant transformation, but present difficulties in terms of sourcing, and propagation to clinically relevant cell numbers during which loss of function is often observed. Organoid cultured primary cells (discussed in more detail in the section on cholangiocyte recellularisation below) may offer a realistic solution to expanding primary cells *in vitro* without loss of desirable phenotype.

Thus, there are a multitude of studies reporting hepatic scaffold repopulation using a variety of cell types, introduced into scaffolds *via* different routes, and using various cell combinations, and reporting different means of assessing the repopulated scaffold. The sections below deal with this heterogeneity by describing progress in recellularisation by considering each main hepatic cell type. It is entirely acknowledged however, that optimal function will be achieved by simultaneous co-recellularisation of a variety of cell types, as cell interactions are critical for optimal cell function[171]. Key examples of this concept in the liver recellularisation literature include the reports of (1) Baptista *et al*[90] showing that human foetal liver cells and human umbilical vein endothelial cells (HUVECs) exhibited better function when infused together in scaffold than individually; (2) Barakat *et al*[168], showing that human foetal stellate cells and human foetal hepatocytes together resulted in the generation of mature hepatocyte phenotype; and (3) Kojima *et al*[172], showing that co-seeding of hepatocytes with liver sinusoidal endothelial cells (LSECs), but not HUVECs, improved hepatocyte function.

Hepatocyte recellularisation: The first report of liver tissue decellularisation and repopulation by Uygun *et al*[86] in a rat model was followed by others in rodent models[172-174], and thereafter on a larger scale in pig[153] and human livers[154].

These and other models have used a variety of hepatocyte sources for recellularisation including mostly primary hepatocytes[175,176], but also primary hepatocytes after spheroid propagation[174], foetal hepatocytes[90] and hepatocyte carcinoma cell lines[87].

The mechanism of re-introduction of hepatocytes has been by means of infusion *via* the portal vein in the vast majority of studies, though infusion *via* multiple vascular routes (Hepatic artery, Portal vein, supra and infra hepatic vena cava)[89] and *via* the *via* bile duct[175] have also been reported. There are few comparative studies to determine whether one or other route is optimal, though one study reports significantly higher parenchymal engraftment of hepatocytes after infusion *via* the biliary tree in comparison to the portal vein[173]. For portal vein infusion of hepatocytes, multiple sequential infusions result in better cell engraftment efficiency, cell proliferation, and cell function than infusion of the same number of hepatocytes in one single infusion[86,164].

Many indicators of function have been used to assess the function of hepatocytes reintroduced into decellularised scaffolds, including: (1) Albumin and urea production[86,90]; (2) elimination of ammonia, consumption of glucose and expression of cytochrome p450 metabolic enzymes[161,164,175]; (3) Immunofluorescence demonstration of expression of hepatocyte-specific marker fumarylacetoacetate [175]; (4) Immunostaining demonstration of hepatocyte viability enzymes such as UDP glucuronosyl-transferase 1, glucose6phosphatase[86]; (5) Expression of dipeptidyl peptidase- 4, a bile canaliculus marker, demonstrating hepatocyte polarity[172]; and (6) Immunofluorescence demonstration of hepatocytic lineage markers α-fetoprotein, CYP2A and CYP3A[90].

Ultimately, however, the most meaningful measure of hepatocyte function is whether a repopulated scaffold can exhibit significant function in the harsh test of *in vivo* physiological environment. Two studies have reported the early stages of such function: Bao *et al*[174] repopulated decellularised and heparin treated rat liver scaffolds with primary rat hepatocytes from spheroid culture. Repopulated scaffolds were implanted heterotopically in rats having undergone 90% hepatectomy, with control animals undergoing 90% hepatectomy without scaffold implantation. At 72 h post-operation, hepatocytes in the implanted scaffolds expressed liver specific genes, including coagulation factor X, albumin, and cytochrome P450. In contrast to control rats whose ammonia levels rose substantially, scaffold implanted rats had significantly slower ammonia increases, and mean survival in this acute liver failure model was increased from 16 h to 72 h.

Anderson *et al*[175] repopulated a decellularised porcine liver using HUVECs infused *via* the vena cava and portal vein and porcine hepatocytes *via* bile duct infusion.

In vitro assessment of the repopulated scaffolds showed increasing production of Von Willebrand factor over time, albumin production, ammonia detoxification and urea production. The presence of HUVEC repopulated vasculature was essential to sustain blood flow in an *ex-vivo* blood circuit. The authors also investigated a porcine heterotopic liver transplant model of acute liver failure. Thus, scaffold portal vein and vena cava were anastomosed to native portal vein and inferior vena cava respectively, and native liver blood flow was entirely abolished by ligation of native portal vein branches and arteries to native liver. The scaffolds sustained flow for 48 h during which intracranial pressure (ICP) and ammonia levels (indicators of acute liver failure) were monitored. Control animals underwent portocaval shunt and liver devascularisation without scaffold implantation. Although no definite differences were seen in ICP measurements, the scaffold transplanted animals showed clear evidence of ammonia level stabilisation in contrast to inexorable increase in control animals. The authors suggested that scaffold functionality was limited by the small size of the grafts (required by the heterotopic implantation) which restricted the number of implanted hepatocytes and resulted in significant small for size syndrome.

Cholangiocyte repopulation: In comparison to hepatocyte repopulation, there are to date few if any reports of repopulation of the biliary tree using primary cholangiocytes. In a rare report in this category, Chen *et al*[177] repopulated a decellularised rat liver scaffold with primary rat cholangiocytes *via* the bile duct and hepatocytes *via* the portal vein, and perfused the repopulated scaffold for 48 h *in vitro*,

with assessments showing expression of a number of cholangiocyte genes including cytokeratin 7, Cystic Fibrosis transmembrane conductance regulator (CFTR), hepatocyte nuclear factor-1 alpha (HNF-1α), gamma glutamyl transferase (GGT).

The reason for the relative absence of studies reporting repopulation with primary cholangiocytes is the longstanding challenge of propagating and maintaining cholangiocytes in conventional cell culture with loss of essential phenotype[178]. As an alternative, driving pluripotent stem cells towards cholangiocytic differentiation requires extensive manipulation[179] and the clinical applicability of such cells remains in doubt in terms of the risk of malignant change[180]. The evolution of organoid culture, however, offers possible opportunities.

Organoid cultures are 3D cell culture systems whereby cells of choice, when placed in the 3D environment of a supporting substrate (typically Matrigel) undergo cell differentiation, self-organization, whilst retaining the ability to propagate[181]. Thus, organoid culture has provided a potential solution to the supply of biliary epithelial cells, allowing expansion of cholangiocytes from small adult tissue samples whilst retaining cholangiocyte phenotype[182] such expression of such as cytokeratins 7 and 19, and epithelial cell adhesion molecule. The technique, first achieved with intrahepatic human cholangiocytes from a liver biopsy by Huch *et al*[183], was then confirmed subsequently using extrahepatic bile duct cholangiocytes[184], and bile derived cholangiocytes[185,186], with demonstrable transcriptomic and phenotypic differences between cholangiocytes of different origin within the biliary tree[187].

The availability of cholangiocytes provided by organoids has allowed their use in repopulation of decellularised biliary tissue in several models. Thus, Willemse *et al*[188] repopulated decellularised human bile duct tissue with intra hepatic, extra hepatic, and bile derived cholangiocytes from organoid culture and analysed expression of cholangiocyte markers and biliary function of the tissue engineered constructs. In contrast to intra hepatic counterparts, extra hepatic and bile derived cholangiocytes repopulated decellularised bile duct efficiently, exhibited tight junctions and polarity with apical cilia, showed a gene expression profile suggesting maturation of cholangiocytes, as well as appropriate expression cholangiocyte-specific transporter genes such as CFTR, which was active in a functional assay. Similarly, Roos *et al*[189] isolated cholangiocytes from human bile collected from gall bladders after cholecystectomy, percutaneous trans-hepatic cholangiography, and endoscopic retrograde cholangio-pancreatography (ERCP), and demonstrated efficient and long-term organoid culture (passage > 15 over > 5 mo). The cholangiocytes in organoids showed transcriptomic patterns consistent with native cholangiocytes, expressed functional ion channel protein MDR1, and efficiently repopulated decellularised human bile duct scaffolds.

The potential of organoid cultured cholangiocytes was further emphasised by Sampaziotis *et al*[184] who cultured biliary organoids using human cholangiocytes from deceased donors as well as ERCP brush samples. Transcriptomic analysis showed maintained genetic stability over passages and expression of key biliary markers, including cytokeratins 7 and 19, HNF-1β, GGT, secretin receptor, sodium-dependent bile acid transporter (SLC10A2), CFTR and SRY-box 9. Electron microscopy revealed the presence of ultrastructural features characteristic of cholangiocytes, including cilia, microvilli, and tight junctions. Finally, several assays demonstrated key functionalities: (1) Rhodamine 123 accumulated in the ECO lumen only in the absence of the MDR1 antagonist verapamil; (2) fluorescent bile acid cholyl-lysyl-fluorescein was actively exported from cholangiocyte organoids; and (3) Secretin promoted water secretion, resulting in distension of the bile duct lumen, whereas somatostatin negated the effects of secretin. Moreover, *in vivo*, the cholangiocytes self-organized into bile duct–like tubes after transplantation into nude mouse kidney capsule. Finally, the cholangiocytes maintained their phenotype in biodegradable polyglycolic acid scaffolds discs and densified collagen cylinders. Respectively, the repopulated structures were used in mouse *in vivo* models to successfully repair gall bladder wall and reconstitute a functional extra-hepatic biliary tree.

In a further analysis of the potential of organoid cultured cholangiocytes, Sampaziotis *et al*[186] isolated human cholangiocytes for intrahepatic, extrahepatic and gall bladder bile. Transcriptomic analysis showed that cholangiocytes from different sites expressed a core of similar genes but differed in others. The cholangiocytes displayed a gradual shift in their transcriptional profile along the biliary tree, suggesting a response to region-specific microenvironments. Thus, when grown in organoid culture, cholangiocytes of different regions of the biliary tree reverted to a single common expression profile but, when exposed to gall bladder bile adopted the expression profile corresponding to the site of origin of bile. Using a mouse model of cholangiopathy induced by 4,4′ methylenedianiline, intraductal delivery of human gallbladder organoids resulted in engraftment of cholangiocytes, correction of cholangiopathy and phenotype rescue, in comparison to 100% fatality amongst the control group. In a human liver model using discarded deceased donor livers with ischaemic biliary injury, injected organoids engrafted in areas of denuded biliary epithelium, and corrected cholangiopathy.

Thus, in conclusion, whilst there has been a deficit in reports of biliary tree repopulation for decellularised liver scaffolds since the first report of this approach in 2010 from Uygun *et al*[86], the advent of organoid culture appears to have provided a novel means of propagating stable, functional cholangiocytes in sufficient numbers. This would appear to be the best current way of progressing with biliary repopulation of decellularised liver scaffolds.

Hepatic vascular recellularisation: Reconstitution of a viable vasculature in a decellularised liver scaffold is of paramount importance, to allow not only function but survival of the other liver cell populations. The objective is complicated in the case of the liver because of its dual blood inflow supply *via* hepatic artery and portal vein, the immensely complex architecture of liver sinusoids, and the uniquely specialist functions of the sinusoidal endothelial cells. This area of research has progressed in terms of the range of cells used, attempts to optimise the quality of endothelial cover to minimise thrombosis, and advancement in large animal blood perfusion models.

In the first report relating to liver scaffold repopulation, Uygun *et al*[86] used commercially sourced rat cardiac microvascular cells to create an endothelial lining, allowing the repopulated scaffold to be perfused in an *ex-vivo* rat blood circuit for 24 h, and in an *in vivo* heterotopic implantation model to renal vessels for an 8 h perfusion period. Subsequent reports have used a variety of cell types to create vascular cover including Ms1 cells[90], HUVECs[90,172,175,176], human EA.hy926 endothelial cell line [87,88], immortalised endothelial cells[190], and primary liver sinusoidal endothelial cells[172].

Functionality of these repopulated vascular cells has been assessed by various criteria including (1) light microscopy to show vascular cover[90], and supplemented with electron microscopy to demonstrate the presence of sinusoidal cell fenestrae[172]; (2) demonstrating the expression of endothelial cell gene product such as of Von Willebrand factor[90,175], endothelial nitric oxide synthase (eNOS)[90], Lymphatic vessel endothelial hyaluronan receptor 1 and stabilin 2 expression[191], Platelet endothelial cell adhesion molecule 1 (PECAM-1), CD34, VE-cadherin (vascular endothelial cadherin), eNOS, VEGF expression[87], sinusoidal endothelial marker (SE- 1) and stabilin-2[172]; (3) platelet adhesion studies[90]; (4)Transcriptomic analysis of infused HUVECs assuming an LSEC phenotype [191]; and (5) Glucose consumption rate[175,191] of infused endothelial cells.

Given the prime importance of preventing thrombosis in the scaffold, several approaches have explored treating the scaffold with anticoagulants and enhancing endothelial cell cover of the decellularised vascular network. Thus, Bao *et al*[174] investigated layer by layer deposition of heparin in decellularised scaffolds, with hepatocyte repopulation, and reported sustained blood perfusion up to 72 h in a heterotopic rat implantation model, in comparison to rapid thrombosis in un-heparinised scaffolds. In a later study, the same group[176] optimised the layer-by-layer technique and showed that heparinisation did not interfere with hepatocyte or endothelial cell repopulation.

Whilst interesting as a possible method of improving initial thrombogenicity, maintaining heparin deposition is not achievable in the longer term, and could present undesirable consequences. Thus, some authors have investigated the use of heparin to maximise endothelial cell cover, rather than chemically bonding it to scaffold. Studies reporting better endothelial cell repopulation in the presence of heparin preparations include that of Hussain *et al*[87], who reported that exposing scaffold to heparin-gelatin mixture improved endothelial cell ability to migrate and cover vessel discs, perhaps by exploiting gelatin's multiple integrin binding sites which facilitate endothelial cell binding. Scaffolds repopulated with Hep G2 hepatocytes and endothelial cells after heparin gelatin coating showed improved *ex vivo* blood perfusion, in comparison to uncoated scaffolds. Similarly, Meng *et al*[190] 2019 used immortalized endothelial cells to repopulate decellularised rat liver scaffolds. Gelatin hydrogels-based perfusion significantly increased the number of cells that were retained in the scaffolds, and Doppler ultrasound detected active blood flows within the re-endothelialised liver scaffolds 8 d post-transplantation.

Adopting a different approach, some groups have investigated the manipulation of endothelial cell attachment to scaffold to improve vascular cover. Devalliere *et al*[88] covalently coupled the cell-binding domain REDV to the vasculature of decellularised rat livers before seeding endothelial cells *via* the portal vein. REDV coupling increased cell attachment, spreading and proliferation of endothelial cells within the scaffold resulting in uniform endothelial lining of the vasculature, and a reduction in platelet adhesion and activation. Ko *et al*[89] conjugated anti-endothelial cell antibodies to liver scaffolds resulting in uniform endothelial attachment and reduced platelet adhesion upon blood perfusion *in vitro* . The re-endothelialised livers, withstood physiological blood flow *in vivo* for up to 24 h in a porcine implant model. Kim *et al*[192] used aptamers (short, single-stranded DNA or RNA molecules that selectively bind to specific targets) with CD31 specificity. Aptamer coated scaffolds showed higher endothelial cell coverage, enabled perfusion with blood for 2 h with reduced platelet adhesion *ex vivo*, and restored liver function in a hepatic fibrosis rat model.

In the most significant advances to date in the area of successful hepatic vascular perfusion of repopulated scaffolds, at least in terms of length of *in vivo* perfusion, Shaheen *et al*[191] seeded decellularised whole porcine livers with HUVECs and showed successful perfusion of the heterotopically implanted scaffolds into for up to 20 d. The same group[175] later co-seeded primary porcine hepatocytes after HUVEC reendothelialisation. Repopulated scaffolds were implanted heterotopically in a pig model and produced improved biochemical function in an acute liver failure model.

In conclusion, the difficult problem of repopulating the vasculature of decellularised scaffolds has seen significant progress, with reports of *in vivo* blood perfusion lasting many days. Whist encouraging, there remain advances to be made in the development of clinically relevant cell populations for this purpose, and the repopulation of the highly specific liver sinusoidal endothelial cells.

Conclusions

The field of hepatic scaffold recellularisation has advanced from *in vitro* rodent liver scaffold models to large animal *in vivo* blood perfusion. Whilst this represents much progress, significant areas of development remain to be investigated. Of the different liver cell types, even in the case of hepatocytes where repopulation results are the most advanced, the degree of hepatocyte function observed to date is still limited. Cholangiocyte recellularisation is far behind, though organoid sourced cells may help with this challenge. Intra-hepatic vascular recellularisation has allowed impressive *in vivo* perfusion but using cells which have limited application beyond experimental models. Minority cell groups such as Kupffer cells and stellate cells, though important in their influence on other cell types, have not been repopulated decellularised scaffolds.

RECELLULARISATION OF EXTRA HEPATIC BLOOD VESSELS

Introduction

If the objective of whole liver recellularisation is the bioengineering of neo-organs is implantation to provide useful function, neo-livers will need to be fully reconnected to the recipient circulation, with both hepatic arterial and portal venous inflow, and hepatic vein outflow. To date, because the focus of investigation has understandably been to achieve viable blood circulation through the sinusoidal network, extra-hepatic vascular inflow has relied exclusively on portal reperfusion of recellularised grafts. Whilst much progress has been made with sustained portal perfusion of up to 20 d in large animal models[191], recellularisation and perfusion of the hepatic artery has not been reported. This gap in the field will need to be addressed, as, unlike hepatocytes which may survive on portal flow alone, the biliary tree is critically dependant on hepatic arterial supply.

Arterial scaffold recellularisation precedents

In addition to thrombogenicity, the hepatic artery presents considerable other difficulties stemming from the biophysical demands of withstanding arterial pressure in the short and long term. In the short term, a recellularised artery and its arterial anastomosis needs to be able to tolerate pressures of 3000 mmHg[193], and then do so in the long term without accelerated atherosclerosis.

The challenges of bioengineering viable arterial conduits[193] is an entire field in itself, with much research motivated by the clinical need represented by the immense burden of cardiac, cerebrovascular and peripheral vascular disease. The research trajectory of vascular biologists and clinicians in vessel bioengineering has followed much the same path as those studying the liver. As a result of the drawbacks of synthetic[194] and allogeneic and xenogeneic grafts[195] (long-term patency issues due to thrombosis, inflammation, and stenosis), there has been an evolution towards cellular repopulation of scaffolds of various types. Thus, following pioneering reports by Weinberg *et al*[196] of early bioengineered vessels containing collagen, Dacron and a combination of smooth muscle and endothelial cells, L'Heureux *et al*. reported the use of extracellular matrix with vascular cells to bioengineer a blood vessel[197], with subsequent reports of successful bioengineered grafts in clinical practice[198,199].

Arterial vessel anatomy is complex and consists of three concentric layers (1) the intima layer, composed of endothelial cells resting on an internal elastic lamina layer of type 4 collagen and elastin, which separates it from media; (2) the media, composed of smooth muscle cells (SMC), type I and type III collagen; and (3) the adventitia, containing fibroblasts embedded in a loose collagen matrix of type I and type II collagen. The ability of arteries to withstand arterial pressure waves stems from the complex tri-layer of cells and ECM above, which therefore likely requires recapitulation to achieve similar function in recellularised grafts. In the context of tubular grafts, this challenge has been investigated using a variety of biofabrication techniques including biomaterial moulding[200], cell sheet engineering [201], bio-ink applications, with tissue maturation[202] under fluid flow[203] in purpose designed bioreactors[193].

In the specific case of arterial scaffolds obtained by decellularisation techniques, followed by repopulation with appropriate cells, there are many examples of successful long term outcomes in a variety of experimental models, reviewed by Krawiec *et al*[204], and including (1) Cho *et al*[205] who used canine bone marrow mononuclear cells differentiated under different culture conditions to generate smooth muscle and endothelial phenotypes. These were reintroduced into decellularised dog carotid arteries sequentially to create media and intimal layers in neo-vessels, which were reimplanted in a canine carotid model. Seeded grafts were patent at 8 wk compared to thrombosis at 2 wk in unseeded controls; (2) Similarly, Zhao *et al*[206] used ovine bone marrow stem cells and differentiated them into endothelial and smooth muscle phenotypes, before seeding them onto decellularised carotid artery scaffolds. Seeded scaffolds were mechanically stable and patent at 5 months, in comparison to unseeded controls, which all occluded at 2 wk or less; (3) Kaushal *et al*[207] isolated endothelial precursor cells from peripheral blood of sheep, expanded them *ex vivo* and then seeded them on decellularised porcine iliac vessels. Seeded grafts remained patent for 130 d as a carotid interposition graft in sheep, whereas non-seeded grafts occluded within 15 d; (4) Borschel *et al*[208] repopulated decellularised rat femoral arteries with primary endothelial cells, which were implanted as interposition grafts. Patency rates at 4 wk were

89% and 29% recellularised grafts and control grafts respectively; (5) Ma *et al*[209] repopulated decellularised foetal pig aortas with canine endothelial cells and demonstrated 6-mo patency after reimplantation in a canine carotid model; and (6) Dahan *et al*[210] repopulated decellularised pig carotid artery with autologous endothelial and smooth muscle cells and demonstrated 6-week patency in a carotid interposition graft model.

Conclusion

Thus, the problem of arterial recellularisation brings very significant and specific challenges, but with some promising possible solutions suggested by long term successful perfusion bioengineered repopulated decellularised arterial neo-vessels in several animal models.

IMMUNOGENICITY OF DECELLULARISED SCAFFOLDS

Introduction

In its most ambitious objective, bioengineering neo-organs by decellularisation and recellularisation would involve the use of allogeneic or even xenogeneic scaffolds repopulated with appropriate cells originating from the intended recipient. The resultant neo-organ would thus in theory be immunologically syngeneic, at least from the perspective of the repopulating cells. The question remains, however, whether non-self scaffold, even if covered by syngeneic cells may elicit an adverse immune or inflammatory reaction.

Scaffold immunogenicity studies

Overwhelmingly, *in vivo* animal studies and human clinical studies examining implantation of decellularised scaffold show non pathological and constructive, functional tissue remodelling with the partial restoration of tissue appropriate to the site of implantation[211]. Examples of such animal studies include that of Mirmalek-Sani *et al*[157], who observed no local or systemic adverse host response to decellularised porcine liver scaffold introduced into rats, and similar report of studies involving further xenogeneic introduction of decellularised scaffolds of goat into mouse[212], rat into rabbit[213] and mouse into rat[214]. These results are matched by successful use of decellularised scaffolds in the clinical setting without adverse effect, such as that used by Lawson *et al*[199] who constructed bioengineered vascular grafts for dialysis in patients with chronic renal failure, and other clinical reports describing favourable results with the use of decellularised scaffolds in oesophageal tissue[215], tendon [216], major cardiac vessel[217], and chronic wound management[218]. However, there have also been reports, albeit in a small minority, of scaffold related inflammatory reactions[219,220], thus raising questions relating to the immunogenicity of decellularised ECM.

Depending on the nature of an implanted material into a host, the host response may broadly be characterised as either (1) Pro-inflammatory, eventually leading to the deposition of non-functional dense scar tissue, or, in contrast; and (2) ‘constructive remodelling’, leading to the controlled incorporation/degradation of the implanted material and its replacement with functional tissue consistent with the site of implantation[221,222].

The factors that determine which of these responses prevails are incompletely understood, but involve the interaction of the implanted material with innate[223] and adaptive immune system cells [224] such as the natural killer cells, macrophages, and lymphocytes, which can be directed to assume very different phenotypes, resulting in either a reconstructive or inflamatory reaction. The constructive remodelling response is characterised by the directing of macrophages towards the M2 (reconstructive) rather than M1 (inflammatory) phenotype, and the presence of T helper cells of Th2 phenotype, with cellullar upregulation and downregulation of anti-inflammatory and proinflammatory genes respectively[225].

In relation to the reaction ellicited by the implantation of decellularised ECM, investigation suggests that decellularised ECM per se does not ellicit an inflamatory reaction, but does stimulate a strong pro-healing phenotype of the innate and adaptive immune systems[66,225,226]. Adverse reactions do result, however, as a consequence of retained cellular products from incomplete decellularisation[67,227], post decellularisation processing of scaffolds such as cross linking[100,228], or remnants of decellularising cells[229], or sterilising agents methods in the implanted scaffold[222].

The mechanism whereby decellularised ECM ellicits a reconstructive response is incompletely understood but likely relates to molecular homology, the effect of bioactive molecules within the ECM, and the influence these biomolecules have on host immune and regenerative cells.

Thus, the constituent biochemicals of ECM, including laminin, collagens, fibronectin, and glycosaminoglycans are amongst the most highly conserved molecules in mamalian species[230]. As a result of this high degree of conservation, allogeneic and even xenogeneic ECM implants ellicit similar ‘self’ recognition and constructive cell responses[225,231]. The infiltration of implanted decellularised scaffold by host cells results in the exposure and release of bioactive molecules inluding cryptic peptides, which modulate the immune response and direct innate and adaptive immune cells towards a reconstructive phenotype[232]. These, and other bioactive molecules within the ECM also act as

chemotactic agents for stem and progenitor cells *in vitro* and *in vivo*[233]. Indeed, cryptic peptides from collagen III can reproduce progenitor cell chemotaxis[40,234].

Conclusion

In conclusion, although there are some reports of adverse reactions to implantation of decellularised ECM, these examples are due to retained cellular products or decellularising agents, rather than the ECM itself, which ellicits a favourable remodelling response, even if xenogeneic. This allows some optimism for the prospect of recellularising appropriate animal ECM scaffolds for clinical use in humans.

CONCLUSION

In the 12 years since the first report of liver decellularisation[86] and repopulation to the present, there has been much progress in the field, which has moved from predominantly *in vitro* small animal models to *in vivo* large animal models sustaining bioengineered liver perfusion for up to 20 d *in vivo*[191]. Despite this, many challenges and areas of investigation remain.

Firstly, even in the restricted domain of a single organ such as the liver, decellularisation protocols remain varied, and more often arrived at in empirical rather than comparative ways. Assessment of the quality of the decellularised scaffold is described according to numerous criteria with only some having been validated in terms of recellularisation efficacy. Standardisation of technique and quality assessment will need to progress significantly not only to facilitate experimental investigation, but also in future to meet clinical application standards. In the anticipation of sourcing human scaffolds from decellularised deceased donor livers, such considerations would apply particularly given the likely variability of scaffold quality, in contrast to the relative reproducibility of scaffolds originating from experimental animals. Should xenogeneic scaffolds ever be considered and repopulated with human cells, zoonotic as well as immunological concerns would have to be addressed.

In the area of recellularisation, the first hurdle remains the establishment of a viable vasculature, as no parenchymal function or survival is possible without it. In the liver, this is a particularly difficult problem because of the dual blood supply, and the uniquely specific functions of the sinusoidal endothelial cells. Thus, hepatic arterial recellularisation, and the fashioning of a neo-hepatic artery capable of withstanding arterial pressure has not been attained, but will be essential, as survival of the biliary tree will not be achieved without it.

Although recellularisation of portal sinusoidal and hepatic venous compartments has much progressed, with the achievement of *in vivo* perfusion albeit with portal hypertension[175], these results have been achieved with cells (often HUVECS) which, whilst providing excellent experimental tools, raise barriers to progress to the ultimate aim of recellularising scaffolds with cells from the intended recipient, and generating a syngeneic organ obviating the need for immunosuppression.

Immune considerations aside, the diversity of cell function in the vasculature of the liver is another area requiring investigation. Whilst HUVECs seem to assume some characteristics of liver sinusoidal cells when introduced into decellularised scaffolds, it remains to be shown that they can carry out the numerous, unique, and vital functions of LSECs. If they do not, a more refined recellularisation population will be required.

Assuming that a viable and fully functional vasculature is achieved, recellularisation of the main parenchymal elements, the hepatocytes and cholangiocytes, is also far from attained. In terms of the former, repopulation of decellularised scaffolds with primary hepatocytes has proved reproducible, but has only provided the beginnings of significant function, with temporary stabilisation of serum ammonia in the most successful *in vivo* models[175]. Amongst many others, endocrine, synthetic, detoxifying, and bile metabolic functions have not yet been demonstrated. Repopulation of the biliary tree is also unattained, till now largely due to the difficulty of propagating cholangiocytes in sufficient numbers, though this challenge may be alleviated by the advent of organoid culture. Other cell types, such as Kupffer cells and stellate cells, present as minorities in terms of numbers but significant in their influential interaction with hepatocytes and cholangiocytes, have not been investigated at all in recellularisation.

Although currently very distant, matters relating to clinical applications will also need much consideration. Thus, the entire process, decellularisation agents and methods, the resultant scaffold, and repopulating cells would need to meet stringent clinical grade standards. Concerns regarding scaffold immune response on the part of the host, though thus far not an observation in the context of experimental models, would have to be addressed more rigorously, as would zoonosis in the scenario of xenogeneic scaffolds.

Finally, it seems difficult to envisage that a clinical grade neo-organ could be generated entirely *in vitro*. More likely a partially recellularised scaffold may be produced, and require completion of repopulation *in vivo*, implying, at least temporarily, an auxiliary role for such neo-organs, rather than the prospect of transplantation in the manner that is practised with retrieved donated organs.

In summary, bioengineering of organs by decellularisation and repopulation remains a fascinating area still in an early phase of investigation, where the last decade has produced major advances but also left vast opportunity for research and development.

FOOTNOTES

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REVIEW

Antioxidant and anti-inflammatory agents in chronic liver diseases: Molecular mechanisms and therapy

Chun-Ye Zhang, Shuai Liu, Ming Yang

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Abstract

Chronic liver disease (CLD) is a continuous process that causes a reduction of liver function lasting more than six months. CLD includes alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), chronic viral infection, and autoimmune hepatitis, which can lead to liver fibrosis, cirrhosis, and cancer. Liver inflammation and oxidative stress are commonly associated with the development and progression of CLD. Molecular signaling pathways such as AMP-activated protein kinase (AMPK), C-Jun N-terminal kinase, and peroxisome proliferator-activated receptors (PPARs) are implicated in the pathogenesis of CLD. Therefore, antioxidant and anti-inflammatory agents from natural products are new potent therapies for ALD, NAFLD, and hepatocellular carcinoma (HCC). In this review, we summarize some powerful products that can be potential applied in all the stages of CLD, from ALD/NAFLD to HCC. The selected agents such as β-sitosterol, curcumin, genistein, and silymarin can regulate the activation of several important molecules, including AMPK, Farnesoid X receptor, nuclear factor erythroid 2-related factor-2, PPARs, phosphatidylinositol-3-kinase, and lysyl oxidase-like proteins. In addition, clinical trials are undergoing to evaluate their efficacy and safety.

Key Words: Chronic liver disease; Alcoholic liver disease; Non-alcoholic fatty liver disease; Hepatocellular carcinoma; Natural products; Inflammation; Oxidative stress; Treatment; Clinical trials

Core Tip: Chronic liver disease (CLD) is a continuous process that causes a reduction of liver function lasting more than six months. CLD can be subclassified into alcoholic liver disease, non-alcoholic fatty liver disease, chronic viral infection, and autoimmune hepatitis, which can lead to liver fibrosis, cirrhosis, and cancer. Liver inflammation and oxidative stress are commonly associated with the development and progression of CLD. Therefore, anti-inflammatory and antioxidant agents are promising drugs for CLD treatment. Clinical trials are undergoing to evaluate their efficacy and safety.

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INTRODUCTION

Chronic liver disease (CLD) is a continuous process of inflammation, destruction, and regeneration of liver parenchyma, with a reduction of liver function that lasts more than six months[1]. According to the spectrum of etiologies of CLD, it can be subclassified into alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), chronic viral infection, and autoimmune hepatitis, which can lead to liver fibrosis, cirrhosis, and cancer[2-4].

The spectrum of ALD includes alcoholic fatty liver, alcoholic hepatitis, fibrosis, and cirrhosis[5]. Alcohol drinking history and volume are direct causing factors for ALD, which can progress into hepatocellular carcinoma (HCC, Figure 1), the most common type of primary liver cancer[3]. In addition, factors such as age, gender, genetic variants, chronic virus infection, and smoking contribute to the development and progression of ALD[6,7]. Development of transgenic mouse models of ALD has provided a powerful tool to understand the disease pathogenesis[8]. Cellular and molecular mechanism studies have advanced our knowledge of the pathogenesis of ALD[8,9]. Multiple processes including excessive accumulation of lipids, reactive oxygen species (ROS) production, mitochondrial dysfunction, and cell inflammation and death are involved in ALD pathogenesis[10]. Despite all these efforts, there are no Food and Drug Administration-approved therapies for ALD[11].

NAFLD is the most common CLD with a broad spectrum, ranging from non-alcohol fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) with the progression of liver inflammation and different degrees of fibrosis[12]. NASH also can progression to HCC (Figure 1)[13]. The global prevalence of NAFLD was estimated to be 29.8% [95% confidence interval (CI): 28.6%-31.1%] in 2019[14], and the prevalence is estimated to be 32.4% (95%CI: 29.9-34.9) in 2022[15]. It affects more than 30% of people in the United States[16]. NAFLD is closely associated with other metabolic disorders, including obesity, diabetes, chronic kidney disease, and cardiovascular disease[17,18]. A new nomenclature for NAFLD has been suggested by a group of experts, namely metabolic dysfunction-associated fatty liver disease (MAFLD), which is based on the evidence of hepatic steatosis plus one of the following three criteria, including the presence of overweight or obesity, or presence of type 2 diabetes mellitus (T2DM), or evidence of metabolic dysregulation[19,20]. However, there are no currently approved medicines for NAFLD or MAFLD treatment[12].

Oxidative stress and inflammation are commonly associated with CLD independent of disease types [21,22]. For example, ethanol consumption can induce alcohol liver steatosis, inflammation, and production of ROS, resulting in the development of ALD with liver inflammation and oxidative stress [23]. In addition to hepatocyte injury, both innate and adaptive immune cells including macrophages, dendritic cells, neutrophils, and lymphocytes are involved in the development of CLD[24,25]. Production of ROS and inflammatory cytokines produced by immune cells under the stimuli of alcohol and diet metabolites, such as cholesterol and acetaldehyde, can further trigger liver oxidative stress, inflammation, and cell apoptosis or death to cause the progression of CLD[26,27].

Treatments, such as lifestyle intervention[28,29], gene editing[30,31], and pharmaceutical therapies [32], can ameliorate or cure CLD at the early stages. However, server condition of CLD requires liver transplantation, which lacks donor availability. Here, the roles of antioxidants and anti-inflammatory agents in CLD treatment, especially for ALD, NAFLD, and HCC, are reviewed. Examples of clinical trials for evaluating the potential efficacies of potential treatment agents are summarized.

DATABASE SEARCHING

The databases of PubMed, Cochrane Library (Wiley), Embase, Web of Science, and Google Scholar from the last five years (from July 2020) were searched for studies by keywords of CLD, ALD, NAFLD, or HCC, and their treatments with anti-oxidative and anti-inflammatory agents. Papers written in English

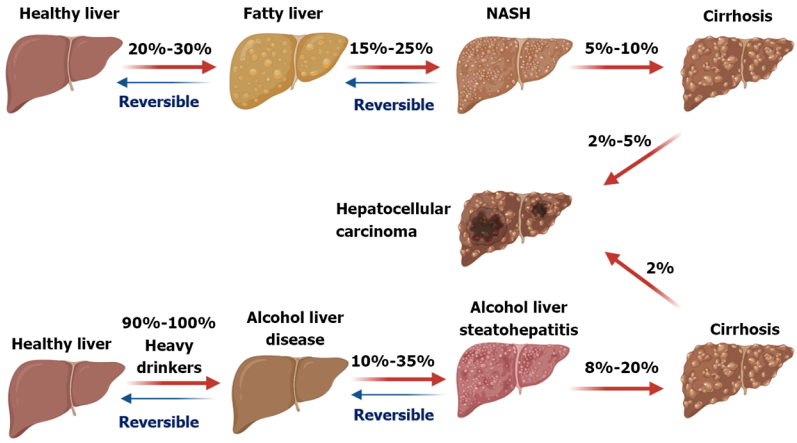

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Figure 1 The development of hepatocellular carcinoma from non-alcoholic fatty liver disease and alcoholic fatty liver disease. The prevalence (20%-30%) of non-alcoholic fatty liver (NAFL) in the world population and the following percentages of NAFL into non-alcoholic steatohepatitis (NASH) (15%-25%), NASH into cirrhosis (5%-10%), and cirrhosis into hepatocellular carcinoma (HCC) (2%-5%) are labeled. Around 90%-100% of heavy drinkers can develop alcoholic liver disease (ALD), then the percentages of progression from simple ALD into alcohol liver steatohepatitis (10%-35%), cirrhosis (8%-20%), and HCC (2%) are shown in the graphic. This cartoon was created using Biorender online tools (https://biorender.com). NASH: Non-alcoholic steatohepatitis.

were studied. When reviewing oxidative stress and/or inflammation-related molecules in CLD, the time restriction of the published data was removed.

INFLAMMATION AND OXIDATIVE STRESS IN CLD AND UNDERLYING MOLECULAR MECHANISMS

Inflammation and oxidative stress are commonly associated with each other in the pathogenesis of CLD [33], including ALD, NAFLD, and HCC. Several common signaling pathways are involved in liver inflammation and oxidative stress, such as Toll-like receptor (TLR)/nuclear factor kappa B (NF-κB) and heme oxygenase-1 (HO-1) signaling pathways[34,35]. Dysregulation of lipid metabolism contributes to the pathogenesis of CLD[36,37], which is commonly associated with liver oxidative stress and inflammation. Molecules such as peroxisome proliferator-activated receptors (PPARs) are involved in alcohol or non-alcohol factors-induced lipid metabolism dysregulation and hepatic steatosis[38,39]. In this section, we review some important signaling pathways involved in liver inflammation and oxidative stress during CLD.

AMP-activated protein kinase

AMP-activated protein kinase (AMPK) as a crucial energy sensor plays an important role in energy metabolism in multiple tissues, including the liver[40]. Activation of AMPK by metformin can reduce induced triglyceride accumulation in the livers of mice treated with ethanol compared to control groups [41]. Activation of sirtuin 1 (SIRT1)/Liver kinase B1/AMPK signaling with botulin (a triterpene) treatment reduces serum aminotransferase and triglyceride levels in mice with chronic-binge ethanol [42]. Activation of the AMPK signaling pathway with plant sterol ester of α-linolenic acid can also attenuate endoplasmic reticulum (ER) stress-induced hepatocyte apoptosis in mice with NAFLD[43]. Similarly, stimulating the activation of AMPK by an activator PXL770 reduces *de novo* lipogenesis in primary mice and human hepatocytes, which can result in the suppression of hepatic steatosis, inflammation, and fibrogenesis in mice with NASH. In addition, PXL770 has a direct inhibitory effect on the production of proinflammatory cytokines and activation of hepatic stellate cells[44].

C-Jun N-terminal kinase

Activation of C-Jun N-terminal kinase (JNK) signaling pathway is involved in lipotoxicity, inflammation, ER stress, and mitochondrial dysfunction. Palmitic acid (PA)-induced activation of JNK/Sab (SH3 domain-binding protein 5) signaling contributes to NASH progression, which is associated with mitochondrial dysfunction, oxidative stress, hepatic steatosis, and inflammation[45].

Deficiency of hypoxia-induced gene domain protein-1α (Higd-1α), a mitochondrial inner membrane protein, promotes free fatty acids (FFAs)-induced apoptosis and oxidative stress in hepatocytes[46]. In this process, the production of cytosolic oxidized mitochondrial DNA (ox-mtDNA) is increased, which induces activation of NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasomes and JNK signaling but decreases fatty acid oxidation (FAO). In contrast, exercise can increase the expression

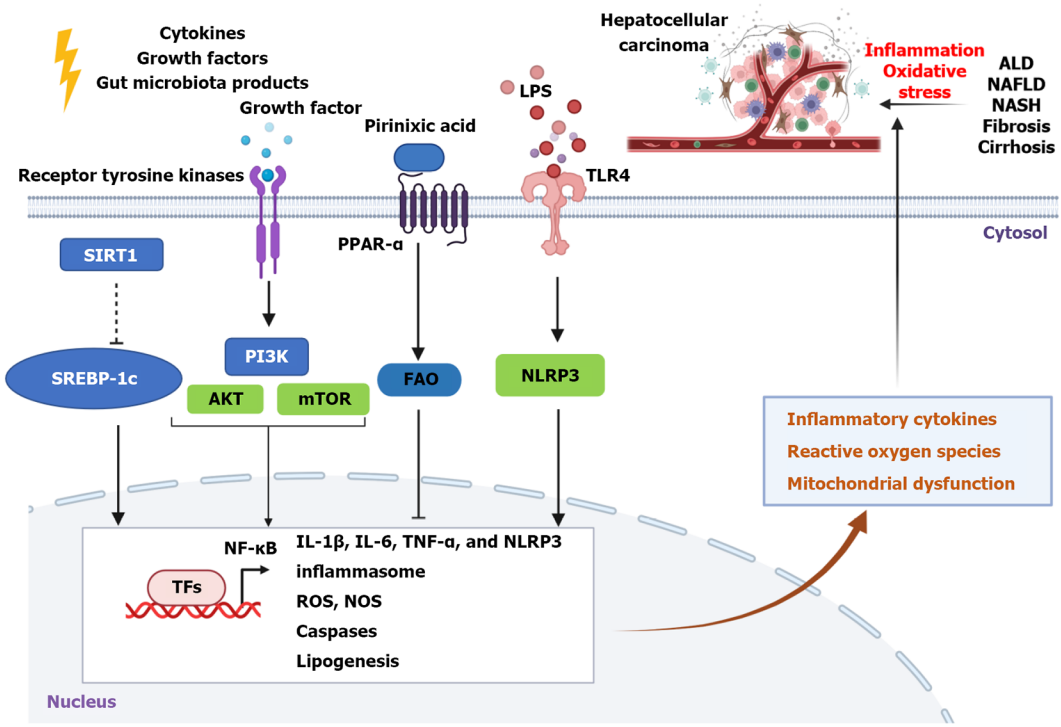

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Figure 2 Molecular signaling pathway in liver inflammation and oxidative stress. Inflammation and oxidative stress are involved in the development of chronic liver diseases such as alcoholic liver disease, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, fibrosis, and cirrhosis into hepatocellular carcinoma. Many factors including cytokines, growth factors, and gut microbiota-derived products such as lipopolysaccharide can activate their receptors such as peroxisome proliferator-activated receptor-α and toll-like receptor 4, resulting in upregulation or inhibition of downstream genes to induce or prevent inflammatory cytokines and production of reactive oxygen species. This cartoon was created using Biorender online tools (https://biorender.com). LPS: Lipopolysaccharide; TLR4: Toll-like receptor 4; ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; PPAR-α: Peroxisome proliferator-activated receptor-α; SIRT1: Sirtuin 1; SREBP-1c: Sterol regulatory element binding protein 1c; PI3K: Phosphatidylinositol-3-kinase; AKT: Protein kinase B; mTOR: Mammalian target of rapamycin; FAO: Fatty acid oxidation; NLRP3: NOD-like receptor family pyrin domain containing 3; NF-κB: Nuclear factor kappa B; IL: Interleukin; TNF-α: Tumor necrosis factor-α; NLRP3: NOD-like receptor family pyrin domain containing 3; ROS: Reactive oxygen species; NOS: Nitric oxide synthase.

of Higd-1α in the liver to ameliorate hepatic steatosis and inflammation by suppressing ox-mtDNA/NLRP3/JNK pathway[46].

Farnesoid X receptor

Farnesoid X receptor (FXR) is a nuclear receptor that metabolically regulates glucose, bile acid, and lipid metabolism[47,48]. Treatment of Lactobacillus reuteri can ameliorate lipid accumulation in mice with ALD by upregulating FXR expression, which is associated with the upregulation of carbohydrate response element binding protein and downregulation of sterol regulatory element binding transcription factor 1 and cluster of differentiation (CD36)[49]. In addition, the FXR/fibroblast growth factors (FGFs) axis (FGF-15 and FGF-19) also plays a key in the regulation of hepatic inflammation, lipid metabolism, and fibrosis[50,51]. Clinically, treatment of FXR agonist vonafexor also shows anti-fibrotic effects in patients with NASH[52].

Nuclear factor erythroid 2-related factor-2/HO-1

Nuclear factor erythroid 2-related factor-2 (Nrf2) is a key transcription factor that plays a critical role in oxidative stress and inflammatory responses. For example, Nrf2 expression is positively associated with oyster peptide-mediated suppression of inflammation mediated by upregulation of NF-κB signaling and upregulation of antioxidant response in mice with ALD[53]. Activation of Nrf2 is involved in the protective effect of diallyl disulfide against chemical (CCl_4)-induced liver injury and oxidative stress [54]. HO-1, an inducible form of antioxidant zyme HO isoforms that regulates heme group degradation, plays an essential role in liver inflammation and oxidative stress[55]. Nrf2 can regulate HO-1 to suppress liver oxidative stress, ER stress, and inflammation[56].

Nrf2 also plays an important role in the pathogenesis of NASH. Activation of Nrf2 can ameliorate liver inflammation, ER stress, iron overload, and lipotoxicity to suppress NASH and oxidative stress, which can be suppressed by transforming growth factor-beta (TGF-β)[57]. Activation of Nrf2 can suppress the expression of ROS and NLRP3 and inhibit Caspase 1/interleukin (IL)-1β and IL-18-mediated inflammation[58]. In addition, pharmacologic activation of Nrf2 by TBE-31, acetylenic tricyclic bis(cyano enone), decreases insulin resistance and liver fat accumulation, inflammation, fibrosis, and

oxidative stress in mice with a high-fat plus fructose diet. However, the TBR-31-mediated effect was abolished in Nrf2-null mice[59].

PPARs

PPARs are a group of nuclear receptor proteins that function as ligand-activated receptors to regulate genes in energy metabolism and inflammation. PPARs comprise three subtypes, PPAR-α, PPAR-β/δ, and PPAR-γ, which are pharmaceutical targets for disease treatments[60,61]. These PPARs play important roles in ALD[62], NAFLD[63], hepatitis virus-mediated liver injury[64], and HCC[65].

Activation of PPAR-α by agonist WY-14643 (Pirinixic Acid, Figure 2) ameliorates ethanol-induced liver fat accumulation by increasing FAO[66]. Sustained activation of PPAR-α can decrease obesity and improve insulin resistance to rebuild glucose homeostasis. However, it increases the risk of HCC development due to liver ER stress[67]. Treatment with GW9662, an antagonist of PPAR-γ, significantly decreased lipopolysaccharide (LPS)/TLR4-mediated expression of IL-1β, IL-6, inducible nitric oxide synthase, and nitrite (NO_2^-) concentration[68].

Treatment with a dual PPAR-α/γ agonist Saroglitazar is able to reduce serum transaminases and 63% of overweight patients with NALFD reduced bodyweight (> 5%)[69]. In addition, many clinical trials have been performed to evaluate the effects of PPARs in ALD. For example, pemafibrate can improve liver function and glucose metabolism in patients with hypertriglyceridemia[70] and decrease liver stiffness in patients with NAFLD measured by magnetic resonance elastography (ClinicalTrials.gov, number: NCT03350165)[71]. Treatments that target PPAR-α such as pemafibrate[71], PPAR-β/δ such as seladelpar[72], and PPAR-γ such as pioglitazone[73,74] show promising efficacy in the clinic for CLD treatment (Figure 3). Meanwhile, a dual PPAR-α/δ agonist elafibranor and a pan-PPAR regulator lanifibranor show promising efficacy for CLD treatment in the clinic[75,76]. For example, a phase 2b clinical trial reveals that treatment of lanifibranor (1200 mg) compared with the placebo can decrease at least 2 points of steatosis, activity, and fibrosis score that incorporates scores for ballooning and inflammation[76].

Phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin

The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB or AKT)/mammalian target of rapamycin (mTOR) signaling pathway is implicated in the pathogenesis of liver disease and therapy[77,78]. For example, this signaling pathway is involved in the anti-steatosis effect of D-mannose in ALD [79]. Activation of PI3K/AKT/mTOR signaling pathway by arecoline (2.5 μM), an alkaloid ester found in the betel nut palm seeds, promotes the proliferation and migration of HepG2 cells[80]. Acid-sensitive ion channel 1α can upregulate the activation of PI3K/AKT/mTOR signaling pathway to enhance the expression of matrix metalloproteinase (MMP)2 and MMP9 to promote liver cancer cell (HepG2 and SK-Hep1 cells) migration and invasion[81]. One human study also indicates that PI3K is more strongly expressed in tumors than that in cirrhotic livers but not AKT and mTOR, and the expression of PI3K in tumor tissues is independent of etiology[82]. In addition, activation of growth factor receptor protein tyrosine kinases (Figure 2) can result in autophosphorylation on tyrosine residues and subsequent binding and activation of PI3K[83], playing an important role in cancer development. Inhibition or blockade of this signaling pathway can suppress liver fibrosis[84,85] and cancer progression[86,87].

Furthermore, lysyl oxidase family members (LOX) and LOX-like proteins (LOXL1-4) play important roles in liver fibrosis and cancer[88]. Insulin resistance can promote extracellular matrix stabilization by upregulating hepatic production of LOXL2 through upregulation of the expression of Forkhead box protein O1 in NAFLD[89]. In addition, galectins such as galectin-3 also play an essential role in CLD[90-92], including liver fibrosis and cancer. Overall, these molecular signaling pathways are involved in liver inflammation and oxidative stress to promote the development of CLD to HCC (Figure 2).

ANTIOXIDANT AND ANTI-INFLAMMATORY AGENTS IN ALD

Many ingredients from natural products or plants have both antioxidant and anti-inflammatory functions, which are good candidates for CLD treatment. Some of these products may have preventive effects on hepatic steatosis in ALD and NAFLD. For example, diallyl trisulfide (DATS) is a bioactive compound isolated from garlic and can reduce serum levels of aspartate transaminase (AST) and alanine aminotransferase (ALT) and decrease alcohol-induced liver injury[93]. DATS can upregulate PPAR-α expression and down-regulate sterol regulatory element binding protein 1c (SREBP-1c) expression to inhibit hepatic steatosis. Meanwhile, it can reduce liver oxidative stress by increasing antioxidant products and reducing ROS and malondialdehyde (MDA) production in the fatty liver[93]. In this section, we review some promising agents in ALD treatments either in animal models or clinical trials.

β-sitosterol

β-sitosterol is isolated from the roots of *Panax ginseng*[94]. As a plant sterol, β-sitosterol can reduce alcohol-induced liver injury and oxidative stress *via* restoration of erythrocyte membrane fluidity,

upregulation of glutathione (GSH) activity, and reduction of MDA production. In addition, β-sitosterol can suppress apoptosis-related gene expression by increasing the phosphorylation of PI3K and AKT[95].

Curcumin

Curcumin is an orange-yellow component of turmeric or curry powder isolated from the rhizome of *Curcuma longa*[96,97]. Supplementation of curcumin can significantly increase the activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) to reduce swimming-induced oxidative stress in mice, by activating Nrf2 signaling pathway[98]. Treatment of curcumin significantly decreases serum levels of ALT, AST, alkaline phosphatase (ALP), gamma-glutamyl transferase, Arginase I, and blood urea nitrogen, while it increases serum levels of Albumin and total protein in ethanol-treated rats compared to the control group[99]. Development of self-assembled micelles of curcumin can be administered by oral delivery to enhance its anti-oxidative stress ability to prevent ALD and gastric mucosa damage[100]. Encapsulation enables to improve the adsorption of curcumin in intestinal epithelial cells and enhance its hepatoprotective effects in rats, *via* increasing the activity of GPx and decreasing high levels of MDA in the liver[101]. Furthermore, a combined treatment of curcumin and bacicalin shows more protective effects on ALD in rats by reducing liver oxidative damage through activation of the Nrf2/HO-1 signaling pathway[102].

Empagliflozin

Empagliflozin (EMPA) has benefits in cardiovascular, renal, and cerebral diseases, which is potentially mediated through its antioxidant and anti-inflammatory activities. Treatment with EMPA can decrease serum levels of ALT, AST, and ALP. It also increases the activities of GSH and SOD in the liver homogenates and decreases the liver content of MDA and nitric oxide (NO)[103]. Moreover, EMPA can downregulate NF-κB signaling to suppress the expression of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-6, which is associated with the upregulation of PPAR-γ, Nrf2, and their target gene HO-1[103].

Gastrodin

Gastrodin is the main bioactive component of *Gastrodia elata Blume* and displays anti-inflammatory and antioxidant properties. For example, administration of gastrodin (50 or 100 mg/kg) in mice significantly inhibits concanavalin A (ConA)-induced acute hepatitis, partly by suppressing IL-6/Janus Kinase 2/signal transducer and activator of transcription 3 signaling pathway[104]. In addition, treatment with gastrodin ameliorated acetaminophen-induced liver injury in mice. The anti-inflammatory and anti-oxidative stress functions of gastrodin are mediated through the inhibition of signal-regulated kinase/JNK/mitogen-activated protein kinase signaling pathways and hepatic MDA activity, as well as activation of Nrf2 expression and SOD activity[105].

Genistein

Genistein is an isoflavone first isolated from the brooming plant Dyer's *Genista tinctoria*, which is widely distributed in the Fabaceae family[106-109]. Treatment of genistein at a dose of 0.3 mmol/kg of bodyweight can ameliorate liver fibrosis and apoptosis in mice by suppressing the expression of proinflammatory cytokines such as TNF-α, IL-6, profibrotic cytokines such as TGF-β1, and cell caspase 3 [110]. In contrast, another study shows that supplementation of soy proteins significantly decreases serum ALT concentrations and hepatic TNF-α and CD-14 expression and decreases NF-κB protein in casein-based 35% high-fat ethanol liquid diet (EtOH)-treated mice by inhibiting β-catenin signaling [111]. More functional studies of genistein have been performed in NAFLD models, which are discussed in the following section.

Lactoferrin

Lactoferrin (LF) is an iron-binding protein found at relatively high concentrations in mammalian milk [112]. LF displays multiple functions, including antioxidant, anti-cancer, and anti-inflammatory activities. For example, LF treatment can decrease the levels of liver superoxide and suppress liver inflammation in male mice with alcoholic-induced liver injury (ALI) by upregulating the expression of aldehyde dehydrogenase-2 and suppressing overexpression of cytochrome P450 2E1 (CYP2E1)[113]. LF treatment also displays a protective effect in female mice with acute ALI by regulating redox-stress response capacity[114]. The protective effect of LF on ALI is associated with the manipulation of gut microbiota and the modulation of hepatic alcohol metabolism[113].

Selenium

Selenium plays an essential role against oxidation, which is part of the catalytic center of different antioxidant selenoproteins including GPxs and selenoprotein P[115]. The serum levels of selenium are decreased in adult patients with acute and chronic alcoholic-related diseases, accompanied by liver damage and the severity of oxidation[115,116].

Silymarin

Silymarin is an active compound from the extracts of milk thistle (*Silybum marianum*)[117]. Silymarin displays antioxidant, antifibrotic, anti-inflammatory, and hepatoprotective properties in different types of CLD[118,119], such as ALD. Simultaneous supplementation of silymarin with alcohol treatment can reduce the ethanol-induced increase of serum ALT levels and hepatic microvesicular steatosis and TNF-α expression[120]. Another study on non-human primates also shows that silymarin can prevent the development of alcohol-induced liver fibrosis by decreasing the production of type I collagens[121].

Taraxasterol

Taraxasterol (TAS) is an active ingredient of *Taraxacum officinale*, which has protective effects on the liver and kidneys by reducing serum levels of ALT and AST, increasing serum and liver SOD and GPx, and maintaining the balance of ion homeostasis[122]. TAS also displays anti-inflammatory function in cultured mouse primary lymphocytes stimulated with Con A and in mice with Con A-induced acute hepatitis[123]. Mechanism studies reveal that TAS inhibits T cell activation and proliferation by suppressing IL-2/IL-2 receptor-mediated downstream signaling pathways[123].

Telmisartan

Telmisartan (TEL) exhibits similar effects with EMPA on ALD. Treatment of TEL (10 mg/kg/day) decreased serum levels of ALT, AST, and ALP in mice with ALD[124]. In addition, TEL displays anti-inflammatory and antioxidant properties in mice with ALD by increasing the activity of SOD and GPx to reduce liver contents of NO and MDA, upregulating the expression of Nrf-2, PPAR-γ, and Hmox-1, and downregulating NF-κB expression[124].

ANTIOXIDANT AND ANTI-INFLAMMATORY AGENTS IN NAFLD

Hepatic inflammation and oxidative stress are also associated with NAFLD pathogenesis[125]. Therefore, many above-discussed products also display similar bioactive functions against NAFLD.

β-sitosterol

Treatment with β-sitosterol can prevent high-fructose diet-induced macrovesicular hepatic steatosis and inhibit the progression of NAFL to NASH in male rats[126]. Meanwhile, it is also able to inhibit high-fructose diet-induced visceral obesity, hypertriglyceridemia, plasma insulin concentration, and homeostatic model assessment of insulin resistance (HOMA-IR) but increase plasma levels of adiponectin in female rats[127]. Another study shows that in combination with stigmasterol, a dietary phytosterol, β-sitosterol can alleviate a high-fat western-style diet-induced NAFLD in mice post-17-wk treatment, by decreasing hepatic di- and tri-acylglycerols and circulating ceramide levels[128].

Curcumin

Curcumin is a natural polyphenol, which shows anti-inflammatory and antioxidant activities. It can improve insulin resistance and reduce hepatic fat accumulation in dietary obese rat models[129]. Accumulating evidence identifies that curcumin can attenuate hepatic steatosis by suppressing hepatic expression of CD36, PPAR-γ, SREBP-1c, and fatty acid synthase (FAS) in NAFLD mice, through upregulation of Nrf2 and FXR expression and downregulation of liver X receptor α expression[130,131]. In addition, curcumin can induce activation of AMPK and upregulation of PPAR-α, and suppress the high-fat diet (HFD)-induced increase in the expression of SREBP-1, acetyl-CoA carboxylase 1, FAS, and CD36 [132]. Meanwhile, curcumin is able to prevent intestinal permeability and suppress LPS/TLR4/NF-κB-mediated inflammatory response to protect against diet-induced hepatic steatosis and inflammation [133]. In addition, curcumin can also suppress NLRP3 inflammasome (Figure 2) and pro–IL-1β synthesis by suppressing LPS-mediated activation of NF-κB signaling pathway[134].

Ex vivo studies also show that treatment of curcumin decreases linoleic acid-induced ROS production and leptin-induced TNF-α expression in human peripheral blood mononuclear cells[135]. A randomized controlled trial in Iran demonstrates that supplementation with curcumin in a phytosomal form (1000 mg/day) significantly reduces body mass index (BMI), waist circumference, and serum levels of AST and ALT[136]. This dose was safe and well tolerated in NAFLD patients[136]. Another double-blind, randomized, placebo-controlled trial displays that daily supplementation of low-dose phospholipid curcumin (250 mg) for 2 mo can significantly decrease hepatic steatosis and serum AST levels in NAFLD patients compared to placebo[137]. In addition, a combined therapy of curcumin (500 mg/day) with piperine, an alkaloid in black pepper with many pharmacological effects on chronic diseases[138], also decreases the severity of NAFLD and serum ALP levels[139]. Large clinical trials are needed for further evaluation of the efficacy of curcumin and its synergistic treatments.

EMPA

EMPA is an inhibitor of sodium-glucose co-transporter 2 (SGLT2), which plays an important role in

NAFLD. EMPA treatment can inhibit PA-induced lipid deposition in hepatocytes (HepG2 cells) and HFD-induced hepatic lipid accumulation and inflammation in mice by upregulating the expression of a stress-inducible protein Sestrin2 and activating AMPK-mTOR signaling pathway[140]. Another study demonstrates that EMPA can upregulate the expression of medium-chain acyl-CoA dehydrogenase in NASH liver and PA and glucose-treated hepatocytes by activating AMPK/forkhead box A2 signaling pathway, resulting in a reduction of hepatic lipid deposition *in vivo* and *in vitro*[141]. A meta-analysis shows that EMPA can significantly reduce BMI, HOMA-IR, AST, and liver fibrosis in patients with NAFLD[142].

In addition, other SGLT2 inhibitors or gliflozins, such as licogliflozin[143,144] and dapagliflozin[145, 146], also can control glycemic production and bodyweight, normalize serum ALT levels, and reduce Fibrosis-4 NAFLD patients with T2DM.

Gastrodin

Gastrodin has been shown to significantly decrease lipid accumulation and inflammatory response in primary mice and human hepatocytes treated with 0.5 mmol/L PA along with 1.0 mmol/L oleic acid. In addition, it ameliorates diet-induced hepatic steatosis and inflammation in mice by activating the AMPK signaling pathway[147]. Gastrodin can also regulate lipid metabolism and display antioxidant effects in larval zebrafish with high-cholesterol diet-induced NAFLD[148].

Genistein

Genistein has been shown to play an important role in NAFLD and NASH treatment. Treatment of genistein reduces the levels of TNF-α and reduces TLR4 mRNA and protein expression and inflammation in the livers of rats with NASH[149]. A combination of genistein with metformin (0.2% + 0.23%) for 3 mo shows a synergistic effect on the reduction of AST, ALT, and TG, liver TG and number of macrophages, and NAFLD activity score (NAS) in HFD-fed mice[150]. The reduction of hepatic steatosis is associated with decreased mRNA levels of lipogenic-related genes *SREBP-1c* and *FAS* and upregulated mRNA expression of FAO-related gene *carnitine palmitoyl transferase 1*[150]. Genistein treatment (16 mg/kg BW/day) for 5 wk can significantly decrease hepatic steatosis, inflammation, and hepatocyte ballooning in ovariectomized rats with high-fat and high-fructose diet-induced NASH[151].

Consumption of dietary isoflavones including genistein is reversely associated with NAFLD, hypertension, and hyperlipidemia in a study on Chinese adults[152]. Molecular mechanism studies show that genistein can suppress the activation of SREBP-1c in FFA-induced fat accumulation in primary human hepatocytes, whereas genistein-mediated upregulation of PPAR-α proteins in normal hepatocytes is abolished in steatotic hepatocytes[153].

LF

LF is an iron-binding protein in mammalian milk and displays multiple functions, including antioxidant, anti-cancer, and anti-inflammatory activities. During NASH progression, LF treatment can inhibit NF-κB activation to downregulate a high-fat diet and chemical dimethylnitrosamine-induced liver injury, inflammation, and fibrosis[154]. Treatment with LF improves insulin sensitivity and reduces hepatic steatosis in ob/ob mice by downregulating SREBP-2. It also regulates hepatocellular iron transport by controlling the hepcidin-ferroportin axis to maintain liver oxidative balance and suppress hepatocyte death[155].

Mastiha

Mastiha is a natural and aromatic resin isolated from the trunk and brunches of mastic trees with antioxidant and anti-inflammatory properties[156]. Mice with diet-induced NASH fed with 0.2% (w/w) Mastiha supplementation for 8 wk can reduce the circulating ALT levels, NAS, hepatic steatosis, and liver collagen production[157]. This study also identifies that Mastiha supplementation changes NASH-induced gut microbiota profile to the diversity and composition of healthy mice. A randomized clinical trial (NCT03135873, www.clinicaltrials.gov) shows that supplementation of Mastiha improves the total antioxidant status (TAS) levels in NAFLD patients with severe obesity compared to that in the corresponding placebo group[158]. The anti-inflammatory function of Mastiha is associated with the expression of microRNA-155 in the plasma of NAFLD patients, which may regulate the differentiation and function of T helper-17 cells[159].

Selenium

Treatment with selenium-enriched green tea extract (200 mg/kg body weight) for 15 wk can significantly reduce body weight gain and visceral fat accumulation in mice with obesity and NAFLD [160]. Reduced serum levels of selenium are independently associated with hepatic fibrosis in NAFLD patients[161]. Another study reveals that selenium deficiency induces hepatic inflammation in pigs by activating the NF-κB signaling pathway, decreasing antioxidant capacity, and increasing ROS levels [162]. Selenium-enriched *Lactobacillus acidophilus* SNZ 86 (probiotic) can decrease western-style diet-induced hepatic steatosis in mice with NAFLD, by activating autophagy through the upregulation of AMPK/SIRT1 signaling pathway[163]. Co-supplementation of selenium with vitamin B6 can reduce

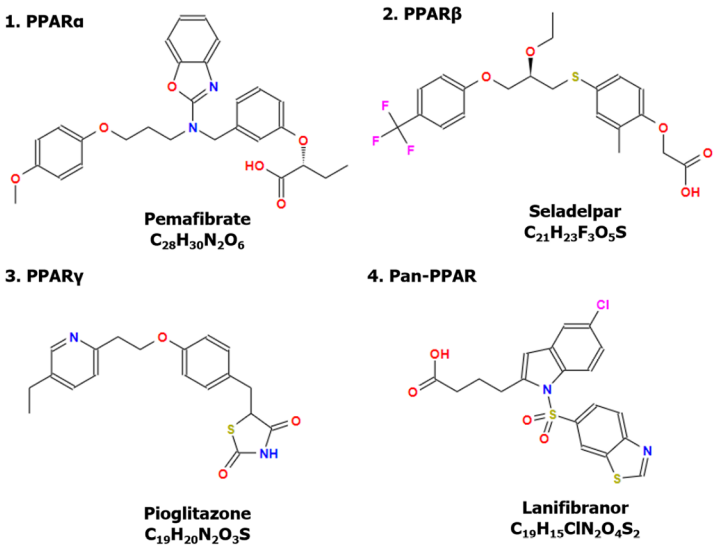

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Figure 3 Structures of peroxisome proliferator-activated receptor agonists or modulators applied for the treatment of chronic liver disease. Many peroxisome proliferator-activated receptor regulators have been evaluated in the clinic, showing promising effects in patients with chronic liver disease. All the chemical structures were collected online from the Chemical Book (https://www.chemicalbook.com, accessed on August 10, 2022). PPAR: Peroxisome proliferator-activated receptor.

liver lipid synthesis and deposition by increasing the expression of SIRT1 to downregulate SREBP-1c expression (Figure 2) and upregulate PPAR-α expression in HFD-fed rats[164].

Silymarin

The major active compound of silymarin is silybin. Treatment with silybin can significantly decrease lipid accumulation in mice with NAFLD by activating PPAR-α[165]. Since it can partially inhibit the effect of PPAR-α agonist fenofibrate, it is not suggested to be simultaneously applied with PPAR-α agonists. Silymarin also displays a synergistic effect with quercetin on the reduction of lipid accumulation in rat hepatocytes[166]. Silymarin treatment significantly ameliorates high fructose-induced oxidative stress and hepatic steatosis in rats[167]. Silymarin supplementation (560 mg daily) for 8 wk significantly improves serum AST/ALT ratio, ultrasound fatty liver grading, and BMI in patients with morbid obesity and NAFLD[168].

TEL

Treatment with TEL significantly improves fibrosis scores and reduces the levels of serum leptin and its expression in liver tissue[169]. As an angiotensin receptor blocker, it significantly decreases fasting serum-FFA levels and triglyceride-glucose index in patients with NAFLD[170]. TEL displays a similar effect as vitamin E on the reduction of NAS, and improvement of hepatic steatosis, but it has a better effect on the reduction of liver lobular inflammation and hepatocyte ballooning[171]. It can function as a PPAR-γ/α dual agonist to simultaneously improve insulin-sensitivity *via* activating PPAR-γ and improve lipid metabolism by activating PPAR-α[172].

Delta-tocotrienol

Tocotrienols are natural compounds that belong to one part of two vitamin E components (Tocopherols as another part), including α, β, γ, and δ tocotrienols[173]. Among them, δ-tocotrienol shows strongly anti-inflammatory activity, which can decrease insulin resistance, hepatic steatosis, and serum triglyceride concentrations in rats with diet-induced obesity[174]. Recent studies also show that δ-tocotrienol has anti-cancer properties by regulating angiogenesis and cell proliferation and apoptosis[175].

A human study indicates that oral supplementation of δ-tocotrienol (300 mg, twice daily) for 12 wk significantly decreases serum aminotransferases, high sensitivity C-reactive protein (hs-CRP), and MDA, and fatty liver index (FLI) score compared to placebo[176]. Clinical trials reveal that δ-tocotrienol supplementation results in a significant reduction in plasma glucose, insulin, glycosylated hemoglobin, MDA, high sensitive C-reactive protein, and proinflammatory cytokines (TNF-α and IL-6), and HOMA-IR in pre-diabetic and diabetic patients[177,178]. Another trial also demonstrates that treatment of δ-tocotrienol (300 mg, twice daily) for 24 wk further significantly reduces FLI score, HOMA-IR, and hepatic steatosis than placebo, except decreased serum levels of hs-CRP, MDA, ALT, and AST, without causing adverse events[179].

ANTIOXIDANT AND ANTI-INFLAMMATORY AGENTS IN LIVER CANCER

Both ALD and NAFLD are major contributors to HCC initiation and progression. Therefore, the above-discussed biomolecules may also exhibit anti-HCC effects. For example, treatment of β-sitosterol niosomes, a form of β-sitosterol with polyethylene glycol modification, shows cytotoxicity to HepG2 cells due to increased cellular uptake and displays *in vivo* anti-HCC ability in *Wistar albino* rats[180]. Treatment of β-sitosterol-assisted silver nanoparticles (BSS-SNPs) significantly inhibits the proliferation of HepG2 cells and their production of ROS and Nrf2, resulting in the regulation of pro-apoptotic genes such as Bcl-2 Associated X-protein and caspases 3 and 9[181]. Similarly, compounds including curcumin [182], EMPA[183], gastrodin[184], genistein[185], LF[186], selenium[187], silymarin[188], TAS[189], TEL [190], and delta-tocotrienol[191] display anti-HCC effects either *in vitro* or *in vivo*, or both (Table 1).

CLINICAL TRIALS

Clinical trials have been started to evaluate the efficacy of these molecules in CLD (Table 2), such as EMPA[192] and silymarin[193,194]. For example, treatment with EMPA can improve liver steatosis in patients with NAFLD without T2DM[192]. Another trial shows that oral supplementation of genistein (250 mg) for 8 wk can decrease insulin resistance, oxidative stress, and inflammation and improve lipid metabolism in patients with NAFLD[195].

CONCLUSION

CLD is a continuous process that causes a reduction of liver function that lasts more than six months. CLD has a broad spectrum with complex cellular and molecular mechanisms. It can be subclassified into ALD, NAFLD or MAFLD, chronic viral infection, and autoimmune hepatitis, which can lead to liver fibrosis, cirrhosis, and cancer. However, there are no currently available treatments for ALD, NAFLD, and liver fibrosis, except the preventive strategies, such as changes in exercise, diet, and alcohol use. Early preventive strategies predict good outcomes. Patients with advanced ALD and NAFLD require liver transplantation, but without enough donor organs. Liver inflammation and oxidative stress are ubiquitously associated with the development and progression of CLD. Molecular signaling pathways such as AMPK, JNK, and PPAR-mediated signaling pathways are implicated in liver inflammation, oxidative stress, and lipid metabolism. Accumulating studies have demonstrated that natural products with antioxidant and anti-inflammatory functions display therapeutic effects against inflammation, fibrosis, and metabolic disorders, including ALD and NAFLD. These products such as β-sitosterol, curcumin, EMPA, gastrodin, and genistein have shown potential application at all the stages of CLD, from ALD/NAFLD to HCC. In addition, clinical trials that are undergoing to evaluate their efficacy and safety are reviewed. Overall, pre-clinical studies in cell and animal models reveal the protective effects of these agents in CLD. However, more clinical trials are required to evaluate their efficacy and safety.

Natural products, especially antioxidant and anti-inflammatory products, show potent therapeutic alternatives for CLD treatment with their efficacy and low side effects. Remarkably, these products also display anti-HCC functions. However, many pharmaceutical dynamic assays have not been tested, and the potential adverse effects of long-term use of these products are not available. In the future, the synergistic effects of different drugs should be evaluated to treat CLD, due to its complex pathogenic factors.

Table 1 Antioxidant and anti-inflammatory agents for the treatment of hepatocellular carcinoma

Molecules	Model	Function	Ref.
β-sitosterol	HepG2 cells; Rat HCC	Treatment of β-sitosterol niosomes displays direct cytotoxicity to HepG2 cells in vitro and anti-HCC ability in rats	[182]
Curcumin	HepG2 and SK-Hep-1 cells. A nude mouse xenograft model bearing HepG2 cells	It can inhibit cell proliferation and increase cell apoptosis and cell cycle arrest at the G0/G1 phase of cancer cells by downregulating the expression of BCLAF1 and inhibiting the activation of the PI3K/AKT/GSK-3β pathway	[183]
Empagliflozin	DENA-induced HCC in mice	It shows a synergistic effect on the control of angiogenesis, invasion, and metastasis of tumor cells in mice with DENA-induced HCC by inhibiting the expression of MAPKs and reducing liver injury enzymes	[184]
Gastrodin	Subcutaneous H22 cells-induced tumor	It can specifically increase the expression of NF-κB	[185]

	in mice	downstream genes such as Bcl-xL, Bcl-2, and IL-2 in CD4 but not CD8 T cells	
Genistein	TAA-induced HCC in rats	It displays antioxidant and anti-HCC effects by suppressing the versican/PDGF bidirectional axis and protein expression of PKC and ERK-1	[186]
Lactoferrin	DEN-induced HCC in rats	It shows a chemopreventive effect against DEN-induced HCC in rats in a dose-dependent manner by suppressing the expression and activation of AKT	[187]
Selenium	TAA-induced HCC in rats	Selenium nanoparticles improve the tumor suppressive effect of sorafenib and overcome drug resistance in rat HCC by inducing apoptosis and targeting AKT/mTOR and NF-κB signaling pathways, as well as epigenetic regulation	[188]
Silymarin	DEN/AAF/CCl_4 induced HCC in rats	It suppresses cancer cell growth in rats with DEN/AAF/CCl_4-induced tumors by inhibiting the expression of Ki-67 and HGF/c-Met, Wnt/β-catenin, and PI3K/Akt/mTOR signaling pathways	[189]
Taraxasterol	HepG2 and Huh7H22 bearing mice	It can suppress tumor cell growth by suppressing Ki67 expression and inducing cell apoptosis *via* suppressing IL-6/STAT3 signaling pathway, as well as promoting T cell infiltration in tumor tissue	[190]
Telmisartan	NDEA-induced HCC in mice	It exerts an anti-HCC effect and increases tumor cell sensitivity to sorafenib treatment by suppressing phosphorylation-induced activation of TAK1 and the ERK1/2 and NF-κB signaling pathways	[191]
Delta-tocotrienol	HCC cell lines SK Hep-1 and Huh7	It promotes the anti-HCC cell activity of IFN-α by increasing ROS and increasing cell apoptosis together with an increased Bax/Bcl-xL ratio. In addition, it can activate Notch1 signaling pathway	[192]

AKT: Protein kinase B; Bax: Bcl-2-like protein 4; Bcl-2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma extra-large; BCLAF1: BCL-2-associated transcription factor 1; CD4: Cluster of differentiation 4; c-Met: Tyrosine-protein kinase Met; ERK-1/2: Extracellular signal-regulated kinases 1/2; GSK-3β: Glycogen synthase kinase-3β; HCC: Hepatocellular carcinoma; HGF: Hepatocyte growth factor; IL-2: Interleukin 2; Ki-67: Marker of proliferation Ki-67; MAPK: Mitogen-activated protein kinase; mTOR: Mammalian target of rapamycin; NF-κB: Nuclear factor κB; PI3K: Phosphatidylinositol-3-kinase; PDGF: Platelet-derived growth factor; SIRT1: Sirtuin 1; SREBP-1c: Sterol regulatory element binding protein 1c; STAT3: Signal transducer and activator of transcription 3; DENA Diethylnitrosamine; TAA: Thioacetamide; ROS: Reactive oxygen species; NDEA: N-Nitrosodiethylamine; AAF: 2-acetylaminofluorene; CCl_4: Carbon tetrachloride.

Table 2 Clinical trials for evaluating the efficacy of compounds in liver disease

Treatment	Trial number	Phase	Aims or results
Curcumin	NCT02908152	2-3	To investigate the effects of curcumin supplements on metabolic factors and hepatic fibrosis in NAFLD patients with T2DM
	NCT04109742	2	To test the effect of curcumin in pediatric patients with NAFLD
Empagliflozin	NCT03867487	2	To evaluate the preliminary feasibility, initial efficacy, and safety of empagliflozin as a SGLT2 inhibitor for treating NAFLD in adolescents with obesity
	NCT04642261	4	To test the effects of empagliflozin on reducing hepatic fat content as measured by MRI-PDFF in NAFLD patients without DM
Gastrodin	NCT04035824	4	To treat hypertension together with Uncaria
Genistein	IRCT201312132480N5	3	Oral supplementation of genistein (250 mg) for 8 wk can decrease insulin resistance, oxidative stress, and inflammation and improve lipid metabolism in patients with NAFLD
Lactoferrin	NCT04335058	None	To test the effect of lactoferrin with iron versus iron alone in the treatment of anemia in CLD
Selenium	NCT00271245	None	To test the effect of selenium in patients with cirrhosis
	NCT01650181	4	To test the impacts using siliphos-selenium-methionine-alpha lipoic acid plus metformin versus metformin in patients with fatty liver and NASH
Silymarin	NCT00389376	1	An increase in silymarin is observed in NAFLD patients, compared to that in patients with HCV
	NCT00680407	2	The effect of silymarin on NASH patients remains inconclusive due to the lack of a

			substantial number of patients
Telmisartan	NCT02213224	4	To evaluate the therapeutic effects of telmisartan and perindopril for NAFLD patients with hypertension

T2DM: Type 2 diabetes mellitus; NAFLD: Non-alcoholic fatty liver disease; SGLT2: Sodium-glucose cotransporter-2; MRI-PDFF: Magnetic resonance imaging-derived proton density fat fraction; CLD: Chronic liver disease; NASH: Non-alcoholic steatohepatitis; HCV: Hepatitis C virus.

FOOTNOTES

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MINIREVIEWS

Galectin-3 inhibition as a potential therapeutic target in non-alcoholic steatohepatitis liver fibrosis

Michael Kram

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Abstract

Nonalcoholic fatty liver disease continues to be one of the major health challenges facing the world, with estimates of non-alcoholic steatohepatitis (NASH) prevalence in over 25 percent of the world's population. NASH represents a spectrum of disease that may lead to hepatic fibrosis and eventual cirrhosis, with the risk of cirrhosis decompensation, and hepatocellular carcinoma. New therapies are desperately needed for NASH, especially for later stages of fibrosis and cirrhosis. Galectin-3 inhibition is being explored as a new liver antifibrotic therapy. This concise review will outline the state of the art of this new therapeutic target.

Key Words: Galectin-3 inhibition; Non-alcoholic fatty liver disease; Fibrosis; Macrophage

Core Tip: Galectin-3 inhibition is being advanced as a therapy for liver fibrosis and cirrhosis. Clinicians need to understand the rationale behind this new advance. This minireview will highlight the basic science, as well as recent advances in the field, including the concept of the "galectin-3 fibrosome" and the galectin-3 positive macrophage that enters the liver from the peripheral circulation in the setting of nonalcoholic fatty liver disease. Galectin-3 appears to be central to the non-alcoholic steatohepatitis fibrosis process, and inhibition of galectin-3 is imperative to curtail liver fibrosis.

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INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is an aggressive form of nonalcoholic fatty liver disease characterized by hepatic steatosis, ballooning hepatocytes, inflammation of hepatic lobules, and excessive deposition of fibrotic tissue. If left untreated, NASH may progress to cirrhosis and hepatocellular carcinoma (HCC), which are major causes of morbidity and mortality[1,2]. The risks for HCC are particularly worrisome in the subpopulation of NASH with diabetes, obesity[3], hypertension and dyslipidemia[4]. Thus far, there are no approved pharmacotherapeutics for the treatment of NASH and the only curative treatment for cirrhosis and early-stage HCC is a liver transplant.

NASH has a complex pathogenesis that is triggered by multiple metabolic factors, including insulin resistance, genetic factors, and lifestyle issues such as unbalanced excessive caloric intake and lack of exercise[5]. Classically, the disease has been divided into early and late stages, and investigative pharmacotherapeutics target different pathogenic metabolic pathways to gain resolution of steatohepatitis or regression of fibrosis, or ideally both processes. Regardless of the etiology or the pathway, the changeover from nonalcoholic fatty liver disease (NAFLD) to NASH leads to liver fibrosis and cirrhosis, *via* the transformation of the hepatic stellate cell (HSC) to an activated myofibroblast that lays down collagen. In liver fibrosis, the interaction of HSCs with other cells is complex. Liver sinusoidal endothelial cells modulate HSCs quiescence as well as fibrosis regression in the homeostatic state[6]. In the fibrotic process, apoptotic hepatocytes increase the inflammatory response and activate macrophages. Chronic liver injury leads to continuous HSCs activation, first *via* the resident liver macrophage, the Kupffer cell, and then *via* myeloid derived liver macrophages which then promote extracellular matrix (ECM) accumulation and tissue structure remodeling and resulting in progressive liver fibrosis[7].

The transforming growth factor (TGF)-β1 has been viewed as the major profibrogenic cytokine released by the liver cell upon injury, turning the HSC into a myofibroblast. A comprehensive review of liver fibrosis has recently been published[8], as well as a review of the signaling pathways and drugs targeting the various pathways in non-alcoholic steatohepatitis[9].

There are many disease processes where the galectin-3 protein has been implicated[10]. Recent data has shown galectin-3 as a direct causative agent in diverse diseases such as endometriosis[11], cardiac fibrosis and atrial fibrillation[12], and Alzheimer's disease[13]. Galectin-3 plays a leading role in cancer progression and in the tumor microenvironment[14]. In HCC, overexpression of galectin-1 and galectin-3 have been noted[15], and galectin-3 favors tumor metastases *via* activation of β-catenin signaling[16].

In cirrhosis, galectin-3 has been proven to be a biomarker, in combination with other scores, to discriminate advanced cirrhosis and predict post-transplant infectious complications[17]. High tissue expression of galectin-3 was also associated with the risk of chronic liver disease and worse overall survival[18]. Blood levels of galectin-3 have not correlated as a biomarker in NASH, since other background diseases such as heart disease can raise galectin-3 levels on their own[19]. This review will focus on the role of galectin-3 in the liver fibrosis associated with NASH.

GALECTIN-3: BASIC FEATURES

Galectins are proteins that are modified in homoeostasis or under pathological conditions by adding glycans to their peptide chains, which in turn modulates their function. These proteins are known as glycoproteins, and the addition of a carbohydrate molecule to a protein molecule is known as glycosylation.

Glycosylation is critical for both cellular and extracellular activities. 'Lectins' are glycan binding proteins capable of recognizing distinct sugar residues, that in turn signal a cascade of molecular events. "S type" lectins, or galectins, selectively bind β- galactosides. Galectins can be found in the nucleus and the cytoplasm, as well as on the cell membrane and in the extracellular space and the ECM[20].

There are fifteen mammalian galectins that have been identified, all of them sharing a structure sequence of 130 amino acids and at least one carbohydrate recognition domain (CRD). Galectins are expressed in practically all immune cells in a constitutive or induced fashion. Galectins 1 and 3 are secreted into extracellular space[21].

Galectin-3 is a 30 kDa protein encoded by a single gene, LGALS3, located on chromosome 14, locus q21-q22. It was initially identified as MAC2 protein and is constitutively expressed on macrophages. Galectin-3 is a 'chimera' type of galectin, presenting one CRD, with a non CRD section of 30 N terminal amino acids, followed by 80 amino acids of tandem rich proline, tyrosine, and glycine[22].

Reviewing the galectin-3 medical literature presents challenges. The original name for galectin-3 was MAC2, and that is still being utilized. At times one also sees 'LGALS3' used, which is the gene that codes for galectin-3. Another term seen in the literature regarding this versatile protein is the galectin-3 binding protein, also known as MAC2 binding protein, or LGALS3BP. This 90 kD multifocal glycoprotein is a receptor ligand for galectin-3 and is present in human body fluids and appears to have a prognostic and functional role in cancer[23]. The term also seen in the literature is MAC2BPGi, the MAC2 binding protein glycan isomer that is now being used as a serum biomarker for assessing liver

fibrosis in various liver diseases[24].

GALECTIN-3 ROLE IN LIVER FIBROSIS AND CIRRHOSIS, THE GALECTIN-3 FIBROSOME

The role of galectin-3 in liver fibrosis was introduced with the discovery that upregulated galectin-3 expression was temporally and spatially related to the induction of hepatic fibrosis and that disruption of the galectin-3 gene blocks myofibroblast activation and procollagen (I) expression[25]. Further confirmation of upregulation of galectin-3 expression has been obtained in other preclinical models of hepatic fibrosis[26], NASH[27] and cholestatic liver diseases[28].

In liver fibrosis, galectin-3, once secreted by several cells, including monocytes and macrophages, help activate quiescent fibroblasts to become myofibroblasts, which is the hallmark event in tissue fibrosis formation[25]. The function of galectins in fibrosis is based on the formation of oligomeric structures that lead to cross linking and lattice like structures. These lattices form a supramolecular assembly and activate different signaling pathways on the cell surface[29].

This fibrosis lattice process appears to be occurring in many disease states such as kidney fibrosis, cardiac fibrosis, and pulmonary fibrosis. In the lung fibrosis associated with coronavirus disease 2019, a macromolecular assembly on the surface of epithelial and mesenchymal cells that clusters pro-fibrotic factors has been discovered[30]. The researchers coined the term the 'galectin-3 fibrosome' to describe how galectin-3 oligomerizes *via* its N-terminal domain and binds modified glycan chains on glycoproteins on cell surfaces. The galectin-3 interactions anchor two complexes, TGF-βRII and the (CD98hc): β1-integrin complex that mediate inflammatory and fibrotic cellular responses to extracellular stimuli. TGF-βRII is a key receptor for the profibrotic cytokine TGF-β1. The CD98 heavy chain (CD98hc): β1-integrin complex mediates inflammatory cytokine responses to extracellular factors[31]. This discovery was made in idiopathic pulmonary fibrosis, and needs to be proven in hepatic fibrosis, but disruption of this 'gal-3-fibrosome' appears to be a promising target for new anti-fibrotic therapies.

GALECTIN-3 MACROPHAGE ACTIVATION AND LIVER FIBROSIS

The tissue-resident liver macrophages, termed Kupffer cells, represent key phagocytes that closely interact with local parenchymal, interstitial, and other immunological cells in the liver to maintain homeostasis and tolerance against harmless antigens[32]. Upon liver injury, the pool of hepatic macrophages expands dramatically by infiltrating bone marrow/monocyte-derived macrophages. The interplay of the injured microenvironment and altered macrophage pool skews the subsequent course of the liver injury[33].

Liver macrophages are laden with galectin-3[34]. The activated macrophages that enter the liver from the peripheral circulation in the setting of injury are shown to be markedly stained with galectin-3 on immunohistochemistry, and this immunohistochemistry staining is clearly different from normal liver tissue[35] (see accompanying Figure 1). Macrophage plasticity allows changes from an M1 to an M2 subtype, and that subtype performs a protective role in liver injury[36]. CD68 is the pan macrophage marker, and CD 206 is a marker for the M2 macrophage. In a Pediatric NAFLD immunofluorescence histology study, different subpopulations of hepatocytes and galectin-3 positive macrophages were correlated with distinct stages of the NAFLD to NASH disease spectrum. Researchers found that the number of α-SMA/Gal-3+ cells was significantly increased in the NASH fibrosis stage. The data reinforced a direct correlation between an increased fibrosis score and α-SMA/Gal-3+ cells in NAFLD children and supported the profibrogenic role of galectin-3[37].

Reading this literature also presents difficulties with the older terms M1 and M2 macrophage still being used, along with newer terms for macrophages described by their cluster of differentiation (CD 68, CD 206) as well as even newer terms depicting liver macrophages as NAM's; NASH associated macrophages, including a subset that may be triggering receptor expressed on myeloid cells 2 positive (TREM-2+), and may promote the emergence of restorative macrophages during recovery from liver damage[38]. More recent literature involving single cell RNA sequencing has defined a subset of NAM's that are TREM2+ and CD9+ positive and are a macrophage population that expands in fibrosis and differentiates from circulating monocytes. These macrophages are similar to 'LAM's; lipid associated macrophages, which surround adipose tissue and form 'crown-like macrophages'[39].

In addition to the galectin-3 macrophage literature, further proof of the role of galectin-3 in liver fibrosis has come from live cell to cell mapping that has captured galectin-3 glycan interactions amongst live hepatic cells, the macrophage, and the stellate cells[40]. Yet another confirmation has recently been obtained from genomic studies, where a swine model of NASH had elevated levels of the LGALS3 gene that codes for galectin-3. Most interestingly only LGALS3 was associated with lipid droplet areas, suggesting a role for galectin-3 in the transition of NAFLD to NASH[41].

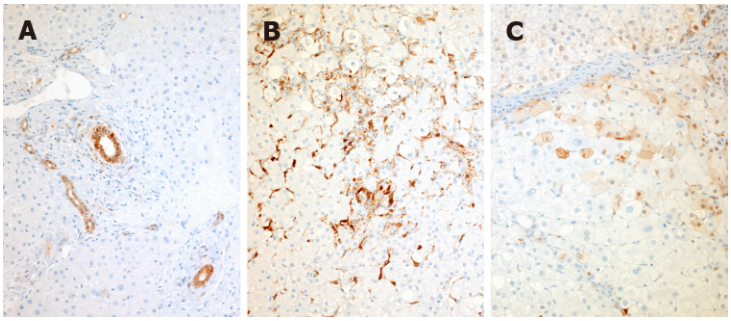

Figure 1 Immunohistochemical staining of normal, non-alcoholic steatohepatitis, and non-alcoholic steatohepatitis cirrhosis liver tissue with a galectin-3 antibody. A: Normal liver: In the normal liver, immunoreactive galectin-3 is present in epithelial cells of all large and small bile ducts and in periportal ductules; B: Non-alcoholic steatohepatitis (NASH) liver: In livers with inflammatory disease, staining appears in activated macrophages both in the parenchyma and in the portal tracts as well as in lymphoid germinal centers when present in chronic hepatitis. Notably there is no galectin-3 positivity in hepatocytes in pre-cirrhotic inflammatory disease, even in the presence of ballooning degeneration, Mallory-Denk bodies or features of apoptosis; C: NASH cirrhosis: Galectin-3 appears in hepatocytes and can be focal or widespread with both cytoplasmic and nuclear activity. A-C: Citation: Goodman Z, Lowe E, Boudes P. Hepatic Expression of Galectin-3, a Pro-fibrotic and Pro-inflammatory Marker: An Immunohistochemical Survey. Proceedings of the American Association for the Study of Liver Diseases meetings; 2022 Nov; Washington, US. Copyright© The Authors 2020. The authors have obtained the permission for figure using from the authors (Supplementary material).

GALECTIN-3 INHIBITION CURRENT THERAPEUTIC APPROACHES

The rationale to block galectin-3 in hepatic fibrosis has been laid out. The conserved homology between galectins makes treatment strategy difficult, and the ubiquity of galectins in our body as well as the need to have normal galectin intracellular function, have confounded galectin inhibition therapy approaches. The role of intracellular galectin-3 in the context of fibrosis development is less well understood and the available data suggest the extracellular component is likely the main driver of its pro-fibrotic effects[42]. Although the best target area of inhibition remains unclear, blocking galectin-3 in the extracellular space and avoiding intracellular galectin-3 inhibition might indeed be the best approach[43], especially for late-stage liver disease patients who are tenuous from their liver disease and other comorbidities.

Galectin-3 inhibition involves complex sugar organic chemistry. Galectin-3 inhibitors employ either small molecules that can be given orally, or large molecules that are administered parenterally[44]. The traditional approach has been to block the CRD, as there are specific structural features in small molecule oligosaccharides that promote stronger binding to the CRD[45]. Additionally, small molecule inhibitors offer another potential advantage to be engineered to selectively bind each of the known fifteen galectins[43].

Large polysaccharide molecules can bind not only to the galectin-3 CRD, but also the N-terminal[46]. Studies have suggested that inhibiting the galectin-3 CRD alone might be inadequate, and several researchers have contended that both the N- and C- terminus of galectin-3 should be targeted to combat fibrosis[47]. Thus far there does not appear to be any safety or tolerability issues in humans associated with inhibiting galectin-3 both extracellularly and intracellularly[44]. A recent hepatic impairment study of galectin-3 inhibition revealed no safety concerns, even when administered in late-stage cirrhosis[48].

Galectin blockers are now a focus of intensive research. Studies are now integrating galectin research with a transdisciplinary approach that includes the disciple of complex sugar chemistry known as 'Glycobiology', along with material science, and a variety of galectin targeted biomaterials. These studies remain preclinical and have been recently reviewed[49]. For now, galectin-3 inhibition for hepatic fibrosis is moving forward in trials with several agents.

GALECTIN-3 INHIBITORS CURRENTLY IN TRIALS

The most advanced inhibitors currently in trials are belapectin and GB1107. Belapectin is a large molecule galactoarabino-rhamnogalacturonan polysaccharide inhibitor derived from natural sources. Post hoc analysis of a phase 2 belapectin study in compensated cirrhosis showed that belapectin prevented esophageal varices formation in a subgroup analysis of patients without esophageal varices at baseline, and reduced hepatic venous pressure gradient after 52 weeks of therapy[50]. A follow up international adaptive P2b/3 trial is now ongoing using the clinical endpoint of preventing esophageal varices based on endoscopic evaluation[51].

GB1107 is a small molecule thiogalactoside oral inhibitor targeting the CRD. It is advancing in P2 with a trial in cirrhotics and a first in human study with GB1211, an analogue of GB1107, is proceeding into a P2 study with cirrhosis of all etiologies[52].

CONCLUSION

This review centered around the evolving role of the galectin-3 and the hepatic Gal3+ macrophage at the center of the liver fibrotic pathway. Cell to cell interactions between the hepatocyte, the macrophage, and the stellate cells initiate the transformation of the stellate cell into a myofibroblast that lays down collagen in the ECM. Genomics, transcriptomics, proteomics, immunohistochemistry staining, and live cell to cell mapping have confirmed the vital role of galectin-3 in liver fibrosis. The concept of the 'galectin-3 fibrosome' has been illuminated, and the role of the galectin-3 positive macrophage in liver fibrosis continues to evolve. The picture is filling in but is by no means complete.

A confounding factor for those researching this topic is that the medical literature is confused by older terms still being employed for the same process, both for galectin-3, and for the hepatic Gal3+ macrophage. The author believes an international consensus needs to be achieved on nomenclature as this field moves forward. It is apparent that galectin-3 inhibition for liver fibrosis and cirrhosis will continue to be a fertile target of clinical research. Given galectin-3's role in HCC and HCC metastatic spread, it is intriguing to speculate that galectin-3 inhibition might have protective effects against HCC development in cirrhosis, as well as a potential future role in adjunctive HCC therapy. In the next few years, data from upcoming galectin-3 inhibition trials will determine whether the future of NASH therapy includes this promising antifibrotic approach.

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MINIREVIEWS

Clostridioides difficile infection in patients with nonalcoholic fatty liver disease-current status

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease, leading to fibrosis, cirrhosis and hepatocellular carcinoma and also associated with increased cardiovascular disease mortality. The pathogenesis of NAFLD is not fully understood, although NAFLD is thought to be a hepatic form of metabolic syndrome. There is an increasing understanding of the role of microbiota disturbances in NAFLD pathogenesis, and as with many other conditions affecting the microbiota, NAFLD may be a novel risk factor for *Clostridioides difficile* (*C. difficile*) colonization (CDC) and *C. difficile* infection (CDI). CDI is an emerging nosocomial disease, and community-acquired cases of infection are growing, probably due to an increase in CDC rates. The association of NAFLD with CDI has been shown in only 4 studies to date, three of which included less than 1000 patients, although the frequency of NAFLD in these studies was observed in almost 20% of the total patient cohort. These data revealed that NAFLD is a risk factor for CDI development and, moreover, is a risk factor for intestinal complications of CDI. More studies are needed to investigate this

association and move forward CDC and CDI screening efforts for this group of patients.

Key Words: Nonalcoholic fatty liver disease; *Clostridioides difficile*; *Clostridioides difficile* colonization; *Clostridioides difficile* infection; Minireview

Core Tip: The association of nonalcoholic fatty liver disease (NAFLD) with *Clostridioides difficile* (*C. difficile*) infection (CDI) has been shown in only 4 studies to date, three of which included less than 1000 patients, although the frequency of NAFLD in these studies was observed in almost 20% of the total patient cohort. These data revealed that NAFLD is a risk factor for CDI development and, moreover, is a risk factor for intestinal complications of CDI. More retr-ospective studies and systematic reviews are needed to examine this group of patients as a risk factor for CDI, make recommendations to prevent CDI, and effectively screen and diagnose *C. difficile* colonization within NAFLD patients.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized as a chronic liver disease with ≥ 5% hepatic fat accumulation and a natural progressive course from nonalcoholic fatty liver (NFL) to nonalcoholic hepatitis (NASH) and cirrhosis. The current epidemiology of NAFLD is not totally understood due to its low diagnostic rates, as patients may remain asymptomatic even after the formation of cirrhosis and escape medical evaluation; however, NAFLD is thought to affect approximately 25% of the adult population, and the incidence of NAFLD is expected to increase in the future[1-3]. In NFL, the fibrosis progression rate averages 14 years *per* each stage of fibrosis *vs* 7 years *per* each stage of fibrosis in NASH. There are also rapid progressors with NASH in whom fibrosis progresses in less than 7 years. Among NAFLD patients, approximately 20% have NASH, and these patients should be diagnosed and receive proper treatment, as they can develop cirrhosis within 2-3 decades[4].

Patients with NAFLD are at risk for hepatocellular carcinoma (HCC), the fourth leading cause of cancer death worldwide, which may occur in the absence of cirrhosis in up to 50% of NAFLD patients, leading to late diagnosis and increased mortality[3,5,6]. In addition to cirrhosis and HCC, NAFLD is associated with an increased risk of cardiovascular disease (CVD), as these patients tend to have obesity, type 2 diabetes mellitus, and dyslipidemia, the hallmark of metabolic syndrome. Thus, these patients are at a higher risk for hypertension, coronary heart disease, cardiac arrhythmias, cardiomyopathy development and increased cardiovascular morbidity and mortality[7]. Nonobese patients with NAFLD have significantly lower rates of CVD than obese patients with NAFLD; however, even in the absence of obesity, patients with NAFLD are at a higher risk of CVD, with an incidence rate of 18.7 *per* 1000 persons-years[1]. In addition to the association of NAFLD with CVD and HCC, recent studies have shown that patients with NAFLD are at risk for *Clostridioides difficile* (*C. difficile*) infection (CDI) development[8-11].

ABOUT *C. DIFFICILE*

C. difficile is a gram-positive, spore-forming bacterium with transmission by the fecal-oral route. It is widespread in the environment and human population, may persist in the intestinal tract of asymptomatic carriers and animals and contaminate ambient objects, and can cause mild to severe diarrhea and colitis. In the last 30 years, CDI has become one of the most significant nosocomial infections and the leading cause of antibiotic-associated diarrhea, with increased severity, rate of recurrence (*i.e.*, up to 10%-30%) and mortality[12,13]. In 2011, 453000 new cases of CDI and 29300 associated deaths were identified in the United States; in 2017, the incidence was estimated at 223900 with 12800 deaths[14]. In Europe, the annual estimated number of cases is up to 189256, according to a 2016-2017 study[12]. The increased incidence and severity associated with CDI can be attributed to the emergence and spread of a strain known as ribotype 027 (NAP1/BI/027) among hospitalized patients

[15]. NAP1/BI/027 is highly resistant to fluoroquinolone, has increased toxin A and B production, produces a strain-specific binary toxin and persists in the United States and Europe; however, in Asia, the dominant strains include ribotype 017, 018 and 014[16]. Of note, drug resistance and severity of CDI also vary by ribotype and region. Developing diagnostic methods have led to an understanding of the heterogeneity of *C. difficile*, while molecular typing studies have demonstrated the presence of up to 98 different ribotypes in a single country[17,18].

C. difficile toxins cause acute colonic inflammation *via* epithelial disruption and the release of proinflammatory cytokines and chemokines, resulting in CDI, which is clinically heterogeneous. The severity of CDI is thought to be dependent on both the host and strain characteristics[17,19]. The distal colon is the most frequently affected organ in CDI, resulting in mild diarrhea with spontaneous recovery after antibiotic withdrawal. However, some patients manifest profuse diarrhea, colonic ileus, pseudomembranous colitis and toxic megacolon, followed by fever, abdominal pain, sepsis, *etc.* Clinical and laboratory findings may vary between patients depending on CDI severity including dehydration, peritonitis, leukocytosis, a positive fecal occult blood test, *etc.*; therefore, CDI should be suspected in any patient with acute diarrhea, recent antimicrobial exposure and a prolonged hospital stay[20,21]. Risk factors for CDI include non-CDI-active antimicrobial use, prolonged hospitalization, advanced age (≥ 65 years.) and recent intake of acid-suppressive therapy[20,22-24].

Recurrent CDI (rCDI) is a new CDI episode occurring within eight weeks after a previous episode. Etiologically, rCDI may be due to relapse of the same strain as the first infection or reinfection by a different strain, and it develops in 15% to 30% of patients after initial CDI. The risk of further recurrence is much higher, as approximately 40% of patients with one episode of rCDI will develop the second episode, whereas the third episode will develop in 45%-65% of patients. Thus, prevention of rCDI remains very important[25]. Risk factors for rCDI include advanced age (> 76 years), antibiotic exposure, gastric acid suppression, CDI caused by a highly virulent strain (NAP1/BI/027), severe underlying diseases and a prolonged hospital stay[25,26].

CDI diagnosis depends on clinical findings and the detection of *C. difficile*, its toxin or toxin-producing gene in a stool specimen taken before the initiation of *C. difficile*-specific treatment to avoid false-negative results. The European Society of Clinical Microbiology and Infectious Disease recommends a 2-step diagnostic algorithm for CDI confirmation. The first step is a highly sensitive screening method (*i.e.*, the nucleic acid amplification test and the glutamate dehydrogenase assay). Positive results are followed by the performance of a second step which includes detecting free toxins in stool (*i.e.*, the enzyme immunoassay for disease causing toxins or the cell cytotoxicity neutralization assay)[20,27].

Recently, there has been an interest in asymptomatic colonized individuals, acting as a reservoir for CDI and being at increased risk (*i.e.*, 51.9 cases *per* 100000 persons) of developing CDI[20]. *C. difficile* colonization (CDC) stands for the detection of *C. difficile* in the absence of CDI symptoms for 12 wk pre- or post-specimen collection; however, many studies use the simple definition of a *C. difficile*-positive stool and the absence of CDI symptoms[28]. *C. difficile* colonizes the gut of 5% of the adult population and up to 70% of infants and does not affect the intestinal tract while the gut microbiome is intact; however, administration of antibiotics affects its composition and promotes the growth of vegetative forms, the germination of spores, and the production of toxins[15,18]. Approximately 4%-10% of patients are colonized with *C. difficile* at the time of hospitalization, and the number of colonized patients increases during their stay[17]. Therefore, asymptomatic hospitalized patients require *C. difficile* screening to prevent microbe transmission and the development of strategies to mitigate the risks for developing active CDI[28,29].

C. DIFFICILE AND LIVER DISEASES

It is widely known that cirrhosis is associated with an increased risk of CDI and a severe disease course as cirrhotic patients have a high rate of hospitalization, an immunocompromised state, and are often prescribed to take antibiotics due to an increased risk of infection[30-32]. The average hospitalization stay in patients with CDI and cirrhosis is 14 d, inpatient mortality is ≥ 14%, and 30-d readmission rates occur in 35% of patients compared to the results for noncirrhotic patients, which are 13 d, 8% and 20%, respectively[33]. CDI is an independent mortality risk factor in cirrhotic patients as evident from the fact that mortality in a cohort of patients with cirrhosis and concurrent CDI were demonstrated to be higher (13.8%) than mortality in cirrhosis (8.2%) and CDI (9.6%) patients alone[34]. Moreover, hypoalbuminemia and admission to the intensive care unit are independent predictors for short-term mortality [35]. Sahra *et al*[36] revealed that patients with cirrhosis were more likely to develop CDI than noncirrhotic patients. Interestingly, the etiology of cirrhosis also affects CDI prevalence. For instance, patients with cirrhosis due to alcoholic liver disease (ALD) and NAFLD were more prone to CDI than patients with viral hepatitis B and C cirrhosis (174.0 *vs* 184.9, *vs* 81.7 *vs* 117.9 persons *per* 100000, respectively) [36].

In contrast to cirrhosis, the association between NAFLD and CDI is not fully understood. To the best of our knowledge, there are currently only four studies examining this question, even though NAFLD is

the most common cause of chronic liver disease and CDI is one of the most common nosocomial infections.

In November 2019, Nseir *et al*[9] published their retrospective cross-sectional study, revealing that NAFLD is a risk factor for *C. difficile*-associated diarrhea (CDAD). Patients with NAFLD accounted for 66% of all patients with confirmed CDAD. Moreover, the authors revealed that metabolic syndrome, which is commonly seen in patients with NAFLD, is associated with severe CDAD[9]. A similar retrospective study by Papić *et al*[8] confirmed that NAFLD is a risk factor for inpatient CDI, with an incidence rate of 16.9% *vs* 7.4%, as seen in the control group.

In 2021, Jiang *et al*[10] presented a large retrospective study that included 7239 patients with CDI and coexisting NAFLD (with a total of 94.5% that were noncirrhotic) and compared them to patients with coexisting ALD and viral liver disease (VLD). The analysis showed that patients in the NAFLD group had a lower incidence of respiratory failure (2.7%), septic shock (0.5%), acute kidney injury (13%), hospital mortality (0.8%) and length of stay (5.75 ± 0.16 d) than those in the ALD and VLD groups; however, the rates for intestinal complications were increased in the NAFLD group. Specifically, intestinal obstruction was seen in 4.6% of patients with NAFLD compared to 2.2% of patients with ALD. Additionally, a higher rate of intestinal perforation was observed in the NAFLD group compared to the VLD group[10].

Recently, Šamadan *et al*[11] revealed that NAFLD is not only a risk factor for inpatient CDI in elderly patients exposed to systemic antibiotics but also a risk factor for rCDI (47.4% in the NAFLD group compared to 27.9% in the non-NAFLD group). Interestingly, the authors found a decreased rCDI ratio in patients taking statins in both the NAFLD and non-NAFLD groups, possibly due to their modulatory effect on the microbiome[11].

GUT MICROBIOTA DISTURBANCES IN NAFLD AND CDI PATHOGENESIS

Although the association of NAFLD with CDI has not been fully studied, biological plausible links may lie in their shared pathogenesis (*i.e.*, the gut microbiota disturbances).

It is widely accepted that microbiota disturbances play a main role in *C. difficile* colonization and infection; therefore, it is not surprising that most patients develop CDI after a course of antibiotics. The pathogenesis of *C. difficile* colonization and infection includes intermicrobial interactions. For instance, *C. difficile* produces quorum signals, inducing Proteobacteria metabolite production leading to Bacteroidetes inhibition. *C. difficile* can also produce inhibitors of indigenous microbiota, such as proline-base cyclic dipeptides[37]. Secondary bile acids have been shown to inhibit toxin activity and the growth of vegetative forms of *C. difficile*, while antibiotics affect microbes producing these acids. In contrast, primary bile acids promote *C. difficile* spore germination. Therefore, a low level of secondary bile salts (and consequently a low concentration of secondary bile acid-producing bacteria) and a high level of primary bile salts, results in CDI and its recurrence[38].

Multiple studies have shown that smaller microbial diversity and decreases in certain species are often seen in patients with CDI and CDC. For example, stool samples of patients with CDI revealed an increase in Proteobacteria, Firmicutes and Enterobacteriales, and a decrease in Bacteroidetes and butyrate-producing Ruminococcaceae and Lachnospiraceae families in comparison to healthy individuals[28].

The CDC microbiome disturbances were similar to those of CDI patients; however, in regard to the degree of changes seen, they were closer to healthy individuals. In addition, a higher level of some bacterial families were noted in CDC microbiomes, including Clostridiales family XI incertae sedis, Clostridium, and Eubacterium, but were significantly decreased in the infected individuals[39]. This data confirms that CDI occurrence is dependent on the presence of certain bacterial species and that colonization with these species may prevent CDC and CDI[40]. Studies on murine models have already confirmed that intestinal colonization with Lachnospiraceae significantly reduced CDC and that administration of *Clostridium scindens* prevented CDI development in antibiotic treated mice with *C. difficile* spores[41,42]. From these studies it can be inferred that any condition connected with gut microbiota disturbance is a risk factor for CDC and CDI.

Recently, there has been increasing evidence of the role of microbiota disturbances in NAFLD pathogenesis and progression[43-45]. For example, it was shown that the transfer of the microbiome from mice with fasting hyperglycemia and insulinemia to germ-free mice led to the development of NAFLD. These conventionalized NAFLD mice had Lachnospiraceae bacterium 609 and *Barnesiella intestinihominis* overrepresented in their feces, whereas *Bacteroides vulgatus* (*B. vulgatus*) was underrepresented in comparison to the control group[46].

Changes in the gut microbiota were also found in humans with NAFLD. Moreover, the composition of the gut microbiota varied not only between the control group and patients with NAFLD but also between patients with NAFLD, NASH and NAFLD cirrhosis[47]. Loomba *et al*[48] revealed the dominance of Firmicutes and Bacteroidetes in NAFLD patients; however, the progression of the disease from mild/moderate to advanced fibrosis led to an increase in Proteobacteria and a decrease in Firmicutes. *Eubacterium rectale* and *B. vulgatus* were shown to be the most abundant species in mild/

Table 1 Nonalcoholic fatty liver disease association with *Clostridioides difficile* infection and recurrent *Clostridioides difficile* infection

Ref.	Year of publication	Type of publication	Number of patients	Correlation between NAFLD/CDI
Nseir *et al* [9]	2020	Retrospective study	115	NAFLD was found in 76/115 (66%) patients with CDAD *vs* 35/115 (30.4%) in the control group, $P < 0.001$ Multivariate analysis showed that NAFLD was significantly associated with CDAD (OR: 1.51, 95%CI: $P = 0.05$)
Papić *et al* [8]	2020	Retrospective cohort study	314	CDI was significantly more frequent in patients with NAFLD (14, 16.87% *vs* 17, 7.36%, $P = 0.0156$)
Šamadan *et al*[11]	2021	Retrospective cohort study	329	Multivariable Cox regression analysis showed that age > 75 yr, NAFLD, CACI > 6, chronic kidney disease, statins and immobility were associated with rCDI
Jiang *et al* [10]	2021	Retrospective cohort study	7239	CDI with NAFLD was associated with a higher rate of intestinal perforation ($P < 0.01$) when compared to viral liver disease and a higher rate of intestinal obstruction (4.6% *vs* 2.2%, $P = 0.001$) when compared to CDI with ALD

NAFLD: Nonalcoholic fatty liver disease; CDI: *Clostridioides difficile* infection; ALD: Alcoholic liver disease; CDAD: *Clostridioides difficile*-associated diarrhea; rCDI: Recurrent *Clostridioides difficile* infection.

moderate NAFLD, and *B. vulgatus* (2.2%) and *Escherichia coli* were the most abundant in advanced fibrosis, suggesting a shift toward gram-negative microbes in which lipopolysaccharide is thought to cause the progression of fibrosis. Proteobacteria, Enterobacteria, Escherichia and Bacteroides were found in abundance in patients with NASH, while Gammaproteobacteria and Prevotella were more prevalent in the stool of obese children with NAFLD in comparison to non-NAFLD obese children[46, 49]. Zhu *et al*[50] found an increased representation of an alcohol-producing Escherichia, followed by increased blood alcohol concentration, in NASH patients compared to obese and healthy individuals, which may play a role in NASH pathogenesis. Zhang *et al*[51] showed the association of a high-fat/high-cholesterol diet with progression of NAFLD and the concomitant changes in the microbiota of mice. Thus, enrichment of Mucispirillum schaedleri_Otu038, Desulfovibrio_Otu047, Anaerotruncus_Otu107, Desulfovibrionaceae_Otu073, Clostridium celatum_Otu070, C. ruminantium_Otu059 , C. cocelatum_Otu036 and C. methylpentosum_Otu053, and the depletion of Bifidobacterium_Otu026, Akkermansia municiphila_Otu034, Lactobacillus_Otu009, Bacteroides acidifaciens_Otu032, Bacteroides_Otu012, B. uniformis_Otu080 and B. eggerthii_Otu079 in the microbiota were observed with the progression of NAFLD to NASH and HAFLD-HCC. The authors also revealed a possible role of Helicobacter ganmanii_Otu031 enrichment and Bacteroides_Otu012 depletion in HCC development in mice. Lastly, fecal microbiome transplantation from NAFLD patients to germ-free mice confirmed a role of gut microbiota in NAFLD pathogenesis as these mice showed hepatic steatosis, inflammation and multifocal necrosis on a high-fat diet (HFD), while germ-free mice from the control group only had minor liver inflammation and fat accumulation on the same HFD[52]. Therefore, the gut microbiota disturbances seen in both NAFLD and CDC/CDI and preexisting microbiota changes in patients with NAFLD may explain its association with CDI and rCDI[8-10].

CONCLUSION

NAFLD is the most common chronic liver disease with an estimated prevalence of 20% in the general population. NAFLD is a well-known risk factor for cirrhosis and HCC development and is also associated with cardiovascular mortality. Although the pathogenesis of NAFLD is not fully understood, the past decade of research has led to an understanding of the role of the gut microbiota in NAFLD development and progression toward cirrhosis. As with any condition associated with microbiota disturbance, NAFLD has been shown to be associated with CDI severity. Despite NAFLD being such a common, chronic liver disease and *C. difficile* being an emerging nosocomial infection with increasing community-acquired cases, only 4 studies have examined this issue, to date (Table 1). More retrospective studies and systematic reviews are needed to examine this group of patients as a risk factor for CDI, make recommendations to prevent CDI, and effectively screen and diagnose CDC within NAFLD patients.

FOOTNOTES

Author contributions: Kiseleva YV is responsible for conceptualization, supervision, manuscript first draft preparation, approved final draft; Maslennikov RV is responsible for supervision, data acquisition, approved final

draft; Gadzhiakhmedova AN is responsible for data acquisition, visualization, manuscript writing and editing, approved final draft; Zharikova TS is responsible for data aquisiton, formal analysis, manuscript writing and editing, approved final draft; Kalinin DV is responsible for visualization, formal analysis, manuscript writing, review and editing, approved final draft; Zharikov YO is responsible for supervision, conceptualization, manuscript first draft preparation, review and editing, approved final draft.

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MINIREVIEWS

Sonographic gallbladder wall thickness measurement and the prediction of esophageal varices among cirrhotics

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Abstract

Acute variceal bleeding in patients with liver cirrhosis and portal hypertension (PHT) is the most serious emergency complication among those patients and could have catastrophic outcomes if not timely managed. Early screening by esophago-gastro-duodenoscopy (EGD) for the presence of esophageal varices (EVs) is currently recommended by the practice guidelines for all cirrhotic patients. Meanwhile, EGD is not readily accepted or preferred by many patients. The literature is rich in studies to investigate and validate non-invasive markers of EVs prediction aiming at reducing the unneeded endoscopic procedures. Gallbladder (GB) wall thickness (GBWT) measurement has been found promising in many published research articles. We aim to highlight the validity of sonographic GBWT measurement in the prediction of EVs based on the available evidence. We searched databases including Cochrane library, PubMed, Web of Science and many others for relevant articles. GBWT is associated with the presence of EVs in cirrhotic patients with PHT of different etiologies. The cut-off of GBWT that can predict the presence of EVs varied in the literature and ranges from 3.1 mm to 4.35 mm with variable sensitivities of 46%-90.9% and lower cut-offs in viral cirrhosis compared to non-viral, however GBWT > 4 mm in many studies is associated with acceptable sensitivity up to 90%. Furthermore, a relation was also noticed with the degree of varices and portal hypertensive gastropathy.

Among cirrhotics, GBWT > 3.5 mm predicts the presence of advanced (grade III-IV) EVs with a sensitivity of 45%, the sensitivity increased to 92% when a cut-off ≥ 3.95 mm was used in another cohort. Analysis of these results should carefully be revised in the context of ascites, hypoalbuminemia and other intrinsic GB diseases among cirrhotic patients. The sensitivity for prediction of EVs improved upon combining GBWT measurement with other non-invasive predictors, *e.g.*, platelets/GBWT.

Key Words: Sonographic; Gallbladder wall thickness; Prediction; Esophageal varices; Portal hypertension; Esophago-gastro-duodenoscopy

Core Tip: Ruptured varices is a medical emergency and is associated with high mortality. Hence, it was recommended by the current practice guidelines to screen cirrhotic patients with portal hypertension for the presence of varices and eradicate the risky varices early. However, many issues exist with this policy. This directed the clinicians to search for non-invasive assessment tools aiming to refer only indicated cases for endoscopic examination. Among the promising tools is sonographic measurement of gallbladder wall thickness that was found related not only with the presence of esophageal varices but also with the degree of varices and portal hypertensive gastropathy.

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INTRODUCTION

Acute bleeding from ruptured gastro-esophageal varices (EVs) is a serious and potentially fatal outcome of portal hypertension (PHT) particularly among cirrhotic patients. Although the management of PHT has evolved dramatically, ruptured EVs still represents a major medical emergency with high morbidity and mortality rates[1]. Therefore, the current practice guidelines recommend screening of all cirrhotics by esophago-gastro-duodenoscopy (EGD) for the presence of EVs and to deliver management if large risky varices were detected[2,3].

Over the last few decades, non-invasive prediction has become the focus of interest for many researchers and clinicians. Many composite scores were proposed for early prediction of liver cirrhosis and its complications, particularly PHT. These predictors ranged from very simple tests such as the platelet count or prothrombin index that are readily available, affordable, and routinely used as part of cirrhotic patients' regular care to much more specific, costly, and not-readily available ones such as hyaluronic acid or type IV collagen assay. Many of these were correlated with the presence of EVs of various degrees, but their accuracy in diagnosis were not consistent[4-6].

To increase the diagnostic accuracy of these non-invasive predictors for EVs detection, combinations of markers were investigated, tested and some of them were proved useful, such as aspartate transaminase (AST) to alanine transaminase ratio[7], AST to platelet ratio index (APRI)[8], or platelet count to spleen diameter ratio[7].

Among the studied predictors, gallbladder (GB) wall thickness (GBWT) measurement by ultrasonography has been found promising in many of the published research articles. The relation of GBWT to PHT and EVs have been spotted late in the last century[9,10].

The aim of this review is to evaluate the validity of the sonographic GBWT measurement in the prediction of EVs based on the available evidence.

LITERATURE SEARCH

We searched databases including Cochrane library, Web of Science, Ovid, Science Direct, Scopus, Directory of Open Access Journals, EBSCO HOST, ProQuest, Institute for Scientific Information, EBESCO, MEDLINE /PubMed, Egyptian knowledge bank, Google scholar, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) and the Research Gate for relevant articles. We retrieved a number of studies focusing on sonographic GBWT measurement and PHT or EVs. The articles were

analyzed for delineating the relationship to PHT, EVs or portal hypertensive gastropathy (PHG). In our search strategy, we used the relevant keywords of "gallbladder wall thickness" and "gastro-esophageal varices", "gastric varices", "esophageal varices", "portal hypertensive gastropathy", "PHT", and "cirrhosis".

WHY NOT ENDOSCOPY?

EGD is the gold standard procedure in the management of EVs due to the possibility of both diagnostic and therapeutic potentials[11]. However, the application of EGD screening among cirrhotic patients–as advised by many of the current guidelines-carries the burden of performing large numbers of unnecessary endoscopies. Moreover, it is of an invasive nature with possible procedure associated adverse events, unavailable in the remote areas, requires special skills and experience with a formal training program. Furthermore, endoscopy is refused by a reasonable number of patients[3]. Hence, several trials to investigate and validate non-invasive predictors for detection of EVs were tried[3,12] with the aim to pick up appropriate candidates for the screening endoscopy.

RATIONALE FOR GBWT MEASURMENT (PATHOPHYSIOLOGY)

The question that pops up here is, why GBWT measurement is used to predict the presence of EVs although its main function is bile storage. The answer is inferred from our knowledge of four points. First, ultrasonography either the grey scale or the color Doppler mode is a non-invasive imaging technique used to evaluate cirrhotic patients. Furthermore, it is part of the hepatologists' and gastroenterologists' day-to-day practice. Second, there is growing evidence documenting validity of GBWT measurement in predicting the presence of varices[7,12-15]. Third, measuring GB wall could easily be calculated in the out-patient clinic, it is non-invasive, and is reproducible. Fourth, the GB is drained through veins of the portal circulation. This means that, it will be affected by the conditions influencing the portal venous pressure. The possible explanation for the increased GBWT in patients with EVs, is the impairment reported in the portal venous blood out flow that could precede the significant changes in the portal vein velocity[16], and it was concluded in a study by Li *et al*[13], that the degree of PHT among patients with liver cirrhosis could be predicted through the measurement of GB wall.

GB venous blood is drained through 2 pathways. First, through small veins directly into the liver. Second, through small veins toward the veins of the cystic duct and then with vessels from the common bile duct, terminating in the portal venous system. Consequently, in cases of PHT the venous drainage is impaired, and congestion of the GB wall do occur and hence the wall thickness is increased and that is why it is referred to as congestive cholecystopathy[17] in some studies.

Indirect evidence supporting this assumption is that cirrhotic patients treated with propranolol developed a significant reduction in portal pressure that subsequently was associated with a decrease in GBWT measurements[18].

OPTIMIZATION OF GBWT MEASUREMENT

The increase in GBWT may be a focal increase due to intrinsic GB diseases or diffuse[15,16,18,19]. The diffuse thickness may be related to intrinsic GB disease or diseases not related to the GB. Among the intrinsic gall bladder diseases are acute cholecystitis, chronic cholecystitis, and GB tumors. However, extrinsic diseases that may also affect the GBWT include hypoalbuminemia, sepsis, AIDS, right sided heart failure, and chronic kidney diseases[20]. Determination of GBWT measurement at different locations could differentiate focal from diffuse thickening, while revising the clinical, laboratory as well as sonographic data would differentiate intrinsic from extrinsic GB affection. In fact, among patients with liver cirrhosis, the diffuse non-inflammatory thickening of the GB wall is multifactorial and is related to PHT[9], hypoalbuminemia and the presence of ascites[21,22].

For perfect evaluation of the GBWT, sonographic assessment should be done in the fasting state. The fasting may be for 6-8 h[23], or sometimes evaluation can be done on the same day of endoscopy but before it following an overnight fasting[24,25]. In case of diffuse GBWT increase, measurements in more than one area of the GB wall are advised and the average is then taken. The position of the patient during examination was also focused on in the studies[26,27]. It would be beneficial to shift the patient from the classic supine position to the left lateral position. This position displaces the GB below the ribs and minimizes the gas interference from the colon[26,27]. The issue of gaseous interference was focused in some studies[24,25] where overnight simethicone was given to the patients prior to examination in an attempt to adsorb gases.

GBWT MEASUREMENT CAN PREDICT THE PRESENCE OF VARICES

The prediction of PHT and EVs through the GBWT measurement got attention of hepatologists around the globe over the last decades (Table 1). Li *et al*[13] figured out an inverse relationship between wall thickness of the GB and both portal vein blood flow and its mean velocity. The authors recommended that the degree of PHT in patients with liver cirrhosis could be predicted *via* measuring the GB wall.

De Alcantara *et al*[15] noticed a correlation between the increased wall thickness of the GB and the presence of GB varices as well as extra-hepatic portal vein obstruction that was favorable to correlations reported for cirrhotic patients with PHT. Meanwhile, Tsaknakis *et al*[12] found that the increase in the GBWT has occurred more significantly among cirrhotic patients with EVs despite its low sensitivity.

Elkerdawy *et al*[24] evaluated the diagnostic accuracy of GBWT measurement in comparison to several readily available and easily calculated indices (*e.g.*, platelet count and platelet count/splenic diameter ratio index) and they found GBWT measurement to have a comparable diagnostic accuracy to many of these parameters.

Khan *et al*[28] found that patients with EVs had significantly increased GBWT of 4.96 ± 0.85 mm compared to 2.54 ± 0.76 mm among patients without EVs. Among the cirrhotic group with varices, 81.25% of patients had GBWT > 4 mm compared to 10% among cirrhotic non-variceal patients (P < 0.0001). The authors concluded that measuring GBWT is very useful for the detection of EVs in cirrhotic patients.

Shehata *et al*[29] found a significant correlation between GBWT and PHT and they recommended GBWT to be used as a non-invasive predictor of EVs in cirrhotic patients. They reported GBWT as an independent predictor for varices in both univariate (GBWT OR: 0.408, CI: 0.264–0.854, P < 0.001) and multivariate logistic regression analysis (OR: 0.352, CI: 0.068–0.604, P < 0.005).

Recently in 2022, Afifi *et al*[14], focused GBWT measurement in comparison with platelet/splenic diameter ratio in predicting the presence of varices among cirrhotic patients of different Child classes. They reported GBWT at a cut-off value ≥ 3.350 to predict the presence of EVs. However, GBWT at a cut-off value ≥ 3.350 was less sensitive and less specific than platelet count to spleen diameter ratio at cut-off level ≤ 1391.00 for detection of EVs, while GBWT at cut-off level ≥ 3.950 was a predictor for the presence of large varices with a 92% sensitivity and furthermore GBWT at cut-off level ≥ 3.950 was more specific and more sensitive than platelet count to spleen diameter ratio at the same cut-off level.

GBWT AND THE DEGREE OF VARICES

The relationship of the GBWT to the endoscopic grade of varices was described in a few studies as shown in Table 2. Shehata *et al*[29] reported positive correlation (OR: 0.634, P = 0.001) between GBWT and the grade of EVs among cirrhotic patients. Elkerdawy *et al*[24] in their study grouped the varices as advanced (grades III and IV) and non-advanced (grades I and II). The authors reported the ability of the GBWT measurement to predict the presence of advanced varices (P ≤ 0.001). GBWT predicted advanced EVs at a cut-off level of > 3.5 mm, with 45%, 90%, and 77.1% sensitivity, specificity, and accuracy, respectively. In the same study both platelet count and spleen length were also independent predictors for advanced EVs. Platelet count predicted advanced EVs at a cut-off level of < 115, with 80%, 76%, and 74.3% sensitivity, specificity, and accuracy, respectively. Spleen length was a valuable predictor of advanced EVs at a cut-off level of > 15 cm, with 90% sensitivity, although it had a 60% and 71.4% specificity and accuracy, respectively.

Begum *et al*[26] observed that the mean GBWT was significantly increased (P < 0.05) in chronic liver disease (CLD) with grade III and IV varices (6.1 ± 0.8 mm) than in grade I and II varices (3.9 ± 0.7 mm).

One study published in 2011 by Yousaf *et al*[23], surprisingly reported that GBWT was most profound in patients with smaller (F1) and moderate (F2) EVs. Most of the patients with no varices in that study had normal GBWT and the authors concluded that the evolving nature of PHT causing gradual congestion of the GB stands behind this[23]. However, this study recruited patients with Child B and C cirrhosis in whom hypoalbuminemia and ascites were seen, making these conclusions unsafe.

More recently, GBWT at a cut-off level ≥ 3.95 mm was a predictor for the presence of large varices with a 92% sensitivity, 95% specificity, 86.7% positive predictive value (PPV), and 97.1% negative predictive value (NPV), with area under the curve (AUC) = 0.986. It was more superior than (more sensitive 92% *vs* 80% and more specific 75% *vs* 70%) platelet count to spleen diameter ratio at the same cut-off level ≤ 1391.00[14].

It seems that the GB wall diameter increases with evolving stages of liver diseases and its associated EVs grades. In patients with CLD with advanced varices the GBWT was 6.1 ± 0.8 mm, in compensated cirrhotics it was ≥ 3.5 mm while in advanced cirrhosis GBWT was ≥ 3.95 mm. The variability in these measurements may be related to the underlying etiologies of liver diseases.

Table 1 Studies focusing gallbladder wall thickness measurement in the prediction of varices

Ref.	Target patients	Number of patients	GBWT cut-off	Reported sensitivity	Conclusions
Li *et al*[13]	Cirrhotic	152			GBWT is closely related to hemodynamic parameters. It is feasible to predict the degree of portal hypertension through the observation of GBWT
Begum *et al*[26]	CLDs	61			GBWT among CLD patients with EVs was 5.6 ± 0.2 mm compared to 2.7 ± 0.1 mm in non-variceal group ($P < 0.05$). GBWT may be considered as an important marker for the presence of esophageal varices in CLD patients
de Alcantara *et al*[15]	Children and adolescents younger than 20 years with CLD and extrahepatic portal venous obstruction (EHPVO)	53	≥ 4.35 mm	For group I ($n = 35$; patients with CLD): 60%. For group II ($n = 18$; patients with EHPVO): 90.9%	The presence of SS and greater LOT were indicative of EVs in patients with CLD. The presence of gallbladder varices and greater GBWT indicated the presence of EVs in patients with EHPVO. The presence of an SS and a greater LOT indicated the presence of PHG in patients with CLD
Pathak *et al*[21]	Alcoholic Cirrhosis	60	> 4 mm		Thus, the presence of increased GBWT on ultrasonography in patients of cirrhosis without intrinsic gallbladder disease should be considered as an early sign of portal hypertension
Tsaknakis *et al*[12]	Chronic hepatic diseases of variable etiologies	194	≥ 4 mm	46%	GBWT occurs significantly more often in patients with EVs. However, because of the low sensitivity, combination with other non-invasive parameters such as platelet count is recommended
Elkerdawy *et al*[24]	Post-viral cirrhosis with portal hypertension	105	≥ 3.1 mm	54.29%	GBWT was associated not only with the presence of EVs, but also with advanced EVs. Although, the reported sensitivity of GBWT in prediction of EVs was low, its diagnostic accuracy was comparable and even superior to some simple non-invasive predictors
Khan *et al*[28]	Liver cirrhosis of Child-Pugh class A (80% were due to HCV)	160	> 4 mm	Not calculated	Patients with esophageal varices had significantly increased gallbladder wall thickness 4.96 ± 0.85 mm as compared to patients without esophageal varices 2.54 ± 076 mm. In group A, 65 (81.25%) patients had GBWT > 4 mm while in group B, 8 (10%) patients had GBWT > 4 mm and significant difference was observed between both groups with P value < 0.0001
Shehata *et al*[29]	Cirrhosis (multiple etiologies; causes not mentioned)	120	4	82%	Significant correlation was observed between GBWT and portal hypertension, they recommend that GBWT can be used as a non-invasive predictor of esophageal varices in cirrhotic patients
Amer *et al*[25]	Liver cirrhosis	100	> 3.5 mm	64%	Sensitivity and specificity of GBWT in prediction of PHG were 64% and 68%
Afifi *et al*[14]	Cirrhosis (causes not mentioned)	100	3.35 mm	68%	GBWT was significantly higher in EVs patients compared to the non-EVs group (mean: 4.2 mm *vs* 2.7 mm, $P < 0.001$)

CLD: Chronic liver diseases; EHPVO: Extra-hepatic venous obstruction; EVs: Esophageal varices; GBWT: Gallbladder wall thickness; HCV: Hepatitis C virus LOT: Lesser omental thickness; PHG: Portal hypertensive gastropathy; SS: Splenorenal shunt.

GBWT MEASUREMENT CAN PREDICT PORTAL HYPERTENSIVE GASTROPATHY

The relation of the GBWT measurement to the PHG was investigated in only one study. Amer *et al*[25] reported that GBWT was significantly higher in the PHG group than non-PHG ($P < 0.001$) and this difference exists irrespective of the prevalence of varices in both groups. The significant difference ($P < 0.001$) was still seen when the ratio of Platelets/GBWT was compared between both groups which was lower in the PHG group. Furthermore, Platelets/GBWT was significantly decreased in the severe grade of PHG than in the mild group ($P < 0.001$). Similarly, GBWT was significantly higher ($P = 0.003$) with severe PHG than with mild PHG.

CUT-OFFS OF GBWT MEASUREMENTS

The cut-off in GBWT measurement varied in the published literature and this had an impact on the reported indices of diagnostic accuracy. In the study of Shehata *et al*[29], GBWT ranged from 2.5 mm to 7 mm in cirrhotic patients with EVs while in cirrhotic patients without EVs, it ranged from 1.5 mm to 5 mm. Mean GBWT of cirrhotic patients with EVs was 4.56 ± 1.08 and in cirrhotic patients without EV was 2.97 ± 0.88. They reported a cut-off value of 4 mm, hence GBWT > 4 mm is a predictor of EVs with a

Table 2 Studies focusing gallbladder wall thickness measurement and the degree of esophageal varices

Ref.	Target patients	Number of patients	GBWT cut-off	Reported sensitivity	Conclusions
Yousaf *et al* [23]	Child B and C cirrhosis	103	4 mm	Not reported	GBWT most profound in the patients with smaller (F1) and moderate (f2) esophageal varices. Most of the patients with no varices had normal gall bladder wall
Begum *et al* [26]	CLDs	61			The mean GBWT was significantly ($P < 0.05$) higher in CLD patients with grade III and IV varices (6.1 ± 0.8 mm) compared to grade I and II (3.9 ± 0.7 mm).
Elkerdawy *et al*[24]	Post-hepatitis cirrhosis with portal hypertension	105	≥ 3.1 mm	54.29%	GBWT was associated not only with the presence of EVs, but also with advanced EVs. Although, the reported sensitivity of GBWT in prediction of EVs was low, its diagnostic accuracy was comparable and even superior to some simple non-invasive predictors
Afifi *et al*[14]	Cirrhosis (Child A, B and C)	100	≥ 3.950	92%	GBWT at cut-off level ≥ 3.950 had 92% sensitivity, 95% specificity, 86.7% PPV, and 97.1% NPV for detection of large-sized EVs, with AUC = 0.986

AUC: Area under the curve; CLD: Chronic liver disease; EVs: Esophageal varices; GBWT: Gallbladder wall thickness; NPV: Negative predictive value; PPV: Positive predictive value.

sensitivity of 82%, specificity of 77%, PPV of 78%, NPV of 81% and accuracy of 79%. In the study of Khan *et al*[28], the cut-off value that discriminated variceal from non-variceal group was 4 mm. Another study by Elkerdawy *et al*[24] used 3.1 mm as a cut-off to predict the presence of EVs among cirrhotic patients of viral etiology with 54.29%, 97.14%, 97.4%, 51.5%, and 68.5% sensitivity, specificity, PPV, NPV, and diagnostic accuracy, respectively. One study focusing on adult cirrhotic patients found that GBWT had 46%, 89%, 70%, 73% sensitivity, specificity, PPV, and NPV, respectively in the prediction of EVs[12] but with higher cut-off of ≥ 4 mm. Among children and adolescents with cirrhosis at a cut-off of ≥ 4.35 mm, GBWT had a sensitivity, specificity, PPV, and NPV of 60%, 90%, 85.7%, and 69.2%, respectively, while its diagnostic accuracy was 67.5%[15]. One recent study by Afifi *et al*[14] reported GBWT at a cut-off of ≥ 3.350 mm and ≥ 3.950 mm to predict the presence of varices and to a large degree varices with reasonable sensitivities, respectively (Tables 1 and 2).

For PHG, Amer *et al*[25] showed that GBWT, with a cut-off > 3.5 mm predict PHG, with a sensitivity of 64%, specificity of 68%, PPV of 66.7%, NPV of 65.4%, AUC was 0.736, and *P* value was < 0.001. Amer *et al*[25] found that both GBWT and Platelets/GBWT were significantly associated with PHG in the univariate logistic regression analysis however both were non-significant in the multivariate analysis.

The differences of the GBWT cut-offs and the subsequent reported indices may be related to the underlying causes of cirrhosis. All cirrhotic patients in Elkerdawy *et al*[24] were of viral etiology, while only 20% of patients in Tsaknakis *et al*[12] study were of viral etiology, and none of the patients in de Alcantara *et al*[15] study were cirrhotics of viral causes. While Shehata *el al*[29] and Khan *et al*[28] did not report the underlying causes of cirrhosis, despite the high prevalence of viral hepatitis in the Egyptian and Pakistani community, respectively.

Patients in Tsaknakis *et al*[12] and the de Alcantara *et al*[15] studies were predominantly alcoholics and those with autoimmune hepatitis, respectively, while the study carried out by Pathak *et al*[21] recruited only patients with alcoholic cirrhosis. The degrees of associated hepatic fibrosis are different from those of viral hepatitis and this probably justified the lower cut-offs of the GBWT which emerged out of the viral cirrhosis studies.

GBWT in comparison to other non-invasive predictors

In many studies, GBWT measurement was compared to many non-invasive predictors of EVs. Elkerdawy *et al*[24] reported in multivariate logistic regression analysis GBWT ($P \leq 0.001$) and APRI ($P \leq 0.046$) as the independent predictors for the presence of EVs. They also reported Platelet count/Splenic diameter ratio at a cut-off level of ≤ 8.64 and predicts the presence of EVs with 61.4%, 80%, 86%, 50.9%, and 67.6% sensitivity, specificity, PPV, NPV, and the accuracy, respectively. These findings match those of Tsaknakis *et al*[12] who reported GBWT ($P < 0.04$) and platelet count ($P < 0.001$) as the independent predictors for EVs.

Other simple and easily calculated parameters for prediction of EVs, with sensitivities ranging from 60%-70% were evaluated in an Egyptian study[24] including the splenic length (cut-off 14.9 cm), PV diameter (cut-off 14.6 mm), and APRI score (cut-off 0.9). However, when these parameters were compared to GBWT, it was obvious that the GBWT measurement had the highest area under ROC curve (0.09) with the highest diagnostic accuracy (68.5%). These simple parameters were shown in different studies to predict the presence of EVs with variable sensitivities[3].

GBWT COMBINATION WITH OTHER PARAMETERS

Many authors reported improved sensitivity in prediction of varices upon combining GBWT with other non-invasive parameters. Tsaknakis *et al*[12] reported that the platelet count/GBWT ratio (cut-off > 46.2) achieves a sensitivity of 78%, a specificity of 86%, 76% PPV, 87% NPV and an AUC of 0.864 in predicting EVs. In that study, ROC analysis showed that the platelet count/GBWT ratio performed at a comparable level to the platelet count/spleen (cut-off > 909) diameter ratio.

Amer *et al*[25] reported that platelets/GBWT ratio, using a cut-off of < 40 predict PHG, with a sensitivity of 68%, specificity of 78%, PPV of 75.6%, NPV of 70.9%, AUC was 0.861 and *P* value was < 0.001, although it was significant in the univariate logistic regression analysis but was non-significant in the multivariate analysis.

LIMITATIONS

Despite the favorable results of the current studies, there are many considerations that should not be overlooked. First, the inter-observer variability. The subjective nature of sonographic assessment of GBWT can be reduced by rendering specialized experienced sonographer/radiologist/physicians rather than hepatologists who should examine the patients as demonstrated in some studies[21,24]. Optimal examination of the GB requires the patient to come fasting. This was considered in the individual studies. Fasting for 8 h was advised by Begum *et al*[26], while overnight fasting was advised by others [24,25]. Following the initial scan in the supine position, patients were turned onto the left decubitus position, as this position allows the liver and GB to medially fall away from the ribs, unfolding the GB and moving the overlying bowel away from the region of interest. GBWT was measured in its thickest portion preferably at the anterior wall[26]. In addition, some sonographic features (*e.g.*, GB wall varices) may be detected during examination in those patients especially with pre-hepatic PHT.

Secondly, the time interval. In an attempt to reduce the time effect on either the GBWT or the varices both sonography and endoscopy should be performed in the same period of time and this was considered in some studies[24,25].

Thirdly, many confounding factors may affect the GBWT, *e.g.*, ascites and hypoalbuminemia. It was clear in some studies (*e.g.*, Shehata *et al*[29]) that cases with severe hypoalbuminemia of 2.2 gm/dL were excluded. In the study of Pathak *et al*[21] cirrhotic patients with ascites and hypoalbuminemia were not excluded and as expected a correlation between GBWT, both serum albumin and ascites was observed and hence the relationship between GBWT and both PHT and EVs is questionable.

Fourthly, the relationship between GBWT and portal vein parameters (*e.g.*, diameter and flow velocity out) and the remaining parameters were not thoroughly investigated.

Lastly, liver cirrhosis is a heterogeneous group and in the current review we did not differentiate between different etiologies and grades of cirrhosis. This should trigger future studies focusing specific types of liver cirrhosis with different stages of functional decompensation.

CONCLUSION

Among cirrhotic patients with PHT of different etiologies, GBWT is associated with the presence of EVs. The cut-off of GBWT that can predict the presence of EVs varied in the literatures and ranges from 3.1 mm to 4.35 mm with variable sensitivities of 46%-90.9% with lower cut-offs in viral cirrhosis compared to non-viral. However, GBWT > 4 mm in many studies is associated with an acceptable sensitivity up to 90%. Furthermore, a relationship was also noticed with the degree of varices and PHG. Among cirrhotics, GBWT > 3.5 mm predicts the presence of advanced (grade III-IV) EVs with a sensitivity of 45%; the sensitivity increased to 92% when a cut-off ≥ 3.95 mm was used in another cohort. Analysis of these results should be carefully revised in the context of ascites, hypoalbuminemia and other intrinsic GB diseases before those cirrhotic patients are referred to endoscopy. The sensitivity for prediction of EVs improved upon combining GBWT measurement with other non-invasive predictors, *e.g.*, platelets/GBWT. Consequently, there is a need to standardize the criteria for GBWT measurement and its utility among those patients.

FOOTNOTES

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ORIGINAL ARTICLE

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Progressive changes in platelet counts and Fib-4 scores precede the diagnosis of advanced fibrosis in NASH patients

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Abstract

BACKGROUND

Cirrhosis and its complications develop in a subgroup of patients with non-alcoholic fatty liver disease (NASH). Early detection of liver fibrosis represents an important goal of clinical care.

AIM

To test the hypothesis that the development of cirrhosis in nonalcoholic fatty liver disease patients is preceded by the long-term trends of platelet counts and Fib-4 scores.

METHODS

We identified all patients in our healthcare system who had undergone fibrosis staging by liver biopsy or magnetic resonance elastography (MRE) for non-alcoholic fatty liver disease during the past decade (n = 310). Platelet counts, serum glutamic-pyruvic transaminase and serum glutamic oxalacetic transaminase values preceding the staging tests were extracted from the electronic

medical record system, and Fib-4 scores were calculated. Potential predictors of advanced fibrosis were evaluated using multivariate regression analysis.

RESULTS

Significant decreases in platelet counts and increases in Fib-4 scores were observed in all fibrosis stages, particularly in patients with cirrhosis. In the liver biopsy group, the presence of cirrhosis was best predicted by the combination of the Fib-4 score at the time closest to staging ($P < 0.0001$), the presence of diabetes ($P = 0.0001$), and the correlation coefficient of the preceding time-dependent drop in platelet count ($P = 0.044$). In the MRE group, Fib4 score ($P = 0.0025$) and platelet drop ($P = 0.0373$) were significant predictors. In comparison, the time-dependent rise of the Fib-4 score did not contribute in a statistically significant way.

CONCLUSION

Time-dependent changes in platelet counts and Fib-4 scores contribute to the prediction of cirrhosis in NASH patients with biopsy- or MRE-staged fibrosis. Their incorporation into predictive algorithms may assist in the earlier identification of high-risk patients.

Key Words: Non-alcoholic fatty liver disease; Liver fibrosis; cirrhosis; Prediction; Liver biopsy; Magnetic resonance elastography

Core Tip: Our study is based on the well-known phenomenon of declining platelet counts in patients who develop cirrhosis, including those with underlying non-alcoholic fatty liver disease (NASH). This phenomenon has resulted in several recent publications using large health registries to show that progressive changes in non-invasive fibrosis scores preceded the ICD 9-based diagnoses of cirrhosis. These studies raised the issue of "predictability" of cirrhosis development. Our analysis extends these studies by examining a smaller, well-defined NASH patient population. Unlike previous studies, we included ALL fibrosis stages, provided that patients had undergone definitive staging by liver biopsy or magnetic resonance elastography. Our data unequivocally confirm that progressive thrombocytopenia and an increase in the Fib-4 scores precedes the diagnosis of cirrhosis. Moreover, the kinetics of the platelet drop add to the prediction of cirrhosis, suggesting that the time-dependent decrease in platelet counts may have true predictive power.

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INTRODUCTION

The incidence of non-alcoholic fatty liver disease (NAFLD) induced cirrhosis and its complications are rising in the US and worldwide[1,2]. Due to its indolent clinical course, advanced fibrosis and cirrhosis may go undiagnosed for years or even decades[3]. Frequently, patients are first referred to a hepatologist when they already present with signs and symptoms of decompensation, including portal hypertension, synthetic dysfunction, or hepatocellular cancer. At such stage, patients are typically older and suffer from multisystem comorbidities[4], resulting in their ineligibility for liver transplantation or aggressive cancer treatment regimens with poor outcomes[5]. An early diagnosis of liver fibrosis would allow for appropriate disease surveillance, timely interventions for complications, and improved long-term survival[6].

Due to the advent of electronic medical record (EMR) systems, physicians can easily analyze long-term trends of patients' demographic, clinical, and laboratory data. Automated machine learning methods are being developed to predict and monitor progression of a wide range of disease states[7]. Recent reports suggest that time-dependent trends of platelet count and Fib-4 scores – extracted from the patients' electronic medical record system - might help predict the occurrence of advanced liver fibrosis and cirrhosis[8-10]. We tested our hypothesis in a long-term follow-up study of patients in our healthcare system prior to undergoing a liver biopsy or magnetic resonance elastography for staging of their non-alcoholic fatty liver disease.

MATERIALS AND METHODS

Human subjects

The study protocol (EH 21-163) conformed with the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board of NorthShore University Health System. Informed consent and HIPAA authorization requirements were waived.

Patient Identification

We searched the NorthShore EPIC patient database to identify all patients who had undergone a liver biopsy or magnetic resonance elastography for the assessment of non-alcoholic fatty liver disease during the time period between February of 2010 and October of 2020. A chart review was performed for each patient to ascertain the correct diagnosis, and to extract clinical, demographic, laboratory, liver biopsy, and magnetic resonance elastography (MRE) data. When necessary, liver biopsies were reviewed by a trained hepato-pathologist (N.J.) to determine the fibrosis stage, using the NASH Clinical Research Network criteria[11]. Samples subclassified as fibrosis stages 1A, 1B, or 1C were combined under "stage 1". The MRE measurements were performed using a Siemens Magnetom Aera 1.5T scanner. MRE liver stiffness measurements were stratified into five groups (0-2.9 kPa, 2.9-3.5 kPa, 3.5-4.0 kPa, 4.0-5.0 kPa, and >5.0 kPa), following published guidelines[12].

Data analysis

Time-dependent changes in platelet counts and Fib-4 scores were analyzed in subgroups of patients in whom data were available for a minimum time period of five years (biopsy group: n = 120 for platelet count, n = 105 for Fib-4, MRE group: n = 79 for platelet count, n = 75 for Fib-4, respectively). Representative values were calculated as the mean of all available measurements for each year. No attempts were made to replace missing data.

In the statistical analysis, liver fibrosis scores constituted the primary outcome variable. In two separate groups of patients, liver fibrosis was ranked 0 through 4 based on histopathology of liver biopsy specimens, or on numeric scores obtained through magnetic resonance elastography (MRE). The presence or absence of categorical variables, such as gender or hypertension, in patients with different fibrosis scores were compared using chi-squared tests. In the final analysis, smoking was also entered as dichotomous variable, with former and current smokers being grouped together. Differences in body mass index (BMI) or laboratory values between two patient subgroups were compared using t-tests. For each individual patient, linear regression analysis was used to calculate the correlation coefficients between passage of time and consecutive platelet counts or Fib-4 scores, respectively. Multivariable least-squares linear regression analyses were used to test the joint influence of multiple predictor variables on the occurrence of the outcome variable (liver fibrosis). The list of predictor variables included the last Fib-4 score, BMI, sex, two individual correlation coefficients associated with time-dependent changes of platelet counts or Fib-4 scores, presence or absence of diabetes mellitus, hypertension, and smoking. Patients with follow-up periods shorter than one year or with less than 3 consecutive platelet counts or Fib-4 scores were excluded from the time trend analyses.

RESULTS

Study population

A total of 317 patients were identified in the initial search of the patient data file. Seven patients assigned to the liver biopsy group were excluded from the analysis, due to inadequate tissue sampling (n = 3) or lack of procedural documentation (n = 4).

The remaining 310 patients were entered into the analysis. Between February 2010 and October 2020, 203 patients underwent liver biopsy for a diagnosis of non-alcoholic fatty liver disease. Between April 2015 and May 2021, 107 patients underwent MRE for the same indication. In 165/203 (81%) of patients with liver biopsy, the overall length of follow-up within the health system was longer than one year, with an average (SD) of 7.9 ± 3.9 years. In 94/107 (88%) of patients with MRE, the overall length of follow-up was longer than one year, with an average of 10.2 ± 4.6 years, resulting in a final combined sample size of 259.

Biopsy cohort: Patient characteristics

Patients undergoing liver biopsies were stratified by their fibrosis scores and their demographic and clinical characteristics (Table 1). Except for random fluctuations, the five subgroups did not differ with respect to gender, ethnicity, and smoking habits. Patient ages tended to increase with fibrosis stages. The frequency of diabetes mellitus and hypertension appeared to increase with rising fibrosis stage. No obvious pattern was revealed with respect to BMI, serum glutamic oxalacetic transaminase (SGOT), or serum glutamic-pyruvic transaminase (SGPT). Average platelet counts decreased, and average Fib-4 scores increased, respectively, with increasing fibrosis stages. The average correlation coefficient for the

Table 1 Characteristics of patients undergoing liver biopsy

	Fibrosis 0		Fibrosis 1		Fibrosis 2		Fibrosis 3		Fibrosis 4	
	(% or SD)		(% or SD)		(% or SD)		(% or SD)		(% or SD)	
Total (N)	36	(100)	41	(100)	27	(100)	26	(100)	73	(100)
Follow-up > 1 yr	29	(81)	32	(78)	22	(81%)	23	(88%)	59	(81%)
Follow-up (mean, yr)	7.1	(4.32)	7.3	(4.03)	8.6	(3.61)	8.4	(3.36)	8.2	(3.99)
Age (mean, yr)	44.6	(15.7)	47.6	(14.7)	55.9	(10.6)	55.6	(13.2)	58.0	(11.7)
Sex										
Male (N)	16	(44)	25	(61)	13	(48)	12	(46)	45	(62)
Female (N)	20	(56)	16	(39)	14	(52)	14	(54)	28	(38)
Ethnicity										
White (N)	23	(64)	22	(54)	15	(56)	20	(77)	54	(74)
African American (N)	0	(0)	0	(0)	1	(4)	0	(0)	0	(0)
Hispanic (N)	6	(17)	6	(15)	4	(15)	3	(12)	9	(12)
Asian American (N)	5	(14)	11	(27)	5	(19)	3	(12)	9	(12)
Other (N)	2	(6%)	2	(5)	2	(7)	0	(0)	1	(1)
Smoker										
Yes (N)	5	(14)	1	(2)	5	(19)	0	(0)	6	(8)
No (N)	26	(72)	30	(73)	16	(59)	20	(77)	38	(52)
Former (N)	5	(14)	10	(24)	6	(22)	6	(23)	29	(40)
Diabetes mellitus										
Yes (N)	8	(22)	14	(34)	12	(44)	10	(38)	42	(58)
No (N)	28	(78)	27	(66)	15	(56)	16	(62)	31	(42)
Hypertension										
Yes (N)	14	(39)	20	(49)	16	(59)	15	(58)	49	(67)
No (N)	22	(61)	21	(51)	11	(41)	11	(42)	24	(33)
BMI (last)	30	(7)	32	(7)	31	(7)	31	(7)	33	(7)
Laboratory results										
SGOT (last, IU/L)	90	(82)	78	(39)	69	(36)	88	(51)	80	(70)
SGPT (last, IU/L)	126	(86)	124	(57)	89	(52)	113	(87)	79	(68)
Platelets (last, 1000/mL)	259	(87)	242	(64)	229	(75)	206	(73)	167	(65)
Fib-4 score (last)	1.7	(1.8)	1.7	(1.1)	2.4	(2.4)	2.5	(1.1)	3.7	(2.8)
Correlation-plts (mean)	-0.10	(0.48)	-0.22	(0.66)	-0.31	(0.61)	-0.18	(0.52)	-0.56	(0.42)
Correlation-Fib-4 (mean)	0.43	(0.50)	0.36	(0.46)	0.49	(0.45)	0.42	(0.47)	0.43	(0.45)

SGOT: Serum glutamic oxalacetic transaminase; SGPT: Serum glutamic-pyruvic transaminase.

time dependent changes in platelet counts of individual patients was negative in all five subgroups, consistent with an overall drop in the platelet counts. In absolute terms, this drop was most pronounced in patients with stage 4 fibrosis. The average correlation coefficient for the time-dependent changes in Fib-4 scores was positive in all five subgroups, consistent with an overall rise in the Fib-4 scores.

MRE cohort: Patient characteristics

Patient undergoing fibrosis staging by MRE were stratified by their liver stiffness and demographic and clinical characteristics (Table 2). No obvious pattern was discernible among the five subgroups with respect to their demographic and clinical characteristics. The average platelet counts decreased, and the average Fib-4 scores increased with increasing liver stiffness scores. The average correlation coefficient

Table 2 Characteristics of patients undergoing magnetic resonance elastography

	MRE 0-2.9		MRE 2.9-3.5		MRE 3.5-4		MRE 4-5		MRE 5+	
	(% or SD)		(% or SD)		(% or SD)		(% or SD)		(% or SD)	
Total (N)	48	(100%)	15	(100%)	6	(100%)	13	(100%)	24	(100%)
Follow-up > 1 yr (N)	42	(88%)	12	(80%)	6	(100%)	10	(77%)	23	(96%)
Follow-up (mean, yr)	9.8	(4.87)	9.4	(4.87)	12.4	(4.87)	10.7	(4.87)	10.6	(4.87)
Age (mean, yr)	57.8	(15.4)	61.6	(13.5)	63.0	(9.0)	61.5	(8.3)	60.6	(13.4)
Sex										
Male (N)	17	(35%)	5	(33%)	5	(83%)	6	(46%)	11	(46%)
Female (N)	31	(65%)	10	(67%)	1	(17%)	7	(54%)	13	(54%)
Ethnicity										
White (N)	30	(63%)	8	(53%)	4	(67%)	10	(77%)	15	(63%)
African American (N)	2	(4%)	0	(0%)	0	(0%)	0	(0%)	1	(4%)
Hispanic (N)	6	(13)	3	(20)	1	(17)	1	(8)	5	(21)
Asian American (N)	9	(19)	1	(7)	0	(0)	1	(8)	1	(4)
Other (N)	1	(2)	3	(20)	1	(17)	1	(8)	2	(8)
Smoker										
Yes (N)	2	(4)	1	(7)	0	(0)	1	(8)	1	(4)
No (N)	28	(58)	11	(73)	6	(100)	5	(38)	12	(50)
Former (N)	18	(38)	3	(20)	0	(0)	7	(54)	11	(46)
Diabetes mellitus										
Yes (N)	14	(29)	9	(60)	4	(67)	9	(69)	15	(63)
No (N)	34	(71)	6	(40)	2	(33)	4	(31)	9	(38)
Hypertension										
Yes (N)	29	(60)	12	(80)	5	(83)	8	(62)	16	(67)
No (N)	19	(40)	3	(20)	1	(17)	5	(38)	8	(33)
BMI (mean, last)	33	(7)	32	(8)	38	(7)	35	(6)	33	(6)
Laboratory results										
SGOT (last, IU/L)	36	(18)	52	(41)	63	(32)	91	(92)	60	(40)
SGPT (last, IU/L)	48	(29)	57	(48)	79	(34)	101	(88)	59	(43)
Platelets (last, 1000/mL)	244	(60)	231	(62)	205	(39)	201	(74)	182	(75)
Fib-4 score (last)	1.4	(0.9)	1.8	(1.0)	2.6	(1.5)	2.6	(1.1)	3.5	(2.0)
MRE fibrosis score (mean)	2.5	(0.3)	3.2	(0.2)	3.7	(0.1)	4.3	(0.2)	8.2	(3.5)
Correlation-plts (mean)	-0.06	(0.43)	-0.06	(0.43)	-0.28	(0.45)	-0.44	(0.33)	-0.64	(0.35)
Correlation-Fib-4 (mean)	0.44	(0.43)	0.44	(0.43)	0.66	(0.19)	0.61	(0.35)	0.68	(0.32)

BMI: Body mass index; MRE: Magnetic resonance elastography; SGOT: Serum glutamic oxalacetic transaminase; SGPT: Serum glutamic-pyruvic transaminase.

for the time dependent changes in platelet counts of individual patients was negative in all five subgroups, consistent with an overall drop in the platelet counts. In absolute terms, this drop became more pronounced with increasing MRE fibrosis scores. The average correlation coefficient for the time-dependent changes in Fib-4 scores was positive in all five subgroups, indicating an overall rise in the Fib-4 scores. The rise magnitude of the rise was more pronounced in the subgroups with high than low MRE fibrosis scores.

Illustrative individual patient data

We identified two patients (one from each cohort, respectively) for whom longitudinal data were available over a 20-year time period. In both patients alike, platelet counts started to fall, and Fib-4 scores started to rise several years prior to the clinical diagnosis of cirrhosis (Figure 1).

Time-dependent changes in platelet counts and Fib-4 scores: Cohort averages

The temporal changes of platelet counts and Fib-4 scores according to biopsy- or MRE-determined fibrosis stages were analyzed for the ten-year time period prior to staging. Progressive decreases in platelet counts and increases in Fib-4-scores were apparent in patients with the biopsy-documented stage 4 fibrosis or a liver stiffness of > 5.0 kPa on MRE, respectively (Figure 2).

Time-dependent changes in platelet counts and Fib-4 scores: Stage 0 vs Stage 4 fibrosis by liver biopsy

We analyzed the time trends of platelet counts and Fib-4 sores in patients with biopsy-confirmed stage 0 or stage 4 fibrosis who had been followed for a minimum of one year (Figure 3). The overall trends show no significant correlations in stage 0 liver fibrosis, as compared to a significant time-dependent decline of platelet counts in stage 4 liver fibrosis, as well as a significant time-dependent rise of Fib-4 scores. The correlation coefficients shown in the graph vary from those shown in Table 1, because they were based on all patient data analyzed jointly in a single regression analysis, whereas the average values in Table 1 were calculated as an average of many individual correlation coefficients. Based on the varying time intervals of pre-staging testing and the inter-patient variability in time-dependent increases or decreases, the trends of individual patients may mask or cancel each other out in the joint analysis. The results for patients with stage 2 or 3 were similar (data not shown). Their individual data fell within the range outlined by the two extremes shown in Figure 2, as the slopes of the two regression lines for platelet counts and Fib-4 scores tended to become increasingly steeper with increasing fibrosis stage. This pattern supports the conclusion that the occurrence of liver fibrosis was preceded by a long-term gradual fall in platelet counts.

Time-dependent changes in platelet counts and Fib-4 scores: Stage 0 vs Stage 4 fibrosis by MRE

We analyzed the time trends of platelet counts and Fib-4 sores in patients with MRE-determined fibrosis stage 0 (0-2.9 kPa) or stage 4 (> 5.0 kPa) who had been followed for a minimum of one year (Figure 4). The overall trends show no significant correlations associated with fibrosis stage 0, as opposed to a significant decline of platelet counts and a significant rise of Fib-4 scores over time among patients with fibrosis stage 4. The caveats stated above with respect to Figure 3 also apply to Figure 4. Similar results were obtained for MRE fibrosis scores between 2.9 and 5.0 (data not shown). The regression patterns for these patients fell within the range outlined by the two extremes in Figure 4, as the slopes of the two regression lines for platelet counts and Fib-4 scores tended to become increasingly steeper with increasing MRE scores. Similar to the data obtained in the liver biopsy cohort, this pattern supports the impression that in patients with elevated MRE fibrosis scores the occurrence of fibrosis was preceded by a long-term gradual fall in platelet counts.

Multivariable regression analysis for liver biopsy and MRE cohorts

Table 3 contains the results of two separate multivariable regression analyses. Due to the exclusion of patients in whom pre-staging data were available for a time period of less than one year, only 146 and 89 patients were included in the two separate analyses. The outcome variables were fibrosis stage on biopsy or MRE score, respectively. The same set of predictor variables was used in both analyses. In both analyses, the last Fib-4 value and the correlation coefficient of the time-dependent drop in platelet counts contributed in a statistically significant fashion to the overall prediction of cirrhosis. In patients who underwent liver biopsy, the presence of diabetes mellitus was an additional independent and significant predictor. This result supports the hypothesis that a progressive long-term drop in platelet is a feature of progressive liver fibrosis.

In a separate set of multivariable regression analyses, instead of the correlation coefficient associated with platelet counts, the correlation coefficient of the time-dependent rise in the Fib-4 scores was used as predictor variable. The calculation of the Fib-4 requires the simultaneous measurement of SGOT, SGPT, and platelet counts[13]. Because of the requirement for three simultaneous laboratory tests, fewer time points were available for this analysis. Overall, the correlation coefficient of the time-dependent rise in Fib-4 scores failed to function as an independently significant predictor for liver fibrosis in the two patient populations (data not shown).

DISCUSSION

The results of the present illustrate the long-term nature and gradual decline in platelet counts among patients with non-alcoholic fatty liver disease, who subsequently become diagnosed with liver fibrosis

Table 3 Results of least-squares multivariable regression analyses

Predicator variable	Estimate	Std error	*t* value	Prob >|*t*|
Outcome: Fibrosis score on biopsy				
Fib-4 (last score)	0.214	0.048	4.43	**< 0.0001**
Correlation-platelets	-0.461	0.227	-2.03	**0.044**
Diabetes mellitus	-0.436	0.111	-3.94	**0.0001**
Hypertension	-0.181	0.112	-1.63	0.1062
BMI	0.02	0.016	1.28	0.2016
Smoking	-0.173	0.113	-1.53	0.1275
Sex	-0.028	0.11	-0.26	0.7988
R^2 = 0.35, N = 146, P < 0.0001				
Outcome: Fibrosis score on MRE				
Fib-4 (last score)	0.746	0.239	3.12	**0.0025**
Correlation-platelets	-1.582	0.747	-2.12	**0.0373**
Diabetes mellitus	-0.301	0.365	-0.82	0.4133
Hypertension	0.441	0.395	1.12	0.2674
BMI	-0.019	0.039	-0.47	0.6368
Smoking	0.226	0.333	0.68	0.5001
Sex	0.016	0.36	0.04	0.9647
R^2 = 0.24, N = 89, P < 0.002				

BMI: Body mass index; MRE: Magnetic resonance elastography.

or cirrhosis. Such decline in platelet count is also associated with a gradual rise of the Fib-4 scores in the same patient population. The consistency of these patterns is confirmed by their similar occurrence among the two separate subgroups of patients included in the present analysis, that is, those who were diagnosed by liver biopsy *vs* magnetic resonance elastography. In addition to the last Fib-4 score preceding the ultimate diagnosis of liver fibrosis, the preceding decline in platelet count itself was also an independent and statistically significant predictor for the occurrence of advanced liver fibrosis.

The development of thrombocytopenia in patients with chronic liver disease – regardless of its underlying disease etiology – is well known to hepatologists. Several mechanisms have been implicated for this phenomenon, including portal hypertension, and decreases in hepatic thrombopoietin production[14]. The occurrence of progressive thrombocytopenia in NASH patients had previously been described by Liu *et al*[15] in a community-based, cross-sectional study. Interestingly, the authors did not attempt to relate this finding to the patients' disease stage. The first explicit connection between progressive thrombocytopenia and cirrhosis was recently described in a study by Gotlieb *et al*[9]. Using a large computerized data base, the authors identified 5300 cases with an EMR diagnosis of liver cirrhosis of any etiology and compared them to 15700 control subjects. In their retrospective analysis, they noted a significant and progressive drop in platelet counts from 240000 to 190000 up to 15 years prior to diagnosis. The drop became detectable at a time when the patient's platelet counts were still within normal range, preceding the occurrence of abnormal laboratory tests by several years. In a multivariate analysis, the odds of cirrhosis increased 1.3 times for every decrease in platelet counts by 50K. The authors suggested that it might be possible to predict the development of advanced fibrosis or cirrhosis at a much earlier preclinical stage. Similar data were recently reported by Hagstrom and colleagues in a large-scale analysis of a Swedish health care registry[16]. The authors examined the ability of several serum-based fibrosis scoring systems to predict the development of significant liver disease and its complications, as defined by ICD-9 coding. Their analysis included a predefined NAFLD group. In general, high scores (including Fib-4[10]) were associated with an increased risk of cirrhosis development over time, although the positive predictive values were modest.

Our results confirm these previous findings, although our analysis differed in several respects. Our study was smaller in scope as we confined our analysis to a single regional healthcare system. We specifically focused on non-alcoholic fatty liver disease in order to avoid possible confounding effects of different disease etiologies and their potentially unique rates of disease progression. More importantly, we included all fibrosis stages in order to evaluate the specificity of the platelet count changes for

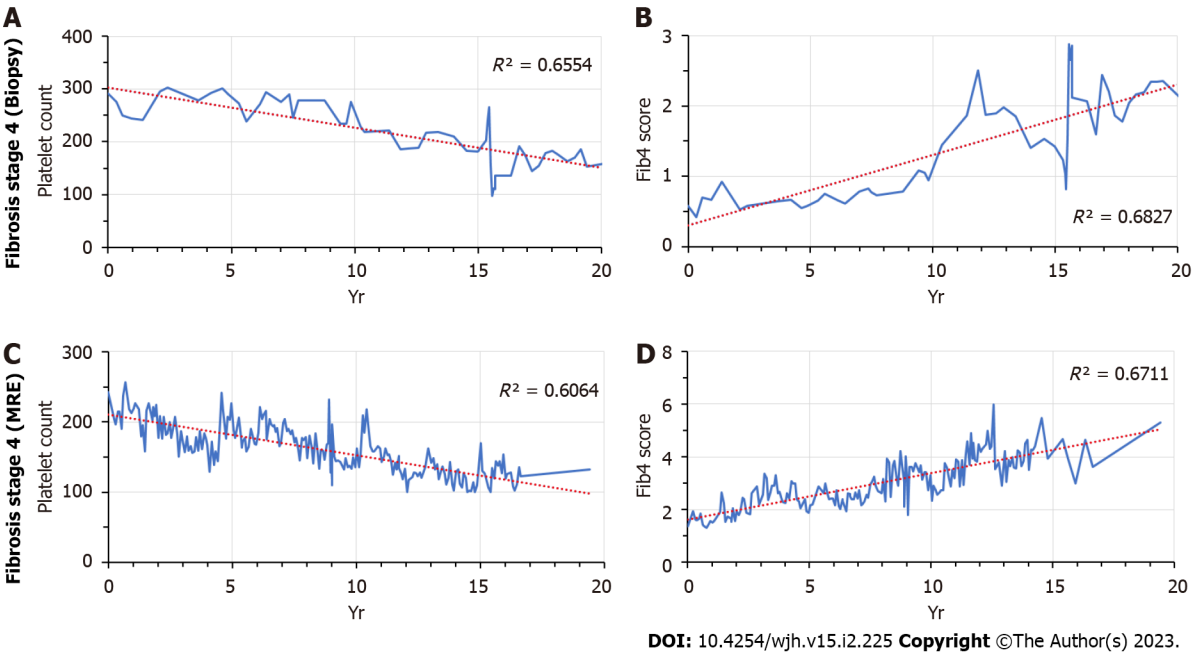

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Figure 1 Long-term changes in platelet counts and Fib4 scores prior to the establishment of cirrhosis. A: Patient 1, platelet counts; B: Patient 1, Fib4 scores; C: Patient 2, platelet counts; D: Patient 2, Fib4 scores. MRE: Magnetic resonance elastography.

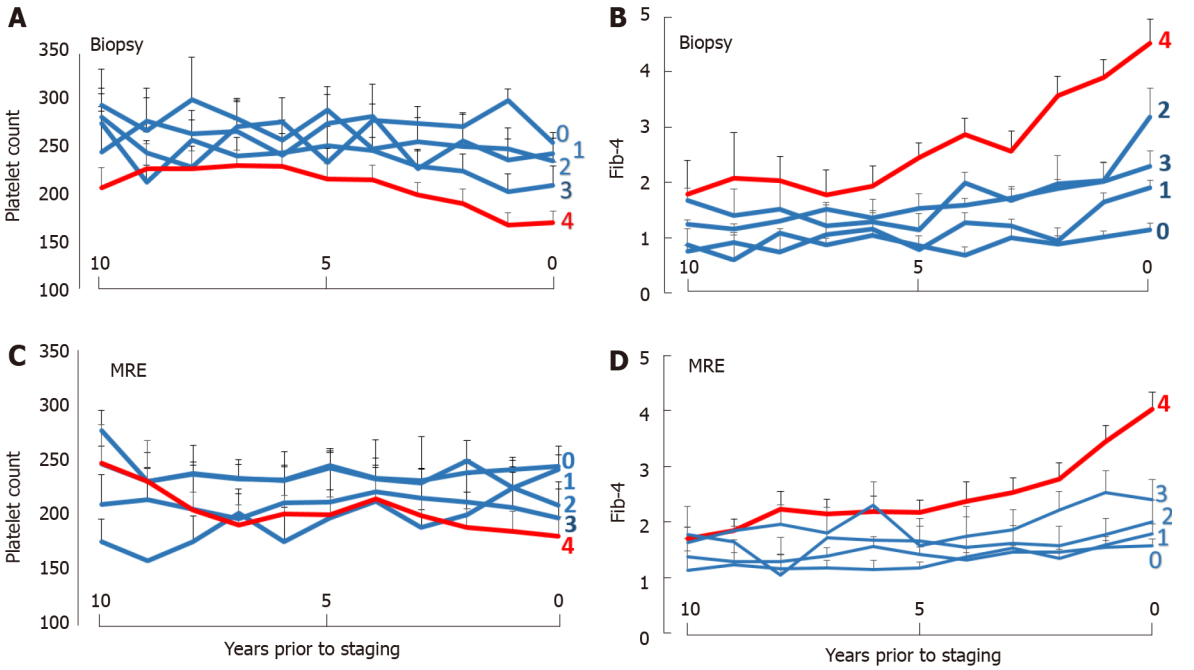

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Figure 2 Time-dependent changes in platelet counts and Fib-4 scores: Cohort averages. A: Biopsy cohort, platelet counts; B: Biopsy cohort, Fib4 scores; C: Magnetic resonance elastography (MRE) cohort, platelet counts; D: MRE cohort, Fib4 scores. Data are shown as mean + standard error. Fibrosis stages 0–4 are marked by the corresponding numerals. For magnetic resonance elastography data, "0" corresponds to a liver stiffness of < 2.9 kPa, "1" to 2.9-3.5 kPa, "2" to 3.5-4.0 kPa, "3" to 40–5.0 kPa, and "4" to > 5.0 kPa. MRE: Magnetic resonance elastography.

"advanced fibrosis", and we only included patients in whom the fibrosis stage had been determined by liver biopsy or MR elastography, the two modalities with the best performance characteristics.

Our analysis demonstrates a significant drop in platelet counts and corresponding rise in Fib-4 scores for all patient groups, including those without or with early-stage fibrosis. This might be partly due to the previously described age-dependent reduction in platelet counts[17] and SGPT[18], or to the onset of fibrogenesis during the early stages of the disease. The most pronounced changes occurred in patients with the highest fibrosis stages, suggesting an acceleration of the underlying disease process.

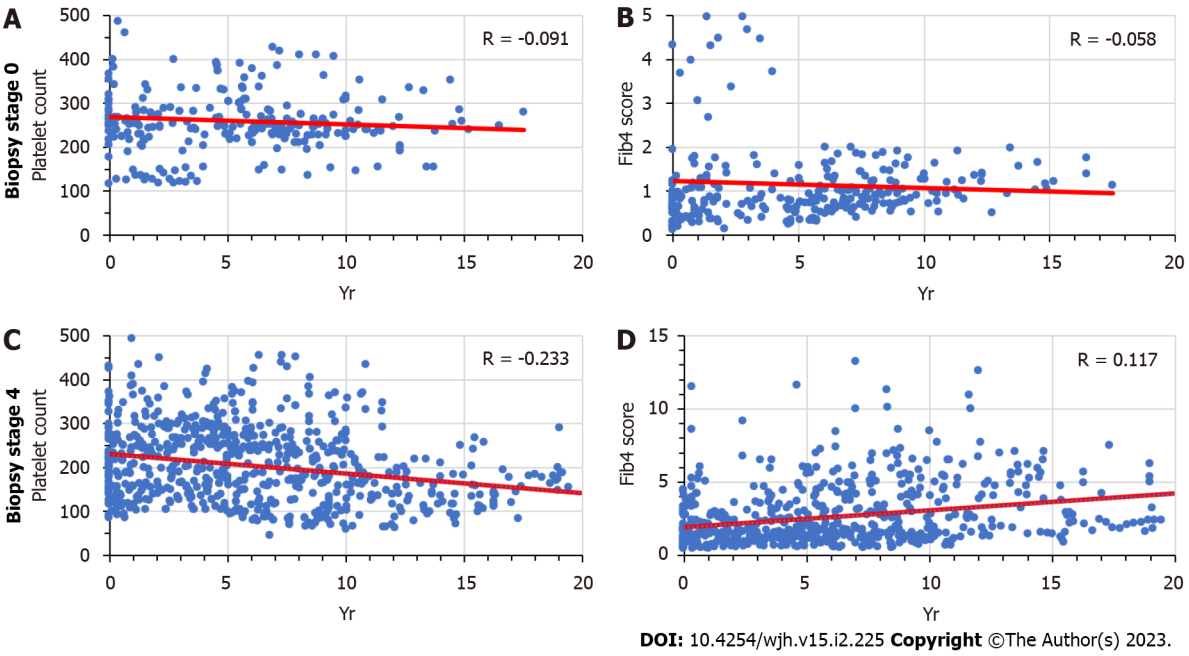

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Figure 3 Time-dependent changes in platelet counts and Fib-4 scores: Stage 0 *vs* stage 4 fibrosis by liver biopsy. A: Stage 0 fibrosis, platelet counts; B: Stage 0 fibrosis, Fib4 scores; C: Stage 4 fibrosis, platelet counts; D: Stage 4 fibrosis, Fib4 scores.

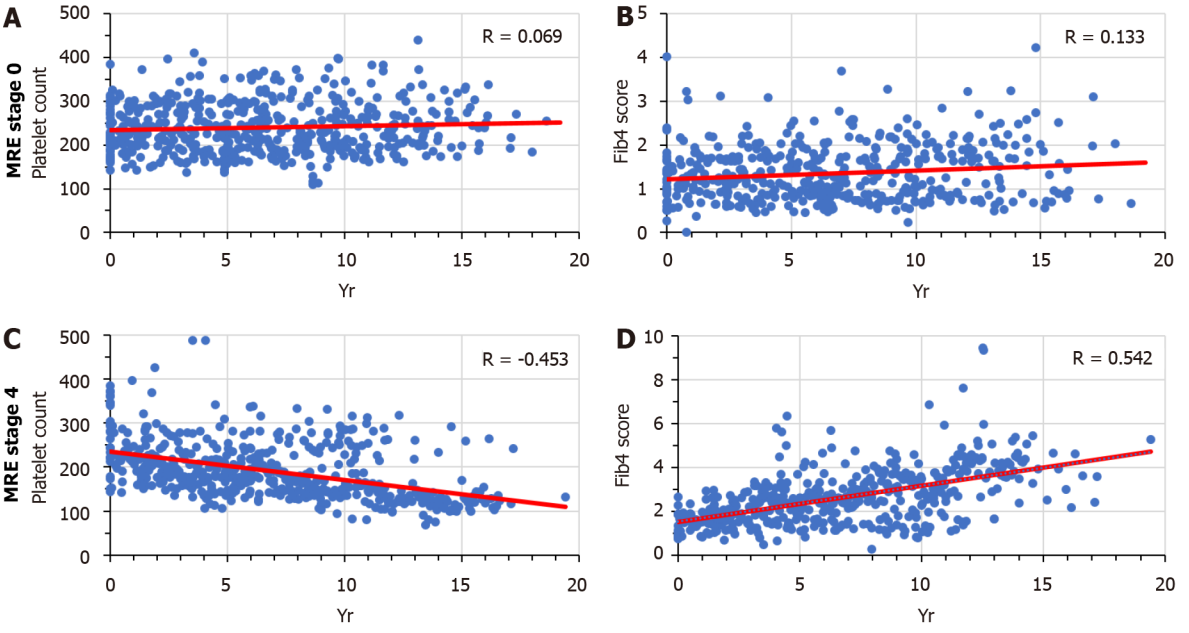

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Figure 4 Time-Dependent changes in platelet counts and Fib-4 scores: stage 0 *vs* stage 4 fibrosis by magnetic resonance elastography. A: Stage 0 fibrosis, platelet counts; B: Stage 0 fibrosis, Fib4 scores; C: Stage 4 fibrosis, platelet counts; D: Stage 4 fibrosis, Fib4 scores. MRE: Magnetic resonance elastography.

In multivariate analyses separately performed for the biopsy and MRE cohorts, the last Fib-4 value prior to staging and the correlation coefficient for the time-dependent drop in platelet counts were both predictive for the severity of liver fibrosis. In addition, a significant association with a diagnosis of diabetes was observed, as previously reported by others[4]. In contrast, the inclusion of the correlation coefficients for the time-dependent increase in Fib-4 scores did not significantly contribute to the overall regression model. Besides the platelet count, serum levels of SGOT and SGPT also serve as separate variables in the formula for calculating the Fib-4 index. Because of the requirement for three simultaneously measured laboratory values, overall, fewer time points could be generated for the Fib-4 scores than for the platelet counts in individual patients. This might explain the lack of statistical significance for the Fib-4 scores. None of the other variables (hypertension, BMI, smoking, or sex) were statistically

associated with advanced fibrosis, regardless of how the fibrosis stage was diagnosed.

The diagnostic utility of low platelet counts or increased Fib-4 scores for the diagnosis of advanced fibrosis has been extensively examined by multiple studies. The main strength of these measurements lies in their excellent negative predictive value, whereas their positive predictive value has generally been less impressive[4]. Because of these performance characteristics, secondary follow-up testing with elastography or liver biopsy is necessary for patients with abnormal Fib-4 scores.

In addition to confirming their diagnostic utility, our data indicate that the dynamic changes in serum fibrosis markers may also harbor some prognostic power. As shown by the two exemplary case patients of Figure 1, a steadily progressive decline in platelet counts may precede the formal staging test by 20 years or more. The consistency of such decline was captured by the magnitude of the correlation coefficient and its impact on predicting the final outcome. In this regard, the statistical analysis supported the underlying hypothesis of the study generated from inspecting the data of individual patients.

A review of trends in all individual patients revealed a high degree of variability within the patient population. In our study, we included all available laboratory data, regardless of the patient's in- or outpatient status, comorbid disease conditions, surgical or other interventions, and medications. Such confounding factors may have contributed to the noticeable fluctuation in our data. However, our approach facilitated automatic data extraction and lends itself to be tested (and utilized) in future prospective studies among high-risk populations. In the future, a potential limitation of the long-term platelet counts may arise from to their overall weaker statistical impact. As shown in Table 3, the significance level for the correlation coefficient of the declining platelet counts was markedly lower than that of the "last Fib-4" or presence of diabetes mellitus.

Eventually, prospective clinical studies in carefully defined populations of patients with non-alcoholic fatty liver disease will be necessary to determine whether the inclusion of "platelet dynamics" can contribute to the prediction of advanced fibrosis. Such studies could be performed in the primary care setting by extracting EMR data from at-risk patients, followed by subsequent fibrosis staging through MRE or liver biopsy.

CONCLUSION

Time-dependent changes in platelet counts and Fib-4 scores contribute to the prediction of cirrhosis in NASH patients with biopsy- or MRE-staged fibrosis. Their incorporation into predictive algorithms may assist in the earlier identification of high-risk patients.

ARTICLE HIGHLIGHTS

Research background

Our study was prompted by the growing public health challenges of non-alcoholic fatty liver disease. One of the key issues is the early detection of significant liver fibrosis. Our study focuses on the use of non-invasive predictors of cirrhosis, using data that can be easily extracted from patients' medical records.

Research motivation

We focused our attention on longitudinal changes in platelet count and Fib-4 scores that precede the development of progressive liver fibrosis. Our results suggest that such changes become apparent several years prior to the establishment of a clinical diagnosis of cirrhosis. Future research should include the prospective evaluation of our predictive algorithms.

Research objectives

The main objective was to determine whether longitudinal changes in platelet count and Fib-4 scores precede the establishment of cirrhosis by liver biopsy or magnetic resonance elastography (MRE). We found that such changes occur, regardless of the method of fibrosis staging. Our results suggest that longitudinal analyses such as those described in our study may have clinical utility for earlier disease detection.

Research methods

We extracted clinical and demographic data from the patients' electronic medical records, and related them to the biopsy- or MRE-documented fibrosis stages. Regression and multivariable analyses were performed to detect significant trends related to fibrosis stage.

Research results

We found that the time-dependent changes in platelet counts and Fib4-scores are correlated with successive fibrosis stages. Our data contribute to the growing field of non-invasive fibrosis prediction in chronic liver disease.

Research conclusions

Our study suggests that longterm, progressive changes in platelet counts and Fib-4 scores occur during the progression of non-alcoholic fatty liver disease (NASH)-related fibrosis. Our results could be incorporated into new predictive algorithms, which in turn would need to be validated prospectively. If successful, our approach might lead to a significantly earlier diagnosis of advanced liver fibrosis in NASH patients.

Research perspectives

The next step in our work will be the prospective evaluation of platelet count and Fib-4 changes to detect stage 4 fibrosis prior to the clinical establishment of the diagnosis.

FOOTNOTES

Author contributions: Zijlstra MK, Gampa A, and Fimmel CJ performed the data extraction and chart reviews; Joseph N performed the pathology reviews, and Sonnenberg A performed the statistical analysis; All authors contributed to the writing of the manuscript; All authors have read and approved the final manuscript.

Institutional review board statement: The study protocol (EH 21-163) conformed with the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board of NorthShore University Health System. Informed consent and HIPAA authorization requirements were waived.

Clinical trial registration statement: Since our analysis was retrospective and de-identified, our study did not meet the criteria for clinical trial registration.

Informed consent statement: The study protocol (EH 21-163) conformed with the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board of NorthShore University Health System. Informed consent and HIPAA authorization requirements were waived.

Conflict-of-interest statement: All authors have no conflict of interest to report.

Data sharing statement: The authors agree to share their original data upon request.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Baseline hepatocyte ballooning is a risk factor for adverse events in patients with chronic hepatitis B complicated with nonalcoholic fatty liver disease

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Abstract

BACKGROUND

Although many studies have investigated the impact of chronic hepatitis B virus (HBV) infection and nonalcoholic fatty liver disease (NAFLD) on liver disease, few have investigated the relationship between nonalcoholic steatohepatitis (NASH) defined by liver pathology and the prognosis of chronic HBV infection. Most patients were followed up for a short time. This study aimed to further explore the impact of NAFLD and the pathological changes confirmed by liver pathology in patients with chronic HBV infection.

AIM

To study the effect of NAFLD confirmed using liver pathology on the outcomes of long-term serious adverse events [cirrhosis, hepatocellular carcinoma (HCC), and death] in patients with chronic hepatitis B (CHB) virus infection.

METHODS

We enrolled patients with chronic hepatitis B virus (HBV) infection who underwent liver biopsy at the Third People's Hospital of Zhenjaing Affiliated Jiangsu University between January 2005 and September 2020. Baseline clinical and pathological data on liver pathology and clinical data at the end of follow-up were collected. Propensity score matching (PSM) was used to balance baseline parameters, Kaplan-Meier (K-M) survival analysis was used to evaluate the risk of clinical events, and Cox regression was used to analyze the risk factors of events.

RESULTS

Overall, 456 patients with chronic HBV infection were included in the study, of whom 152 (33.3%) had histologically confirmed NAFLD. The median follow-up time of the entire cohort was 70.5 mo. Thirty-four patients developed cirrhosis, which was diagnosed using ultrasound during the follow-up period. K-M

survival analysis showed that NAFLD was not significantly associated with the risk of cirrhosis (log-rank test, $P > 0.05$). Patients with CHB with fibrosis at baseline were more prone to cirrhosis (log-rank test, $P = 0.046$). After PSM, multivariate analysis showed that diabetes mellitus, ballooning deformation (BD), and platelet (PLT) were independent risk factors for cirrhosis diagnosed using ultrasound ($P < 0.05$). A total of 10 patients (2.2%) developed HCC, and six of these patients were in the combined NAFLD group. K-M survival analysis showed that the cumulative risk of HCC in the NAFLD group was significantly higher (log-rank test, $P < 0.05$). Hepatocyte ballooning, and severe liver fibrosis were also associated with an increased risk of HCC (log-rank test, all $P < 0.05$). Cox multivariate analysis revealed that hepatocyte ballooning, liver fibrosis, and diabetes mellitus were independent risk factors for HCC.

CONCLUSION

There was no significant correlation between chronic HBV infection and the risk of cirrhosis in patients with NAFLD. Diabetes mellitus, BD, and PLT were independent risk factors for liver cirrhosis. Patients with chronic HBV infection and NASH have an increased risk of HCC. BD, liver fibrosis, and diabetes mellitus are independent risk factors for HCC.

Key Words: Nonalcoholic fatty liver disease; Steatohepatitis; Chronic hepatitis B virus infection; Hepatocellular carcinoma; Cirrhosis

Core Tip: A total of 456 patients with chronic hepatitis B virus infection were included in the study, of whom 152 (33.3%) had histologically confirmed nonalcoholic fatty liver disease (NAFLD). The median follow-up time of the entire cohort was 70.5 mo. Kaplan-Meier (K-M) survival analysis showed that NAFLD was not significantly associated with the risk of cirrhosis. Patients with chronic hepatitis B with fibrosis at baseline were more prone to cirrhosis. After PSM, multivariate analysis showed that diabetes mellitus, ballooning deformation, and platelet were independent risk factors for cirrhosis. A total of 10 patients (2.2%) developed hepatocellular carcinoma (HCC). K-M survival analysis showed that the cumulative risk of HCC in the NAFLD group was significantly higher. Cox multivariate analysis revealed that hepatocyte ballooning, liver fibrosis, and diabetes mellitus were independent risk factors for HCC.

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INTRODUCTION

Chronic hepatitis B (CHB) virus infection and nonalcoholic fatty liver disease (NAFLD) are important causes of liver-related complications and death. With the increasing prevalence of NAFLD, the number of patients with combined NAFLD and hepatitis B virus (HBV) infections is also on the increase. In Asia, the prevalence of NAFLD in patients with hepatitis B virus infection is approximately 14%-67%, which is not different from the data of western countries[1,2]. In recent years, there have been many studies on hepatitis B complicated with NAFLD; however, the interaction between these two diseases is still elusive.

It is understandable that in the case of combined NAFLD, the overall prognosis of these patients seems to be worse. Both NAFLD and CHB can aggravate liver injury and increase the risk of cirrhosis and liver cancer[3-7]. Recently, a cohort study evaluated the FibroScan liver transient elastography results of 459 hepatitis B e antigen (HBeAg)-negative patients over a 10-year period, and found that hepatic steatosis in patients with CHB was associated with the progression of fibrosis[8]. Based on FibroScan examination, a study of 1202 patients with CHB found that the proportion of patients with moderate to severe fibrosis among patients with severe steatosis was significantly higher than that in patients with mild or moderate steatosis (23.2% *vs* 12.6%)[9,10]. A retrospective cohort study of 270 patients with CHB showed that liver steatosis confirmed by biopsy was an independent risk factor for hepatocellular carcinoma (HCC) in patients with CHB[5]. Another large multicenter multi-ethnic cohort study of 1089 patients with CHB showed that liver steatosis confirmed by biopsy was not significantly associated with clinical outcomes (HCC and death).

Although many studies have investigated the impact of chronic HBV infection and NAFLD on liver disease, few have investigated the relationship between nonalcoholic steatohepatitis (NASH) defined by liver pathology and the prognosis of chronic HBV infection. Most patients were followed up for a short time. This study aimed to further explore the impact of NAFLD and the pathological changes confirmed by liver pathology in patients with chronic HBV infection.

MATERIALS AND METHODS

Research objective

All patients with chronic HBV infection who underwent liver biopsy at The Third Hospital of Zhenjiang Affiliated Jiangsu University from January 2005 and September 2020 were selected. Chronic HBV infection was defined as continuous positive serum hepatitis B surface antigen (HBsAg) or HBV DNA results for more than 6 mo. The inclusion criterion was a follow-up time greater than 6 mo. The exclusion criteria were a history of excessive alcohol consumption (defined as alcohol intake ≥ 20 g/day for men and ≥ 10 g/day for women)[11], history of schistosomiasis of the liver, autoimmune hepatitis, primary biliary cirrhosis, malignancy, immunodeficiency virus infection, viral hepatitis C or D, long-term use of drugs that can cause hepatic steatosis (amiodarone, sodium valproate, tamoxifen, dexamethasone, or methotrexate), and incomplete clinical data. This study was approved by the ethics committee of The Third People's Hospital Affiliated to Zhenjiang, Jiangsu University. It was registered in the Chinese Clinical Trial Registry (No: chictr2200060304).

Data acquisition

The demographic data of patients (sex, age, height, and weight) were collected along with their clinical history (diabetes mellitus, hypertension, drug use history, drinking history); antiviral treatment; blood routine, biochemistry, and serological examination of hepatitis B pathogen. Other data collected were tumor index results during liver biopsy, including total bilirubin, albumin, prealbumin (PB), alanine aminotransferase (ALT), aspartate aminotransferase, γ-glutamyl transferase, alkaline phosphatase (ALP), fasting blood glucose (GLU), total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein, platelets (PLT), HBsAg, HBeAg, HBV DNA level, alpha fetoprotein (AFP), other types of viral hepatitis indicators, human immunodeficiency virus antibody, and autoantibody test results. Body mass index (BMI) was calculated using each patient's height and weight with the following formula: BMI = weight (kg)/height $(m)^2$.

The calculation results were graded according to the Asian standard[12], in which overweight and obesity were defined as BMI ≥ 23 kg/m^2 and ≥ 25 kg/m^2, respectively. The detection limit of HBV-DNA was 500 IU/mL.

Pathological evaluation

All liver specimens were evaluated by experienced pathologists and scored according to the nonalcoholic steatohepatitis clinical research network[13] for hepatic steatosis (0-3), lobular inflammation (0-2), portal inflammation (0-3), and ballooning degeneration (0-1). The degree of fibrosis was divided into F0-4 stages according to the METAVIR evaluation system, and the F4 stage was defined as cirrhosis[14]. NAFLD occurs when more than 5% of hepatocytes with steatosis are present in a specimen. The activity score (NAS) of nonalcoholic fatty liver disease was calculated according to the scores of steatosis, lobular inflammation, and ballooning degeneration. NAS ≥ 5 indicates the presence of NASH[13].

Follow up and clinical outcome judgment

The start time of follow-up was the date when the patient underwent liver biopsy. The follow-up endpoint was the date of the last follow-up or the date of the occurrence of clinical outcomes (cirrhosis, HCC, or death). Follow-up of the entire cohort ended in August 2021. The electronic medical record was consulted to obtain the date of the last follow-up, test results (blood routine, HBV pathogen serology, liver function, blood lipids, AFP), and imaging results. The treatment of patients with an interval of more than 6 mo between the last follow-up date and the research deadline (telephone follow-up, regular follow-up) is recommended, and the clinical outcome of those who do not wish to follow up is determined according to the last follow-up record. If there was an out-of-hospital follow-up, the out-of-hospital examination results were obtained, and if there was a death, the time and cause of death were obtained. Those who did not have contact information or could not be contacted were regarded as being lost to follow-up.

The diagnosis of cirrhosis was made by experienced sonographers according to the ultrasound diagnostic criteria of cirrhosis, and computed tomography and magnetic resonance imaging were applied when necessary.

The diagnosis of HCC was based on histological or imaging findings, and the latter was positive lesions detected by at least two imaging techniques (ultrasound, computed tomography, or magnetic

resonance imaging), or the use of imaging technology combined with AFP > 400 ng/mL.

Statistical analysis

The data were analyzed using SPSS version 22.0 (SPSS Inc., Chicago, IL, United States). Continuous variables are expressed as mean ± SD or median (interquartile range), and categorical variables are expressed as percentages. *T*-test was applied when continuous variables were normally distributed, and the Mann–Whitney *U* test was used when they were non-normally distributed. Categorical variables were analyzed using the chi-squared test. Kaplan-Meier (K-M) survival analysis was used to evaluate clinical events, and Cox proportional hazards regression was applied for univariate and multivariate analyses. This study also used 1:1 propensity score matching (PSM) to match the NAFLD and non-NAFLD groups, and the caliper value was set to 0.01. All tests were two-tailed, and statistical significance was set at $P < 0.05$.

RESULTS

Study population

A total of 981 patients with chronic HBV infection underwent liver biopsy at The Third People's Hospital of Zhenjiang between January 2005 and September 2020. After screening based on the inclusion and exclusion criteria, 456 patients were included in the final study, 67 of whom had histologically confirmed cirrhosis at baseline. Figure 1 shows the specific process.

Baseline features

Basic information of the general population: The total number of study patients was 456; of these patients, 152 (33.3%) had histologically confirmed NAFLD. The median follow-up time of the entire cohort was 70.5 mo. The average age of the population was 41 years and 45.1% were female. Regarding BMI, 43% had a normal BMI, 27.9% were overweight (BMI ≥ 23 kg/m^2, < 25 kg/m^2), and 29.2% were obese (BMI > 25 kg/m^2). There were 42 patients with diabetes and 38 with hypertension, accounting for 9.2% and 8.3% of all patients, respectively. Most of the patients (72.4%) received antiviral therapy. At baseline, 358 patients were HBV DNA positive, with a median detection value of 3.63×10^4 ($10^{4.56}$) IU/mL, while in 98 (21.5%) patients the HBV DNA level was below the detection limit. Among all HBV-infected patients, 66% were HBeAg-negative. Table 1 shows the basic information of the total population.

Comparison of baseline data of chronic HBV infection with and without NAFLD: There were 152 patients with chronic HBV infection complicated by NAFLD and 304 patients without NAFLD. Table 2 shows the demographic and main clinical indicators of the two groups. The proportion of female patients in the NAFLD group was higher than that in the NAFLD group ($P < 0.05$), and the median follow-up time was longer than that in the NAFLD group (73 *vs* 63 mo, $P < 0.05$). Compared with the non-NAFLD group, the NAFLD group had a higher prevalence of diabetes and higher BMI ($P < 0.001$), and its LDL, TG, PB, ALT levels were also significantly higher ($P < 0.05$). However, there were no differences in age, prevalence of hypertension, proportion of liver cirrhosis, HBV DNA, and other indicators ($P > 0.05$).

Comparison of clinical characteristics of patients with chronic HBV infection without cirrhosis at baseline with NAFLD and those without NAFLD: Taking patients without cirrhosis at the time of liver biopsy as the research object, the demographic and main clinical indicators of the NAFLD and non-NAFLD groups were compared (Table 3). There were significant differences in sex, BMI, prevalence of diabetes, follow-up duration, HBV DNA, ALT, TC, TG, LDL, and other indicators between the two groups (all $P < 0.05$). However, there was no difference in the antiviral status and other indicators ($P > 0.05$). The prevalence of diabetes, follow-up duration, and ALT levels in the two groups were 1:1 PSM. There were 109 patients in the NAFLD and non-NAFLD groups. After PSM, there were no significant differences in sex, diabetes prevalence, follow-up duration, HBV DNA, ALT, and other indicators between the two groups ($P > 0.05$); however, there were differences in BMI, PA, and TG ($P < 0.05$). Moreover, the NAFLD group was divided into the NASH (21 cases) and non-NASH (88 cases) groups.

Comparison of the pathological characteristics of chronic HBV infection with and without NAFLD: Among patients with NAFLD, 58.6% had mild hepatic steatosis, 31.6% had moderate hepatic steatosis, and 9.9% had severe hepatic steatosis. Differences were observed between the NAFLD group and non-NAFLD group in the degree of liver fibrosis, portal inflammation, and NAS score ($P < 0.05$). There was no difference in lobular inflammation or ballooning deformation (BD) between the two groups ($P > 0.05$) (Table 4).

Antiviral conditions

There was no significant difference in the proportion of patients receiving antiviral treatment, types of antiviral drugs, and duration of antiviral treatment between the NAFLD group and non-NAFLD group

Table 1 Basic information of total chronic hepatitis B (*n* = 456), *n* (%)

Parameters	Results
Age (yr)	41.08 ± 10.41
Sex female	160 (45.1)
BMI (kg/m^2)	
< 23	196 (43.0)
23-25	127 (27.9)
≥ 25	133 (29.2)
Diabetes	42 (9.2)
Hypertension	38 (8.3)
NAFLD	152 (33.3)
Duration of follow-up (mo)	70.5 (29-133)
Antiviral therapy	330 (72.4)
HBV DNA (+), $\log_{10}$ (IU/mL)	4.56 (3-6.92)
HBV DNA (-)[1]	98 (21.5)
HBeAg (-)	301 (66.0)

[1]Hepatitis B virus DNA (-): < 500 IU/mL.
NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

(P > 0.05). At the last follow-up, 302 (91.2%) patients who received antiviral therapy were HBV DNA-negative, including 102 (78.9%) in the NAFLD group and 200 (93%) in the non-NAFLD group. There was no significant difference in the proportion of HBV DNA negativity between the two groups (P > 0.05). A total of 275 (83.1%) patients had normal ALT levels: 90 (77.6%) in the NAFLD group and 185 (56%) in the NAFLD group. There was no significant difference in the proportion of normal ALT levels between the two groups (P = 0.05) (Table 5).

Risk of cirrhosis in patients with chronic HBV infection

Occurrence of cirrhosis: Patients without cirrhosis at the time of liver biopsy were selected as research participants, and the risk of progression to cirrhosis was observed. During the follow-up period, 34 patients developed liver cirrhosis diagnosed by ultrasound, with a median follow-up time of 72 (30-134) mo, including 10 (7.8%) in the NAFLD group and 24 (9.2%) in the NAFLD group. This study was conducted during the follow-up period.

K-M survival analysis of NAFLD and the risk of cirrhosis: The results of the K-M survival analysis showed that there was no significant increase in the risk of liver cirrhosis diagnosed using ultrasound in the combined NAFLD group before and after PSM (log-rank, P = 0.69). The results of the K-M survival analysis after PSM are shown in Figure 2A. F0, 1, 2 was regarded as mild fibrosis, while F3, 4 was regarded as severe fibrosis. Patients with fibrosis after PSM had an increased risk of cirrhosis diagnosed using ultrasound (log rank, P < 0.05). The results of K-M survival analysis after matching are shown in Figure 2B. Figure before PSM. In the NAFLD group, three out of 21 cases in the NASH group and seven out of 88 patients in the non-NASH group developed cirrhosis. The results of K-M survival analysis after PSM are shown in Figure 2C. There was no statistical difference between the two groups (P = 0.17).

Univariate and multivariate Cox regression analysis of cirrhosis: Cox regression univariate analysis showed that age, antiviral duration, lobular inflammation, BD, liver fibrosis, ALP, LDL, and PLT were related to cirrhosis (P < 0.05). Multivariate analysis showed that age, ballooning degeneration, liver fibrosis, and PLT were independent risk factors for cirrhosis diagnosed using ultrasound (all P < 0.05) (Table 6).

Univariate and multivariate Cox regression analysis of cirrhosis after PSM: Cox regression univariate analysis showed that age, hypertension, diabetes mellitus, lobular inflammation, portal inflammation, liver fibrosis, ALT, and PLT were related to cirrhosis diagnosed using ultrasound (P < 0.1). Multivariate analysis showed that diabetes mellitus, BD, and PLT were independent risk factors for liver cirrhosis diagnosed using ultrasound (P < 0.05) (Table 7).

Table 2 Comparison of baseline characteristics of chronic hepatitis B patients with and without nonalcoholic fatty liver disease, *n* (%)

Parameters	NAFLD	No NAFLD	*P* value
	n = 152	*n* = 304	
Age (yr)	41.87 ± 9.44	40.69 ± 10.85	0.24
< 40	67 (44.1)	141 (46.4)	0.64
40-60	77 (50.7)	148 (48.7)	
≥ 60	8 (5.3)	15 (4.9)	
Sex female	41 (27)	119 (39.1)	0.01
BMI (kg/m^2)			< 0.001
< 23	31 (20.4)	165 (54.3)	
23-25	49 (32.2)	78 (25.7)	
≥ 25	72 (47.4)	61 (20.1)	
Diabetes	27 (17.8)	15 (4.9)	< 0.001
Hypertension	18 (11.8)	20 (6.6)	0.07
Cirrhosis	24 (15.8)	43 (14.1)	0.64
Duration of follow-up (mo)	71 (27-118)	73 (32-114)	0.08
HBV DNA (IU/mL)			0.08
< 500 IU/mL	29 (19.1)	69 (22.7)	
< 4 log10	21 (13.8)	60 (19.7)	
≥ 4 log10	102 (67.1)	175 (57.6)	
HBeAg (-)	91 (59.9)	210 (69.1)	0.05
TBil (μmol/L)	15.69 ± 9.06	15.91 ± 9.1	0.8
ALB (g/L)	44.22 ± 3.42	43.62 ± 3.81	0.1
PB (mg/L)	241.53 ± 67.19	224.74 ± 73.69	0.02
ALT (U/L)	85 (32-275)	77 (26-263)	0.002
≤ 40	67 (44.1)	172 (56.6)	0.012
> 40	85 (55.9)	132 (43.4)	
AST (U/L)	76 (27-248)	83 (26.5-247)	0.28
ALP (U/L)	84.7 ± 26.3	83.92 ± 31.68	0.8
GGT (U/L)	40.88 ± 36.95	36.31 ± 38.17	0.23
GLU (mmol/L)	5.48 ± 1.07	5.34 ± 1.02	0.2
TC (mmol/L)	4.32 ± 0.88	4.17 ± 0.71	0.08
TG (mmol/L)	1.54 ± 1.04	1.23 ± 0.5	0.001
LDL (mmol/L)	2.7 ± 0.68	2.56 ± 0.7	0.049
HDL (mmol/L)	1.32 ± 0.4	1.35 ± 0.31	0.27
PLT (× 10^9/L)	167.05 ± 54.18	159.13 ± 56.02	0.15
AFP (ng/mL)	3.25 (2.15-5.83)	2.93 (2.03-5.81)	0.33

NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; TBil: Total bilirubin; ALB: Albumin; PB: Prealbumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; GLU: Fasting blood glucose; TC: Total cholesterol; TG: Triglyceride; LDL: Low density lipoprotein; HDL: High density lipoprotein; PLT: Platelet; AFP: Alpha-fetoprotein; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

Risk of HCC in patients with chronic HBV infection

Characteristics of patients with HCC: During the follow-up period, 10 patients (2.2%) developed HCC (Table 7), and two died (one died due to HCC and the cause of death in the other case was not related to

Table 3 Comparison of baseline characteristics of chronic hepatitis B patients (without cirrhosis) with and without nonalcoholic fatty liver disease, *n* (%)

Parameters	*P* values before PSM	PSM		*P* value
		NAFLD (*n* = 109)	No NAFLD (*n* = 109)	
Age (yr)	0.58			0.63
< 40		52 (47.7)	47 (43.1)	
40-60		51 (46.8)	58 (53.2)	
≥ 60		6 (5.5)	4 (3.7)	
Sex female	< 0.05	29 (26.6)	37 (33.9)	0.24
BMI (kg/m^2)	< 0.05			< 0.05
< 23		21 (19.3)	58 (53.2)	
23-25		35 (32.1)	30 (27.5)	
≥ 25		53 (48.6)	21 (19.3)	
Diabetes	< 0.05	4 (3.7)	4 (3.7)	1
Hypertension	0.07	12 (11)	6 (5.5)	0.14
Duration of follow-up (mo)	< 0.05	71 (28-119.5)	52 (25-111)	0.3
Antiviral drugs	0.59			0.3
Entecavir		49 (61.3)	56 (71.8)	
Tenofovir		26 (32.5)	19 (24.4)	
Other		5 (6.3)	3 (3.8)	
Antiviral duration	0.48			0.46
Never		29 (26.6)	31 (28.4)	
< 5 yr		50 (45.9)	54 (49.5)	
≥ 5 yr		30 (27.5)	24 (22)	
HBV DNA (IU/mL)	< 0.05			0.06
< 500 IU/mL		18 (19.3)	24 (22)	
< 4 log10		13 (32.1)	21 (19.3)	
≥ 4 log10		78 (48.6)	64 (58.7)	
HBeAg (-)	0.12	65 (59.6)	75 (68.8)	0.16
TBil (μmol/L)	0.94	15.73 ± 7.17	14.25 ± 5.73	0.09
ALB (g/L)	0.21	44.15 ± 3.32	44.29 ± 3.78	0.78
PB (mg/L)	0.28	243.24 ± 65.74	225.67 ± 62.19	< 0.05
ALT (U/L)	< 0.05			0.89
≤ 40		43 (19.3)	44 (40.4)	
> 40		66 (32.1)	65 (59.6)	
AST (U/L)	0.16	36 (27.5-49.5)	37 (26.5-50)	0.95
ALP (U/L)	0.1	85.55 ± 26.37	82.82 ± 22.08	0.41
GGT (U/L)	0.12	37.44 ± 25.34	34.76 ± 33.13	0.5
GLU (mmol/L)	0.06	5.25 ± 0.68	5.41 ± 0.89	0.15
TC (mmol/L)	< 0.05	4.37 ± 0.88	4.22 ± 0.64	0.16
TG (mmol/L)	< 0.05	1.47 ± 0.75	1.22 ± 0.51	< 0.05
LDL (mmol/L)	< 0.05	2.77 ± 0.65	2.62 ± 0.61	0.09

HDL (mmol/L)	0.38	1.31 ± 0.36	1.32 ± 0.3	0.82
PLT (× 10^9/L)	0.12	174.29 ± 51.81	162.83 ± 47.49	0.09
AFP (ng/mL)	0.29	3.1 (2.1-5.3)	2.9 (2.1-5.5)	0.74
NASH		15 (13.7)		

PSM: Propensity score matching; BMI: Body mass index; TBil: Total bilirubin; ALB: Albumin; PB: Prealbumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; GLU: Fasting blood glucose; TC: Total cholesterol; TG: Triglyceride; LDL: Low density lipoprotein; HDL: High density lipoprotein; PLT: Platelet; AFP: Alpha-fetoprotein; NASH: Nonalcoholic steatohepatitis; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

Table 4 Comparison of pathological features of chronic hepatitis B patients with and without nonalcoholic fatty liver disease, *n* (%)

Pathological features	Total (*n* = 456)	NAFLD (*n* = 152)	No NAFLD (*n* = 304)	*P* value
Fibrosis				0.045
F0	109 (23.9)	30 (19.7)	79 (26)	
F1	129 (28.3)	36 (23.7)	93 (30.6)	
F2	93 (20.4)	41 (27)	52 (17.1)	
F3	58 (12.7)	21 (13.8)	37 (12.2)	
F4	67 (14.7)	24 (15.8)	43 (14.1)	
Steatosis				
0	304 (66.7)		304 (100)	
1	89 (19.5)	89 (58.6)		
2	48 (10.5)	48 (31.6)		
3	15 (3.3)	15 (9.9)		
Portal tract inflammation				< 0.001
0	35 (7.7)	8 (5.3)	27 (8.9)	
1	275 (60.3)	79 (52)	196 (64.5)	
2	117 (25.7)	50 (32.9)	67 (22)	
3	29 (6.3)	15 (9.9)	14 (4.6)	
Lobular inflammation				0.13
0	91 (20)	28 (18.4)	63 (20.7)	
1	315 (69.1)	101 (66.4)	214 (70.4)	
2	50 (11)	23 (15.1)	27 (8.9)	
Ballooning degeneration				0.004
0	153 (33.6)	58 (38.2)	165 (54.3)	
1	195 (42.8)	61 (40.1)	96 (31.6)	
2	108 (23.7)	33 (21.7)	43 (14.1)	
NASH	32 (21.1)	32 (21.1)		
NAS score	1.7 ± 1.2	2.7 ± 1.2	1.2 ± 0.8	< 0.001

NASH: Nonalcoholic steatohepatitis; NAS: Activity score of nonalcoholic fatty liver disease; NAFLD: Nonalcoholic fatty liver disease.

liver disease). The median time interval between liver biopsy and HCC diagnosis was 100.5 mo (68.5-128.0). HCC occurred in six patients (3.3%) with NAFLD and four patients (1.6%) without NAFLD. Seven patients developed cirrhosis at the time of liver biopsy, and one of the other three developed cirrhosis before HCC.

Table 5 Comparison of antiviral data of chronic hepatitis B virus infection with and without nonalcoholic fatty liver disease, *n* (%)

Parameters	NAFLD (*n* = 152)	No NAFLD (*n* = 304)	*P* value
Antiviral	116 (76.3)	215 (70.7)	0.21
Antiviral drugs			0.5
Entecavir	73 (62.9)	131 (60.9)	
Tenofovir	35 (30.2)	68 (31.6)	
Others	8 (6.9)	16 (7.4)	
Antivirus duration			0.31
< 5 yr	70 (46.1)	129 (42.4)	
≥ 5 yr	46 (30.3)	86 (28.3)	

NAFLD: Nonalcoholic fatty liver disease.

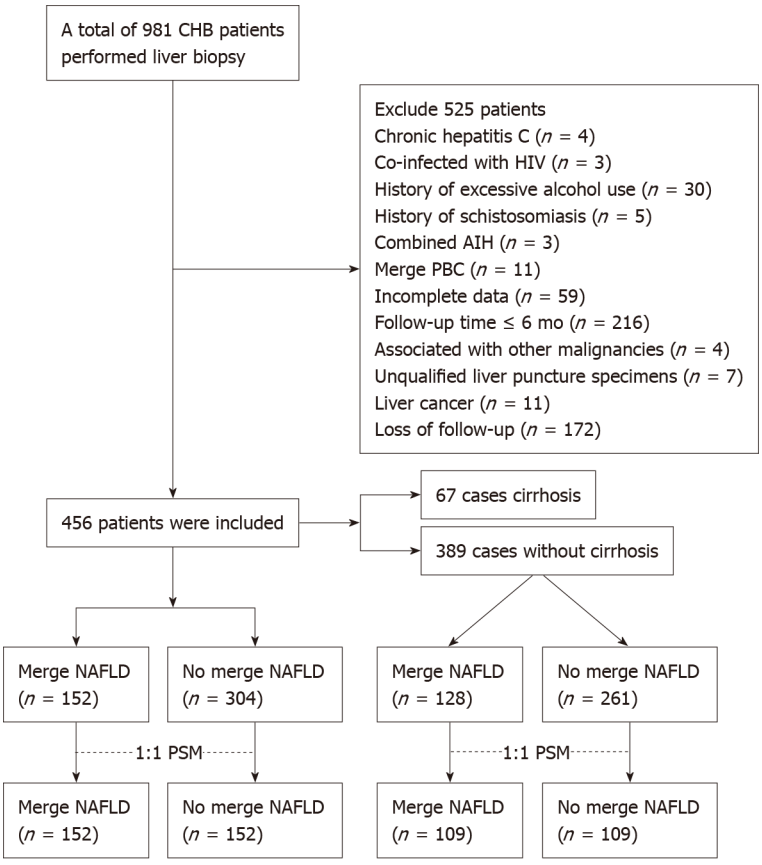

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Figure 1 Flow chart of patient screening and grouping. CHB: Chronic hepatitis B; NAFLD: Nonalcoholic fatty liver disease; HIV: Human immunodeficiency virus; AIH: Autoimmune hepatitis; PBC: Primary biliary cholangitis.

K-M survival analysis of HCC risk: Patients with baseline cirrhosis were included in the K-M survival analysis of HCC risk. It was found that the cumulative risk of HCC in the NAFLD group was significantly higher than that in the non-NAFLD group (log rank, P = 0.02) (Figure 3A). At the same time, the risk of HCC in patients with severe liver fibrosis (F3-4) was also significantly increased (log rank, P = 0.005) (Figure 3B). When the NAFLD group was divided into the NASH group with 32 patients (3 HCCs) and the non-NASH group with 120 patients (3 HCCs), the risk of HCC in the NASH group was increased (log rank, P = 0.03) (Figure 3C), and the risk of HCC in patients with hepatic ballooning was significantly increased (log rank, P = 0.01) (Figure 3D). There was no significant difference in the risk of HCC among patients with steatosis, lobulitis, and portal inflammation (log rank,

Table 6 Univariate and multivariate Cox regression analysis of cirrhosis

Parameters	Univariate	*P* value	Multivariate	*P* value
	HR (95%CI)		HR (95%CI)	
Clinical factors				
Sex	1.36 (0.63-2.92)	0.43		
Age (yr)	1.08 (1.04-1.12)	< 0.001	1.06 (1.02-1.10)	0.003
BMI (kg/m^2)	0.96 (0.64-1.45)	0.86		
Diabetes	2.57 (0.78-8.52)	0.12		
Hypertension	2.71 (0.95-7.76)	0.06		
Duration of antiviral (≥ 5 yr/never or < 5 yr)	1.65 (1.07-2.54)	0.02	1.32 (0.84-2.06)	0.23
Pathological factors				
NAFLD	1.12 (0.53-2.37)	0.76		
NASH	1.38 (0.33-5.86)	0.66		
Lobular inflammation	2.35 (1.19-4.66)	0.01	0.77 (0.33-1.80)	0.54
Portal tract inflammation	1.40 (0.90-2.20)	0.14		
Ballooning degeneration	3.34 (1.65-6.75)	0.001	2.57 (1.05-6.28)	0.04
Fibrosis	1.49 (1.08-2.06)	0.02	1.39 (1.04-1.87)	0.028
Laboratory examination				
TBil (μmol/L)	0.99 (0.95-1.04)	0.8		
ALB (g/L)	0.94 (0.85-1.04)	0.25		
PB (mg/L)	1.00 (0.997-1.00)	0.83		
ALT (> 40/≤ 40U/L)	1.00 (0.996-1.00)	0.64		
AST (U/L)	1.00 (0.99-1.01)	0.54		
ALP (U/L)	1.01 (1.00-1.02)	0.008	1.01 (1.00-1.02)	0.08
GGT (U/L)	1.01 (1.00-1.01)	0.13		
GLU (mmol/L)	1.31 (0.96-1.78)	0.09		
TG (mmol/L)	0.57 (0.28-1.18)	0.13		
TC (mmol/L)	0.95 (0.61-1.49)	0.83		
HDL (mmol/L)	1.71 (0.65-4.49)	0.28		
LDL (mmol/L)	0.46 (0.27-0.79)	0.005	0.76 (0.45-1.29)	0.31
PLT (× 10^9/L)	0.99 (0.98-0.99)	0.001	0.98 (0.97-0.99)	0.001
AFP (ng/mL)	0.99 (0.96-1.03)	0.63		
HBeAg (-)	0.91 (0.43-1.90)	0.79		
HBV DNA (≥ 4 log IU/mL)	0.82 (0.55-1.23)	0.34		

BMI: Body mass index; TBil: Total bilirubin; ALB: Albumin; PB: Prealbumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; GLU: Fasting blood glucose; TC: Total cholesterol; TG: Triglyceride; LDL: Low density lipoprotein; HDL: High density lipoprotein; PLT: Platelet; AFP: Alpha-fetoprotein; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

$P > 0.05$) (figures ignored).

Univariate and multivariate Cox regression analysis of HCC: Cox regression univariate analysis showed that diabetes mellitus, NAFLD, NASH, lobular inflammation, BD, liver fibrosis, GLU, TC, TG, and PLT were correlated with HCC (all $P < 0.05$). Multivariate analysis of factors with $P < 0.10$ in the Cox regression univariate analysis showed that ballooning, liver fibrosis, and diabetes were

Table 7 Univariate and multivariate Cox regression analysis of cirrhosis after propensity score matching

Parameters	Univariate	*P* value	Multivariate	*P* value
	HR (95%CI)		HR (95%CI)	
Age (yr)	1.06 (1.01-1.11)	0.02	1.05 (0.99-1.11)	0.11
Hypertension	3.26 (0.89-11.90)	0.07	0.59 (0.12-2.97)	0.52
Diabetes	6.74 (0.77-58.81)	0.08	12.21 (1.1-134.4)	0.04
Lobular inflammation	2.63 (1.02-6.82)	0.046	1.20 (0.26-5.57)	0.82
Ballooning degeneration	4.33 (1.01-5.15)	0.006	1.80 (0.86-4.48)	0.02
Portal tract inflammation	2.37 (1.18-4.75)	0.02	72.61 (2.16-2436)	0.48
Fibrosis	1.57 (0.96-2.56)	0.07	1.03 (0.48-2.25)	0.93
ALT(U/L)	1.00 (1.00-1.01)	0.06	1.00 (0.99-1.01)	0.26
PLT ($\times 10^9$/L)	0.98 (0.96-0.99)	< 0.001	0.98 (0.96-0.99)	0.001

ALT: Alanine aminotransferase; PLT: Platelet.

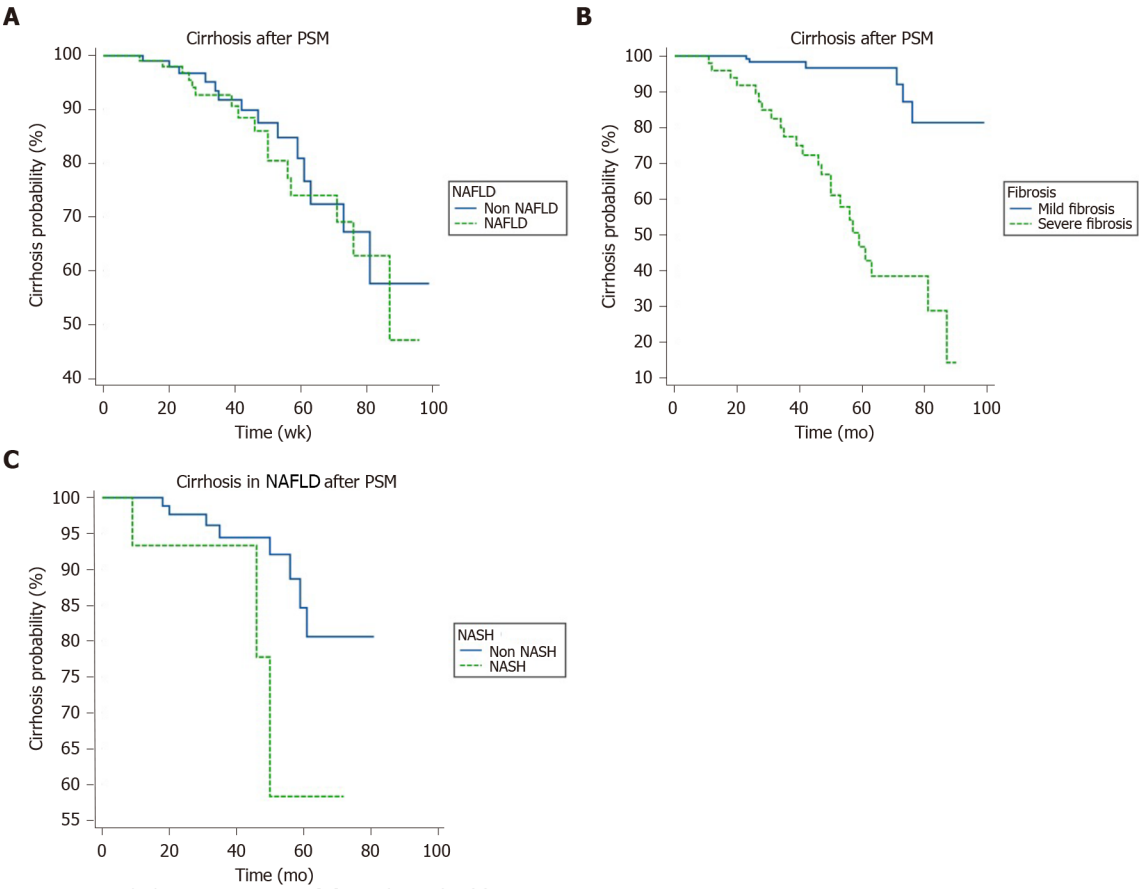

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Figure 2 Kaplan-Meier survival analysis of nonalcoholic fatty liver disease and the risk of cirrhosis. A: Results of the Kaplan-Meier (K-M) survival analysis of the risk of cirrhosis after propensity score matching (PSM) in nonalcoholic fatty liver disease (NAFLD) and non NAFLD with chronic hepatitis B; B: Results of K-M survival analysis of the risk of cirrhosis after PSM in patients with mild fibrosis and in those with severe fibrosis; C: Nonalcoholic steatohepatitis (NASH) and non-NASH stratification of NAFLD and K-M survival analysis of the risk of cirrhosis. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; PSM: Propensity score matching.

independent risk factors for HCC (Table 8).

Table 8 Univariate and multivariate Cox regression analysis of hepatocellular carcinoma occurrence

Parameters	Univariate	*P* value	Multivariate	*P* value
	HR (95%CI)		HR (95%CI)	
Clinical factors				
Sex	40.03 (0.17-9369.3)	0.19		
Age (yr)	1.06 (0.99-1.13)	0.054	0.29 (0.03-2.50)	0.26
BMI (kg/m^2)	0.97 (0.78-1.21)	0.8		
Diabetes	14.36 (4.01-51.47)	< 0.001	34.8 (2.27-534.1)	0.01
Hypertension	1.99 (0.25-15.71)	0.52		
Antivirus duration (≥ 5 yr, never or < 5 yr)	1.32 (0.38-4.56)	0.66		
Pathological factors				
NAFLD (steatosis ≥ 5%)	4.29 (1.20-15.41)	0.025	2.28 (0.44-11.69)	0.33
NASH	4.36 (1.35-24.80)	0.002	0.53 (0.01-1.84)	0.39
Lobular inflammation	7.2 (2.16-23.91)	0.001	5.16 (0.53-49.96)	0.16
Portal tract inflammation	2.02 (0.98-4.15)	0.056	0.49 (0.31-1.85)	0.29
Ballooning degeneration	8.69 (1.57-29.64)	0.008	5.16 (0.83-19.96)	0.03
Fibrosis stage	14.25 (13.68-55.14)	< 0.001	8.37 (1.39-50.44)	0.02
Laboratory examination				
TBil (μmol/L)	1.02 (0.97-1.09)	0.37		
PB (mg/L)	0.998 (0.99-1.01)	0.64		
LB (g/L)	0.97 (0.82-1.14)	0.69		
ALT (> 40/≤ 40 U/L)	0.999 (0.99-1.01)	0.71		
AST (U/L)	0.999 (0.98-1.01)	0.89		
ALP (U/L)	1.01 (0.99-1.03)	0.42		
GGT (U/L)	0.99 (0.96-1.02)	0.46		
GLU (mmol/L)	1.45 (1.08-1.94)	0.01	0.64 (0.37-1.12)	0.12
TG (mmol/L)	0.18 (0.04-0.92)	0.04	0.085 (0.004-1.7)	0.11
TC (mmol/L)	0.36 (0.15-0.85)	0.02	0.39 (0.15-1.01)	0.053
HDL (mmol/L)	0.39 (0.04-3.67)	0.41		
LDL (mmol/L)	0.47 (0.18-1.26)	0.13		
PLT (× 10^9/L)	0.97 (0.96-0.99)	0.004	0.99 (0.97-1.01)	0.26
AFP (ng/mL)	0.99 (0.91-1.07)	0.77		
HBeAg (-) (%)	1.43 (0.40-5.08)	0.58		
HBV DNA (≥ 4 log IU/mL)	1.49 (0.39-5.77)	0.56		

BMI: Body mass index; TBil: Total bilirubin; ALB: Albumin; PB: Prealbumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; GLU: Fasting blood glucose; TC: Total cholesterol; TG: Triglyceride; LDL: Low density lipoprotein; HDL: High density lipoprotein; PLT: Platelet; AFP: Alpha-fetoprotein; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

DISCUSSION

According to the WHO hepatitis report in 2017, 96% of the 1.3 million deaths caused by viral hepatitis worldwide in 2015 were caused by HBV and hepatitis C virus (HCV). In China, 70 million patients with HBV, accounting for 27% of the global population of patients with HBV[14]; furthermore, 68% (95%CI: 60-74) of patients with liver cirrhosis in China are infected with HBV[15]. With the increasing prevalence of obesity and metabolic syndrome, NAFLD has become the most common cause of chronic liver

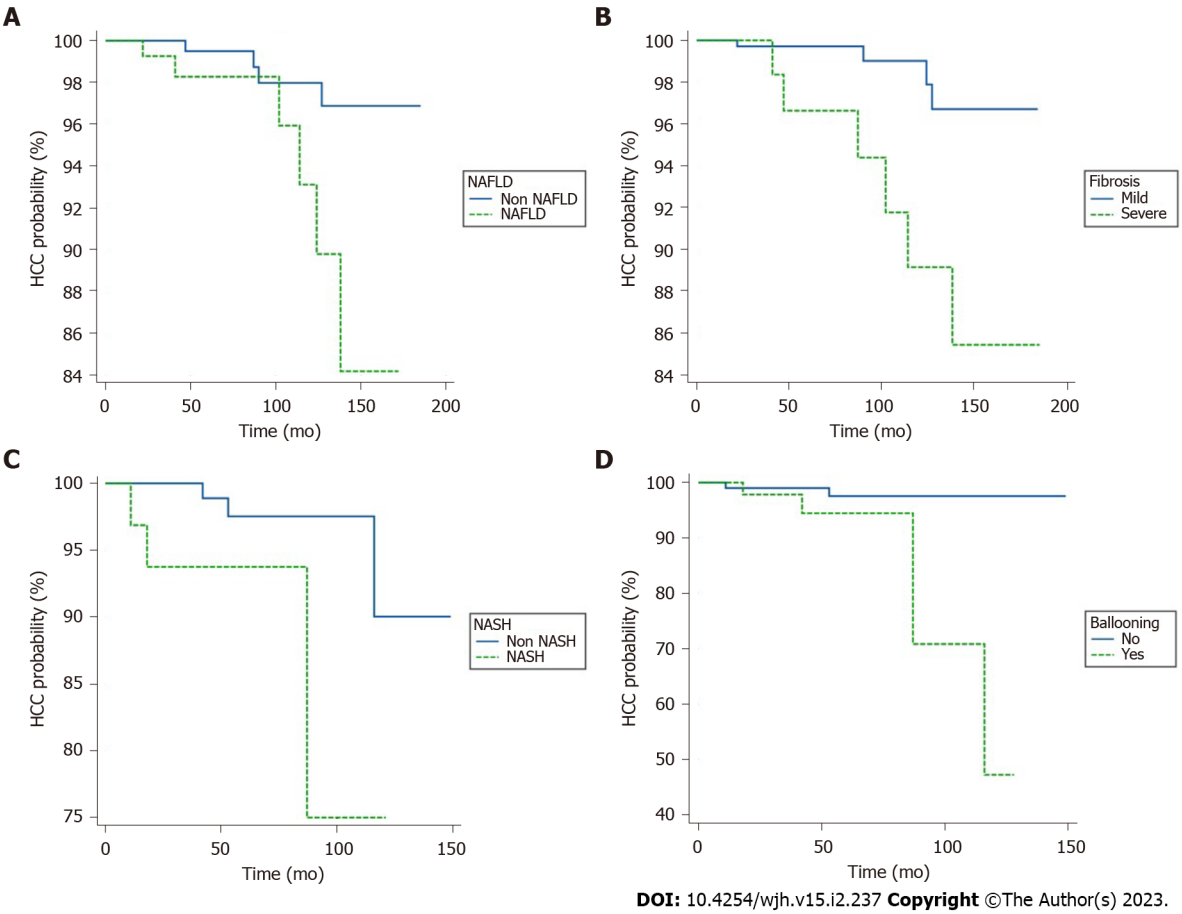

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Figure 3 Kaplan-Meier survival analysis of hepatocellular carcinoma risk. A: Results of the Kaplan-Meier (K-M) survival analysis of the risk of hepatocellular carcinoma (HCC) in patients with chronic hepatitis B and nonalcoholic fatty liver disease (NAFLD) and those without NAFLD; B: Results of K-M survival analysis of the risk of HCC in patients with mild fibrosis and those with severe fibrosis; C: Nonalcoholic steatohepatitis (NASH) and non-NASH stratification of NAFLD and K-M survival analysis of the risk of HCC; D: Ballooning and non-ballooning stratification of NAFLD and K-M survival analysis of the risk of HCC. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

disease worldwide[14]. Therefore, these two liver diseases are often observed, and with the increasing prevalence of NAFLD, the number of patients with combined NAFLD and HBV infections is also on the increase. Studies have reported that the prevalence of NAFLD, confirmed by biopsy in patients with CHB, ranges from 14% to 52%[5,16-22]. The prevalence of NAFLD in this study was 33.3%, which was also within this range.

NAFLD is associated with metabolic syndrome, and this was also reflected in our study. Compared with those without NAFLD, chronic HBV-infected patients with NAFLD have a higher prevalence of diabetes and BMI, and their low-density lipoprotein, triglyceride, and apolipoprotein B levels were also significantly higher. With the aggravation of hepatic steatosis, the proportion of overweight and obese patients and the average BMI gradually increased. Studies have shown that metabolic syndrome can delay the serum clearance of HBeAg, increase the risk of liver fibrosis and cirrhosis, and thus enhance the development of HCC[8,23,24]. Considering that NAFLD is the main hepatic manifestation of obesity and metabolism-related diseases, chronic HBV infection overlapping with NAFLD may further increase the risk of cirrhosis and HCC. NAFLD is not associated with an increased risk of cirrhosis in patients with chronic HBV infection. After propensity matching for follow-up duration, diabetes mellitus, and ALT, there was no difference in diabetes mellitus and most variables between the two groups, while multivariate analysis still showed that diabetes mellitus was an independent risk factor for the occurrence of liver cirrhosis diagnosed using ultrasound.

In addition, NAFLD, obesity, and hyperlipidemia have been found to be associated with accelerated clearance of HBsAg and lower HBV DNA in many clinical studies[9,15,25]. In our study, it was not found that combined NAFLD was associated with decreased HBV DNA levels, both at baseline and at the last follow-up. However, in the cohort of patients without cirrhosis, we found that the proportion of high-level HBV DNA in the combined NAFLD group was lower than that in the non NAFLD group, although the difference between the two groups was not statistically significant after matching, and the comparison was not stratified by antiviral treatment, age, *etc.* However, NAFLD and CHB can jointly aggravate liver injury, and increase the risk of liver cirrhosis and liver cancer[3-7,26].

In this study, 72.4% of patients received antiviral therapy, mainly nucleoside analogs; moreover, the 2017 European Association for the Study of the Liver Guidelines proposed long-term inhibition of HBV DNA as the primary endpoint of chronic hepatitis B treatment[27]. Previously, with the development and clinical application of NAs antiviral drugs, HBV replication was effectively controlled[28-31]. In our study, 91.2% of patients with CHB who received antiviral therapy achieved HBV DNA negativity. However, no significant difference was observed in HBV DNA negativity between the NAFLD and non-NAFLD groups. In addition, 83.1% of the patients had normal ALT levels, and there was no significant difference in the normal ALT levels between the two groups. There are conflicting results regarding whether NAFLD affects the efficacy of antiviral therapy in patients with CHB. A recent meta-analysis showed that the efficacy of antiviral therapy decreases in CHB patients with hepatic steatosis[32]. There were significant differences in virological and biochemical reactions between the subgroups with and that without NAFLD, which may be due to the decreased bioavailability of antiviral drugs caused by fat changes, resulting in a significant reduction in the contact area between hepatocytes and antiviral drugs [32]. However, some studies have failed to find an association between hepatic steatosis and the efficacy of antiviral therapy[20,32-35]. Although the impact of hepatic steatosis on antiviral therapy in these patients remains controversial, it is still recommended to monitor the onset or progression of NAFLD during antiviral therapy to prevent potential negative effects.

In recent years, the proportion of patients with cirrhosis caused by NAFLD has increased rapidly. In particular, NASH, defined as the simultaneous presence of steatosis and inflammatory injury in the liver, with or without liver fibrosis[36,37], is an independent risk factor for the development of cirrhosis [38]. Several retrospective studies with long-term follow-up have found that the unadjusted cumulative probability of liver-related events was significantly higher in patients with NASH than in those without NASH[39,40]. A comprehensive analysis of six studies showed that in 41% of patients with NASH fibrosis progressed, with an average annual growth rate of 0.09%[43]; moreover, the progression of fibrosis can lead to the development of cirrhosis and cause other liver-related diseases, such as HCC. The annual incidence of HCC in patients with NASH is 5.29/1000 person years[36,41]. These findings further prove that the risk of HCC in patients with CHB infection complicated by NASH is significantly increased. In our study, NASH did not increase the risk of cirrhosis, but increased the risk of HCC. Furthermore, we observed that the key factor for NASH was ballooning. Ballooning and fibrosis are pre-PSM cirrhosis and HCC risk factors, whereas post-PSM ballooning is still a risk factor for cirrhosis. A previous large-scale cohort study included 1089 cases of NAFLD confirmed by biopsy in North American and European multiethnic CHB populations. After a median follow-up of 10 years, there was no obvious correlation between CHB combined with NAFLD and the risk of liver-related adverse events (cirrhosis or liver cancer), while CHB combined with NASH still led to a higher risk of liver-related adverse events, but was only significant in the population with advanced liver fibrosis[4]. This large-scale study also pointed out that ballooning degeneration and inflammation were important liver-related adverse events, but steatosis was not related to clinical events[4]. This result supports the conclusion of the present study that the combination of NASH will increase the risk of adverse events in patients with chronic HBV infection. Histological determinants of NASH, such as ballooning and lobular inflammation, are important for liver-related adverse events (cirrhosis and HCC).

First, liver fibrosis and subsequent cirrhosis are generally considered to be the prelude to HCC, which is closely related to the occurrence of HCC[26]. Second, diabetes mellitus, blood glucose, and cholesterol, which are components of the metabolic syndrome, are also closely related to the occurrence of HCC. Previous studies have proposed that obesity, diabetes, and metabolic syndrome are independent risk factors for liver fibrosis, cirrhosis, and HCC in patients with chronic hepatitis B, suggesting that metabolic factors and chronic hepatitis B have a synergistic effect in the pathogenesis of liver cancer[23,24,42,43], and extreme obesity and diabetes increase the risk of developing HBV-related HCC by 12.8-fold[45]. A prospective study in Taiwan showed that a high BMI at baseline was associated with the incidence of cirrhosis and HCC[43]; however, the participants were all male patients with chronic HBV infection. In this study, the baseline BMI was not an independent risk factor for HCC occurrence; however, it may be related to different populations, diets, living habits, and grading standards for BMI. Moreover, this may be due to the complex relationship between BMI and HCC. HCC or cirrhosis as a prelude to HCC has an impact on the nutritional status of patients; moreover, nutritional deficiency and sarcopenia can reduce BMI. The impact of a high baseline BMI on the overall prognosis of patients with HCC has also been controversial: some studies have indicated that patients with a high BMI have a worse prognosis, other studies suggest that they may have a better prognosis, and some studies have indicated that there was no significant correlation between the two[44-46].

This study has some limitations. First, the sample size of this single-center retrospective study was reduced after the application of PSM. Because of the influence of the small sample size, we could not match all factors; hence, larger samples and more rigorous prospective studies are needed for further verification. Second, the conclusion of the study is that CHB combined with NAFLD has little effect on the final outcome of liver cirrhosis, which is inconsistent with the results of mainstream studies, and could be due to our short follow-up duration. The study cohort needs to be observed for a longer time, and the conclusion may change if this is done. Third, in the study, identifying adverse events after the diagnosis of liver cirrhosis relied on ultrasound, and ultrasound itself is subject to the subjective understanding of the ultrasonologists; hence, the diagnostic error could be large.

CONCLUSION

In summary, we conducted a long-term follow-up of a cohort of CHB patients with NAFLD based on a diagnosis of liver pathology, and observed that the PSM of baseline influencing factors revealed that the risk of cirrhosis diagnosed using ultrasound was not significantly higher in the group with NAFLD than in the group without NAFLD. Before PSM, age, BD, liver fibrosis, and PLT were independent risk factors for cirrhosis. After PSM, only BD and PLT were found to be independent risk factors for liver cirrhosis diagnosed using ultrasound. The cumulative risk of HCC was significantly higher in patients with NAFLD or NASH. BD, liver fibrosis, and diabetes mellitus were independent risk factors for HCC.

ARTICLE HIGHLIGHTS

Research background

Chronic hepatitis B (CHB) virus infection and nonalcoholic fatty liver disease (NAFLD) are important causes of liver-related complications and death. With the increasing prevalence of NAFLD, the number of patients with combined NAFLD and hepatitis B virus (HBV) infection is also on the increase.

Research motivation

This study aimed to further explore the impact of NAFLD and the pathological changes confirmed by liver pathology in patients with chronic HBV infection.

Research objectives

To study the effect of NAFLD confirmed using liver pathology on the outcomes of long-term serious adverse events in patients with CHB virus infection.

Research methods

Among 456 cases of chronic HBV infection, 152 were confirmed by liver histology to have NAFLD, and 304 were simple chronic HBV infection. The incidence of serious clinical events at the follow-up endpoint was compared by Kaplan-Meier (K-M) survival analysis at baseline using propensity score matching balance parameters.

Research results

After a median follow-up of 70.5 mo, there were 34 cases of ultrasound-diagnosed cirrhosis and 10 cases of HCC. K-M survival analysis showed no significant difference in the occurrence of CHB complicated with NAFLD cirrhosis, and the cumulative incidence of HCC in the NAFLD group was higher than that in the non-NAFLD group (log-rank test, $P < 0.05$). Hepatocyte ballooning and severe liver fibrosis were also associated with an increased risk of HCC (log-rank test, all $P < 0.05$).

Research conclusions

Baseline hepatocyte ballooning is a risk factor for adverse events in patients with CHB complicated with NAFLD.

Research perspectives

Larger samples and more rigorous prospective studies are needed for further verification. The study cohort needs to be observed for a longer time, and the conclusion may change if this is done, in the study.

FOOTNOTES

Author contributions: YW Tan and JM Wang contribute equally to research; YW Tan and XB Zhou designed the research; YW Tan and JM Wang collected and analyzed the data, and drafted the manuscript; YW Tan performed the liver pathological evaluations; YW Tan and XB Zhou wrote and revised the manuscript; All authors have read and approved the final version to be published.

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Institutional review board statement: This study was approved by the ethics committee of The Third People's Hospital Affiliated to Zhenjiang, Jiangsu University.

Conflict-of-interest statement: The authors declare that there is no relevant conflict of interest.

Data sharing statement: The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Extended criteria brain-dead organ donors: Prevalence and impact on the utilisation of livers for transplantation in Brazil

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Abstract

BACKGROUND

Despite its association with higher postoperative morbidity and mortality, the use of extended criteria donor (ECD) livers for transplantation has increased globally due to the high demand for the procedure.

AIM

To investigate the prevalence of ECD in donation after brain death (DBD) and its impact on organ acceptance for transplantation.

METHODS

Retrospective analysis of DBD organ offers for liver transplantation between 2017 and 2020 in a high-volume transplant centre. The incidence of the Eurotransplant risk factors to define an ECD (ET-ECD) among DBD donors and the likelihood of organ acceptance over the years were analysed. The relationship between organ refusal for transplantation, the occurrence, and the number of ET-ECD was assessed by simple and multiple logistic regression adjustment.

RESULTS

A total of 1619 organ donors were evaluated. Of these, 78.31% (n = 1268) had at least one ET-ECD criterion. There was an increase in the acceptance of ECD DBD organs for transplantation (1 criterion: from 23.40% to 31.60%; 2 criteria: from 13.10% to 27.70%; 3 criteria: From 6.30% to 13.60%). For each addition of one ET-ECD variable, the estimated chance of organ refusal was 64.4% higher (OR 1.644, 95%CI 1.469-1.839, P < 0.001). Except for the donor serum sodium > 165 mmol/L (P = 0.310), all ET-ECD criteria increased the estimated chance of organ refusal for transplantation.

CONCLUSION
A high prevalence of ECD DBD was observed. Despite the increase in their utilisation, the presence and the number of extended donor criteria were associated with an increased likelihood of their refusal for transplantation.

Key Words: Liver transplantation; Extended criteria donors; Donation after brain death; Organ donation

Core Tip: To suffice the demand of patients on the waiting list, the use of extended criteria donor (ECD) organs for transplantation has become a global need. This large retrospective analysis of 1619 donations after brain death (DBD) donor offers to a transplant centre in Brazil applied the Eurotransplant manual criteria to indicate an ECD. The prevalence of ECD was 78.31%. Whilst there was an increase in ECD-DBD liver transplantation over the years. Still, the presence and number of extended donor criteria were associated with an increased chance of donor organ rejection for transplantation.

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INTRODUCTION

Currently, organ shortage is a major limitation in transplantation. Although Brazil is the second country in the absolute number of liver transplants performed worldwide, it still needs to increase its figures. According to the Brazilian Transplant Registry, although 2245 liver transplants were performed in 2019, in that year, the waiting list had yet more 1213 people waiting for an organ[1]. In addition, the same report showed a progressive change in the demographic profile of organ donors, with an increase in the incidence of cerebrovascular diseases as the cause of death-in spite of trauma-and an increase in the proportion of donors older than 60 years old[1].

Although there is still no precise definition by the transplant community, donors who present, among other risk factors, older age, hypernatremia, prolonged time in the intensive care unit (ICU), abnormal liver enzymes, and moderate or severe steatosis are known as extended criteria donors (ECD)[2]. In addition, ECD allografts are associated with an increased risk of delayed graft function, primary nonfunction, and postoperative complications[2-4].

The first international study involving a large sample of patients promoted by the European Liver Intestine Transplant Association (ELITA) and the Eurotransplant Liver Intestine Advisory Committee (ELIAC) reports the following donor risk factors in liver transplantation: Age, ICU time, high body mass index (BMI), steatosis, hypernatremia, elevated alanine aminotransferase (ALT), elevated aspartate aminotransferase (AST), and raised total bilirubin levels[5]. Therefore, the donor is considered an ECD if one of these criteria is present.

To meet the demand of patients on the waiting list, using ECD organs for transplantation has become a global need[3,4]. For example, in the United States of America, from 2000 to 2005, the number of liver transplants increased by 21%[6]. Another study at the same centre reported a growth in the number of transplants with ECD organs (4.5% in 2008 compared to 0.5% in 1999)[7]. Furthermore, organ characteristics, such as ischaemia time and the use of partial grafts, negatively impact postoperative outcomes[3,8].

Despite the relevance of this topic and the numbers described above suggesting a demographic change in the organ donor population, data on ECD prevalence among organ donors and their utilisation rate are scarce. Therefore, this study aimed to evaluate the prevalence of ECD allografts in donation after brain death (DBD) liver transplantation and the likelihood of organ acceptance over the years.

MATERIALS AND METHODS

Design of the study, patient selection, and ethics statement

The study involved a retrospective analysis of data obtained from liver donor offers for the Solid Organ

Transplant Program of the Hospital Israelita Albert Einstein, São Paulo, Brazil, between June 2017 and December 2020. All liver allograft donors offered to our transplant centre over the study period were analysed. There were no exclusion criteria in the study. The study was reviewed and approved by the Research Ethics Committee of Hospital Israelita Albert Einstein with opinion 4.696.905, CAAE: 39704520.0.0000.0071.

Extended criteria donor definition

As previously defined by the Eurotransplant[9], an ECD was defined as the presence of one or more of the following donor characteristics reflective of a high chance of post-transplant complications such as primary nonfunction and early allograft dysfunction (ET-ECD): Age > 65 years old, ICU stay > 7 d, BMI > 30 Kg/m^2, liver steatosis > 40%, serum sodium > 165 mmol/L, ALT > 105 U/L, AST > 90 U/L, and total serum bilirubin > 3 mg/dL. Hepatic steatosis was evaluated by an experienced retrieval surgeon and reported as present when an estimation of more than 40% was observed.

Data collection

In addition to the donor data described above, other variables were collected. This collection included the donor's place of origin (local: Donor in the city of São Paulo; regional: Donor in the state of São Paulo; national: Donor in another Brazilian State), gender, blood type (ABO system), race, cause of death (cerebrovascular accident, trauma, hypoxia, and others), history of alcoholism, and presence of cardiorespiratory arrest among donors. All information was obtained from a retrospective institutional database prospectively maintained by the hospital liver transplantation program management team. This information was delivered anonymised to the researchers.

Outcome variables

The occurrence of the following outcomes over the years was assessed dichotomously (Yes *vs* No): (1) Organ offers acceptance for transplantation; and (2) transplantation of the donor organ. All these variables were considered only once, regardless of the number of times the organ was offered to different recipients of the transplant program.

Statistical analysis

Quantitative variables were described by medians and quartiles, given the distance between mean and median and asymmetry observed in the variables through histograms and normality tests. Categorical variables were described by absolute frequencies and percentages. Simple logistic regression models assessed the relationship between the occurrence and the number of ET-ECD criteria over the years. In addition, the simple Poisson regression adjustment was used to assess the year. The relationship between organ refusal for transplantation, the occurrence, and the number of ET-ECD criteria was also evaluated by simple and multiple logistic regression adjustment. Depending on the expected frequency per category, other associations between qualitative variables were assessed using Fisher's exact or Chi-squared tests. Finally, the nonparametric Mann-Whitney test was used to compare quantitative measures between groups, depending on the distribution of numerical measures. The SPSS statistical program version 22 (IBM Corp, Armonk, NY, United States) was used for analyses, and the significance level adopted was 5%.

RESULTS

A total of 1619 DBD liver donors were evaluated. The distribution of organ donor offers was proportionally similar during the studied period [2017 (6 mo): n = 251 (15.50%); 2018: n = 463 (28.60%); 2019: n = 455 (28.10%); 2020: n = 450 (27.79%)]. The mean donor age was 49.70 years old [standard deviation (SD) 14.74] and the mean donor BMI was 26.66 kg/m^2 (SD 4.68). A detailed descriptive analysis of the donor characteristics by year is presented in Table 1.

There were 351 (21.68%) donor offers without ET-ECD criteria and 1268 (78.32%) with at least one ET-ECD criterion from 2017 to 2020. The frequency of ECD was similar across years [2017 (6 mo): n = 197 (78.49%); 2018: n = 367 (79.27%); 2019: n = 349 (76.70%); 2020: n = 355 (78.89%)]. Of the ECD offers, 57.96% (n = 735) had two or more ET-ECD criteria. A descriptive analysis of the prevalence of ET-ECD features over the years is described in Table 2.

Analysis of extended criteria donor rate and their utilisation for transplantation per year

Every year after 2017, the estimated chance of a donor to be presenting with AST higher than 90 U/L is 17.7% greater [odds ratio (OR) 1.177, 95% confidence interval (CI) 1.068-1.298, P = 0.001]. There was no significant relationship between the year of offering and other ET-ECD risk factors. The results of the simple logistic regression model for the eight ET-ECD variables and the variable indicating the occurrence of at least one ET-ECD criterion are shown in Table 3. There was no significant relationship between the change in the number of ET-ECD characteristics by year of offering (estimated ratio of

Table 1 Descriptive analysis of donor organ characteristics per year, *n* (%)

Variable (year)	2017	2018	2019	2020	Total
Donor's place of origin					
Local	121 (48.21%)	180 (38.88%)	261 (57.36%)	257 (57.11%)	819 (50.59%)
Regional	67 (26.69%)	149 (32.18%)	128 (28.13%)	131 (29.11%)	475 (29.34%)
Nacional	63 (25.10%)	134 (28.94%)	66 (14.51%)	62 (13.78%)	325 (20.07%)
Gender, female	119 (47.60%)	193 (41.87%)	190 (41.76%)	174 (38.75%)	676 (41.86%)
Blood type (ABO system)					
A	82 (32.93%)	150 (32.47%)	157 (34.58%)	168 (37.42%)	557 (34.51%)
B	38 (15.26%)	52 (11.26%)	48 (10.57%)	36 (8.02%)	174 (10.78%)
AB	11 (4.42%)	16 (3.46%)	25 (5.51%)	7 (1.56%)	59 (3.66%)
O	118 (47.39%)	244 (52.81%)	224 (49.34%)	238 (53.01%)	824 (51.05%)
Race					
Black	39 (15.54%)	42 (9.07%)	57 (12.53%)	46 (10.22%)	184 (11.37%)
Mixed-race	80 (31.87%)	164 (35.42%)	159 (34.95%)	178 (39.56%)	581 (35.89%)
White	123 (49.00%)	253 (54.64%)	235 (51.65%)	223 (49.56%)	834 (51.51%)
Others	9 (3.59%)	4 (0.86%)	4 (0.88%)	3 (0.67%)	20 (1.24%)
Age (categories)					
< 40 yr	48 (19.12%)	90 (19.44%)	104 (22.86%)	115 (25.56%)	357 (22.05%)
40 yr to 49 yr	58 (23.11%)	102 (22.03%)	91 (20.00%)	106 (23.56%)	357 (22.05%)
50 yr to 59 yr	77 (30.68%)	134 (28.94%)	136 (29.89%)	123 (27.33%)	470 (29.03%)
60 yr to 69 yr	53 (21.12%)	99 (21.38%)	105 (23.08%)	82 (18.22%)	339 (20.94%)
≥ 70 yr	15 (5.98%)	38 (8.21%)	19 (4.18%)	24 (5.33%)	96 (5.93%)
Age (yr)	50.50 (14.52)	50.77 (15.07)	50.00 (14.09)	47.85 (15.03)	49.70 (14.74)
ICU stay > 5 d	105 (41.83%)	187 (40.39%)	178 (39.12%)	200 (44.44%)	670 (41.38%)
Cause of death					
Cerebrovascular accident	175 (70.00%)	294 (63.50%)	298 (65.49%)	277 (61.56%)	1044 (64.52%)
Trauma	43 (17.20%)	113 (24.41%)	101 (22.20%)	121 (26.89%)	378 (23.36%)
Hypoxia	24 (9.60%)	45 (9.72%)	40 (8.79%)	41 (9.11%)	150 (9.27%)
Others	8 (3.20%)	11 (2.38%)	16 (3.52%)	11 (2.44%)	46 (2.84%)
BMI (kg/m^2)	27.28 (5.04)	26.65 (5.00)	26.69 (4.57)	26.31 (4.20)	26.66 (4.68)
Alcoholism	75 (29.88%)	148 (31.97%)	115 (25.27%)	103 (22.89%)	441 (27.24%)
Cardiorespiratory arrest	61 (24.30%)	99 (21.38%)	82 (18.02%)	77 (17.11%)	319 (19.70%)
Vasoactive drugs in the donor	221 (88.05%)	422 (91.14%)	408 (89.67%)	405 (90.00%)	1456 (89.93%)
AST (U/L)[1]	56.00 (32.00; 102.00)	64.50 (37.00; 125.00)	74.00 (39.00; 151.00)	74.00 (39.40; 141.00)	68.00 (38.00; 132.00)
ALT (U/L)[1]	47.00 (24.00; 93.00)	49.00 (29.00; 96.00)	45.70 (26.00; 103.00)	47.00 (28.00; 89.00)	47.00 (27.00; 96.00)
GGT (U/L)[1]	84.00 (34.00; 182.00)	94.50 (38.55; 200.00)	83.50 (39.00; 207.00)	84.00 (36.00; 197.50)	87.00 (37.00; 198.00)
Total bilirubin (mg/dL)[1]	0.50 (0.32; 0.91)	0.50 (0.30; 0.91)	0.55 (0.35; 0.97)	0.52 (0.35; 0.90)	0.52 (0.33; 0.92)

[1]Categorical variables are presented in absolute numbers (frequency as a percentage). Continuous variables are presented as mean (standard deviation) or median (quartiles).

ICU: Intensive care unit; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase.

Table 2 Descriptive analysis of donor extended criteria over the years, *n* (%)

Variable (year)	2017	2018	2019	2020	Total
Macroscopic assessment of steatosis in the organ	5 (1.99)	0 (0.00)	3 (0.66)	4 (0.89)	12 (0.74)
Age > 65 yr	35 (14.00)	69 (14.90)	67 (14.73)	52 (11.56)	223 (13.78)
ICU > 7 d	81 (32.27)	143 (30.89)	145 (31.87)	153 (34.00)	522 (32.24)
BMI > 30 kg/m^2	61 (24.30)	82 (17.71)	90 (19.78)	80 (17.78)	313 (19.33)
Serum sodium > 165 mmol/L	39 (15.54)	82 (17.71)	73 (16.04)	64 (14.22)	258 (15.94)
AST > 90 U/L	72 (28.69)	172 (37.23)	184 (40.44)	187 (41.65)	615 (38.03)
ALT > 105 U/L	54 (21.51)	108 (23.38)	111 (24.45)	93 (20.71)	366 (22.65)
Total bilirubin > 3 mg/dL	6 (2.39)	13 (2.83)	25 (5.51)	18 (4.01)	62 (3.84)
Number of variables to classify a donor as an extended criteria donor					
0	54 (21.51)	96 (20.73)	106 (23.30)	95 (21.11)	351 (21.68)
1	94 (37.45)	159 (34.34)	122 (26.81)	158 (35.11)	533 (32.92)
2	61 (24.30)	133 (28.73)	133 (29.23)	119 (26.44)	446 (27.55)
3	32 (12.75)	58 (12.53)	68 (14.95)	59 (13.11)	217 (13.40)
4	9 (3.59)	15 (3.24)	24 (5.27)	17 (3.78)	65 (4.01)
5	1 (0.40)	2 (0.43)	2 (0.44)	2 (0.44)	7 (0.43)

Variables are presented as absolute numbers (frequency as a percentage). ICU: Intensive care unit; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 3 Occurrence of donor extended criteria according to the year of offer

Variable	Odds ratio (95%CI)	*P* value
Absence of macroscopic steatosis in the donor organ	0.845 (0.491; 1.455)	0.545
Donor age > 65 yr	0.925 (0.808; 1.059)	0.259
ICU stay > 7 d	1.037 (0.938; 1.147)	0.473
BMI > 30 kg/m^2	0.915 (0.813; 1.030)	0.140
Serum sodium > 165 mmol/L	0.943 (0.830; 1.071)	0.364
AST > 90 U/L	1.177 (1.068; 1.298)	0.001
ALT > 105 U/L	0.979 (0.876; 1.095)	0.717
Total bilirubin > 3 mg/dL	1.223 (0.952; 1.572)	0.116
≥ 1 donor extended criteria	0.991 (0.885; 1.110)	0.878

ICU: Intensive care unit; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

means 1.012, 95%CI 0.973-1.052, $P = 0.551$).

There was a reduction in the likelihood of donor organ refusal for transplantation during the studied period [2017 (6 mo): $n = 193$ (76.89%); 2018: $n = 360$ (77.75%); 2019: $n = 310$ (68.13%); 2020: $n = 319$ (70.89%)]. This reduction was due to the increased acceptance of ECD liver allografts for transplantation. As a result, there was an increase from 23.40% to 31.60% for 1 ET-ECD variable, from 13.10% to 27.70% for 2 ET-ECD variables, and from 6.30% to 13.60% for 3 ET-ECD variables. This growth in using ECD-DBD organs is reflected in the prevalence of ECD per year among the transplants performed, as demonstrated in Figure 1.

Impact of the presence of extended donor criteria on the refusal rate of organs for transplantation

For each addition of one ET-ECD criterion, the estimated chance of organ refusal for transplantation was 64.4% greater (OR 1.644, 95%CI 1.469-1.839, $P < 0.001$). The results of the logistic regression analysis showed that all ET-ECD variables increased the estimated chance of refusing the organ for

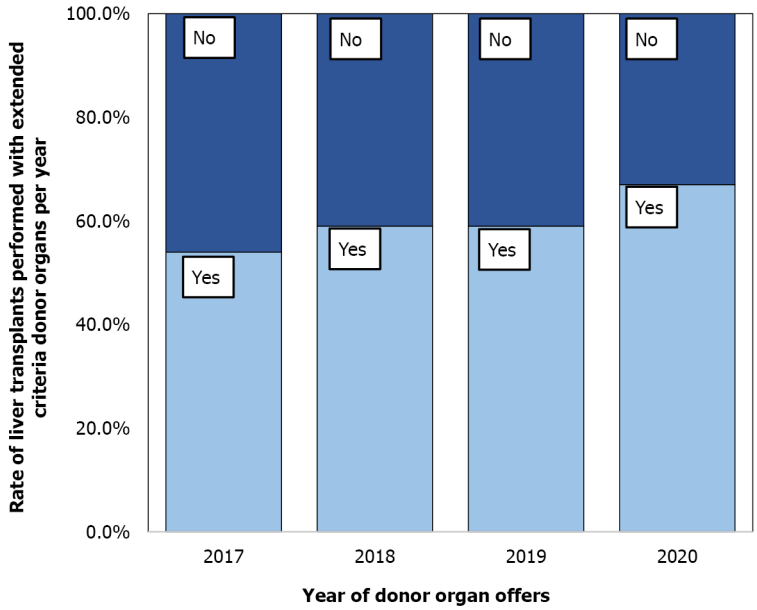

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Figure 1 Percentage of extended criteria donor liver transplantation over the years. Growth in using extended criteria donors after brain death can be noticed over the study period.

transplantation (Tables 4 and 5), except for donor serum sodium > 165 mmol/L (OR 1.173, 95%CI 0.862-1.596, $P = 0.310$).

All significant variables in this analysis were included in a multiple logistic regression model to assess the relationship between organ refusal for transplantation and the occurrence of ET-ECD criteria. A significant association was identified between all measures considered in the model and organ refusal. The results are presented in Tables 4 and 5, along with each category's estimated proportions of refusal. They were evaluated in an adjusted manner in relation to the other variables in the model.

DISCUSSION

Estimating ECD prevalence among DBD donors is critical to developing strategies to expand the use of these higher-risk organs safely. This large retrospective analysis of 1619 DBD organ donors identified a high prevalence of ET-ECD criteria. In addition, the ECD rate remained constant over the studied period. Although an increase in the rate of ECD organ transplantation was identified, the occurrence of these criteria was associated with their refusal for transplantation.

Using ECD organs for transplantation is necessary, even if associated with higher morbidity and mortality[10]. This risk is continuous and progressively more significant with the accumulation of adverse donor and organ characteristics. Several studies have described donor variables associated with an increased risk of graft failure after transplantation, *e.g.*, age, race, height, cerebrovascular accident as a cause of death, and split grafts[11].

By applying the ET-ECD criteria in the Eurotransplant region (Austria, Belgium, Croatia, Germany, Luxembourg, Netherlands, and Slovenia), at least one was present in more than 50% of liver donors [10]. Despite criticism regarding validating the prognostic value of these criteria, they are the only ones applied at the international level[12,13]. In the population investigated in our study, we found that almost 80% of DBD organ donors had at least one of these criteria to be considered an ECD.

Studies applying other criteria to classify an organ donor as an ECD have described their frequencies from approximately 50%[14] to 68%[15] in the United States of America and from 52.8%[16] to 59.9%[17] in Canada. In Brazil, a recent study applying different ECD indicative criteria described the transplantation of 56 ECD livers, representing 51% of the studied sample[18]. Previously, another study conducted in Brazil with data from 178 liver allografts reported an ECD rate of 76.97%[19]. Although these numbers support the high prevalence of ECD found in our study, the diversity of indicative criteria used in each study is a limiting factor for properly interpreting data.

The process of accepting a donor organ for transplantation considers characteristics of the recipient, such as the severity of the liver disease and their comorbidities, and factors of the donor and the donor organ. Non-transplanted livers are often from old donors, those with higher BMIs, viral hepatitis (B and C viruses), and a more significant number of comorbidities[20]. Still, findings in the biopsy are highlighted as a cause for discarding organs for transplantation[20,21]. In Brazil, a recent study reported

Table 4 Analysis of the relationship between the occurrence of donor extended criteria and the refusal rate of liver allografts for transplantation

Logistic regression for donor organ refusal for transplantation (*n* = 1619)		
Variable	**Odds ratio for refusal (95%CI)**	***P* value**
Absence of macroscopic steatosis in the donor organ	0.072 (0.016; 0.332)	< 0.001
Donor age > 65 yr	1.814 (1.264; 2.603)	0.001
ICU stay > 7 d	1.810 (1.408; 2.328)	< 0.001
BMI > 30 kg/m^2	2.215 (1.601; 3.065)	< 0.001
Serum sodium > 165 mmol/L	1.173 (0.862; 1.596)	0.310
AST > 90 U/L	1.713 (1.352; 2.171)	< 0.001
ALT > 105 U/L	2.007 (1.493; 2.697)	< 0.001
Total bilirubin > 3 mg/dL	3.011 (1.361; 6.664)	0.007

ICU: Intensive care unit; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 5 Analysis of the relationship between the occurrence of donor extended criteria and the refusal rate of liver allografts for transplantation

Multiple logistic regression model for donor organ refusal for transplantation (*n* = 1619)			
Variable	**Estimated proportionof refusal (95%CI)**	**Odds ratio for refusal (95%CI)**	***P* value**
Absence of macroscopic steatosis in the donor organ	41.10% (12.04%; 78.06%)	0.064 (0.013; 0.307)	< 0.001
Donor age > 65 yr	79.54% (59.84%; 91.02%)	1.973 (1.360; 2.861)	< 0.001
ICU stay > 7 d	79.18% (60.23%; 90.52%)	1.888 (1.455; 2.450)	< 0.001
BMI > 30 kg/m^2	80.69% (62.19%; 91.39%)	2.279 (1.628; 3.190)	< 0.001
AST > 90 U/L	76.57% (56.61%; 89.11%)	1.394 (1.051; 1.848)	0.021
ALT > 105 U/L	78.44% (58.88%; 90.24%)	1.729 (1.217; 2.456)	0.002
Total bilirubin > 3 mg/dL	82.44% (60.00%; 93.63%)	2.877 (1.282; 6.454)	0.010

ICU: Intensive care unit; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

that problems related to the donor organ (macroscopic pathological changes, visible organ damage, and inappropriate size) were the most common cause for donor organs not being used for transplantation [22].

The present study evaluated a significant sample of DBD organs over three years and a half. Although, probably because of the time interval studied, evolutionary changes in donor characteristics were not identified. The high prevalence of ECD was sustained during the study period. This diagnosis is concerning, especially considering the need to increase the number of transplants to meet the demand for the procedure. Therefore, implementing strategies to use ECD organs safely is necessary.

The routine application of the concept of donor-recipient risk balance (use of organs from higher-risk donors for recipients with lower severity of liver disease and fewer comorbidities) should underpin ECD organ transplantation[23,24]. However, alternative preservation methods may potentially be needed because of the inability of traditional static cold storage to maintain ECD organs effectively[25]. The application of dynamic organ preservation (the machine perfusion of the liver) in this setting is progressively more reported in the literature[3]. Machine perfusion aims to offer superior organ preservation, mitigate ischaemia-reperfusion injury in these highly vulnerable organs, assess their functional capacity, and potentially improve their quality before transplantation[26-29].

There are some limitations to this study. Firstly, this is a retrospective, single-centre study; drawing absolute conclusions based on this methodology may oversimplify the complexities of evaluating a donor organ offer for transplantation. In addition, although policies and the local culture of organ acceptance impact the decision of their use for transplantation, this effect is mitigated by their constancy during the study period. Furthermore, the reasons for discarding the offers were unavailable in our database. Consequently, some of these organs may have been initially declined for the first recipient of the program due to inappropriate size or logistical reasons, and another transplantation team may have

subsequently accepted them, therefore, not returning to a recipient at our institution. However, this effect is random across all subjects and may impact all donors equally-regardless of whether ECD. It is also important to note that due to the Model for End-Stage Liver Disease score-based system of donor organ allocation in Brazil, through a single list according to the severity of liver disease, the refusal rate of ECD organs in our service does not necessarily reflect the percentage of use of these organs for transplantation in the country.

CONCLUSION

This study evaluated a large sample of DBD organ donors and found a high and sustained prevalence of ECD in Brazil, which surpassed the numbers reported in other countries. An increase in the use of these higher-risk organs for transplantation was noticed during the study period, possibly due to the high demand for the procedure. Despite this fact, the refusal rate of DBD organs for transplantation remains high, and the presence and the addition of ET-ECD criteria were associated with an increased chance of them being refused. Therefore, implementing strategies to safely extend the use of ECD organs is critical and demands attention from the transplant community to benefit as many patients waiting for transplantation as possible.

ARTICLE HIGHLIGHTS

Research background

The use of extended criteria donor (ECD) organs for transplantation has become a global need due to the lack of donor organs to attend to the high demand for the procedure.

Research motivation

Knowing the real prevalence of ECD in donation after brain death (DBD) donor organs can pave the way for future research to understand better how to improve their use safely.

Research objectives

To determine the prevalence of ECD allografts in DBD liver transplantation and the likelihood of organ acceptance over the years.

Research methods

This is a retrospective, single-centre study. Liver donor offers for the Solid Organ Transplant Program of the Hospital Israelita Albert Einstein, Sao Paulo, Brazil, were included between June 2017 and December 2020. Multivariate analysis was performed to determine if any Eurotransplant ECD criteria (ET-ECD) were independent risk factors for organ refusal for transplantation.

Research results

The prevalence of ECD among a total of 1619 organ donors analysed was 78.31%. There was an increase in the acceptance of ECD DBD organs for transplantation along the studied period. Despite that, for each addition of one ET-ECD criterion, the estimated chance of organ refusal was 64.4% higher (OR 1.644, 95%CI 1.469-1.839, $P < 0.001$).

Research conclusions

There was a high prevalence of ECD DBD even though an increase in the utilisation rate of these higher-risk organs was noticed. The presence and the number of extended donor criteria were risk factors for their refusal for transplantation.

Research perspectives

Further research is needed to develop more general accepted criteria to indicate ECD donor organs. This must guarantee more reliable data for comparison between countries. Furthermore, based on this diagnosis, strategies to increase ECD liver transplantation safely are urgently needed to attend to the demand for the procedure.

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FOOTNOTES

Author contributions: Boteon YL contributed to study conception and design; Boteon YL, Braga VS, Boteon APCS, and Paglione HB contributed to acquisition of data; Boteon YL, Braga VS, Boteon APCS, Paglione HB, and Pecora RAA contributed to analysis and interpretation of data; Boteon YL, Braga VS, Boteon APCS, Paglione HB, and Pecora RAA contributed to drafting of manuscript; Boteon YL, Braga VS, Boteon APCS, Paglione HB, and Pecora RAA contributed to critical revision of manuscript; all authors contributed to editing and approved the final version of the article.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Hospital Israelita Albert Einstein (Approval No. 4.696.905 CAAE 39704520.0.0000.0071).

Informed consent statement: Informed consent was waived for patients in the study because of the study's retrospective nature and the use of a retrospective database.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prevalence of non-alcoholic fatty liver disease in patients with nephrotic syndrome: A population-based study

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is a global health concern with a prevalence of about 25% amongst United States adults. Its increased prevalence is attributed to increase in patients with obesity and metabolic syndrome, partly due to similar mechanisms of injury. Nephrotic syndrome (NS) is a clinical entity resulting from extensive proteinuria leading to hypoalbuminemia, hyperlipidemia, edema, and other complications. Given its association with hyperlipidemia, there is concern that patients with NS may be at increased risk of NAFLD.

AIM

To perform a cross-sectional population-based study to investigate the prevalence and risk factors of NAFLD in patients with NS.

METHODS

A large multicenter database (Explorys Inc., Cleveland, OH, United States) was utilized for this retrospective cohort study. A cohort of 49700 patients with a diagnosis of "Non-Alcoholic fatty liver disease" using the Systematized

Nomenclature of Medicine-Clinical Terms (SNOMED-CT) between 1999-2022 was identified. Inclusion criteria were age ≥ 18 years, presence of NAFLD, presence of NS. There were no specific exclusion criteria. Univariate and multivariate analysis were performed to adjust for multiple risk factors including age, gender, Caucasian race, NS, type II diabetes mellitus, hypothyroidism, dyslipidemia, obesity, metabolic syndrome and chronic kidney disease. Statistical analysis was conducted using R, and for all analyses, a 2-sided *P* value of < 0.05 was considered statistically significant.

RESULTS

Among the 78734750 individuals screened in this database, there were a total of 49700 subjects with NAFLD. In univariate analysis, the odds of having NAFLD in patients with NS, type 2 diabetes mellitus, hypothyroidism, dyslipidemia, obesity, metabolic syndrome and chronic kidney disease were 14.84 [95% confidence interval (95%CI) 13.67-16.10], 17.05 (95%CI 16.78-17.32), 6.99 (95%CI 6.87-7.11), 13.61 (95%CI 13.38-13.84), 19.19 (95%CI 18.89-19.50), 29.09 (95%CI 28.26--29.95), and 9.05 (95%CI 8.88-9.22), respectively. In multivariate analysis, the odds of having NAFLD amongst patients with NS were increased to 1.85 (95%Cl 1.70-2.02), while the odds were also remained high in patients that have type 2 diabetes mellitus [odds ratio (OR) 3.84], hypothyroidism (OR 1.57), obesity (OR 5.10), hyperlipidemia (OR 3.09), metabolic syndrome (OR 3.42) and chronic kidney disease (OR 1.33).

CONCLUSION

Patients with NS are frequently found to have NAFLD, even when adjusting for common risk factors. Hence, clinicians should maintain a high index of suspicion regarding presence of NAFLD in patients with NS.

Key Words: Non-alcoholic fatty liver disease; Nephrotic syndrome; Chronic kidney disease; Hyperlipidemia; Population-based study; Database

Core Tip: We conducted a population-based study to investigate the prevalence of non-alcoholic fatty liver disease (NAFLD) in patients with Nephrotic syndrome. We screened over 78 million individuals in a nationwide multicenter database. We performed a comprehensive multivariate analysis accounting for multiple cofounding factors including age ≥ 65 years, gender, Caucasian race, obesity, diabetes mellitus type 2, metabolic syndrome, dyslipidemia, chronic kidney disease and hypothyroidism. We found that patients with nephrotic syndrome had a higher prevalence of NAFLD. However, we could not account for certain confounders such as elevated uric acid levels, hormonal therapy, chemotherapy for tumors, and certain drugs such as corticosteroids, which are known to be risk factors for NAFLD. Further studies are required to confirm these findings and assess the utility of surveillance strategies for NAFLD in patients with nephrotic syndrome.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of chronic liver disease worldwide with a prevalence of about 25% in the adult world population. It is characterized by excessive hepatic deposition of fat without any other probable explanation including alcohol, viral hepatitis, inherited liver conditions, or protracted use of steatogenic drugs[1]. NAFLD is seen to occur in a progressive manner from steatosis to nonalcoholic steatohepatitis (NASH), which may lead to fibrosis and cirrhosis[2]. Multiple studies have confirmed that NASH and cirrhosis increase the risk of hepatocellular carcinoma (HCC), which is one of the most common causes of cancer related deaths worldwide [3]. It is thus no wonder that NAFLD and NASH have been recognized as a growing public health problem. The disease burden of NAFLD is influenced by diabetes mellitus type 2, obesity, metabolic syndrome and hypothyroidism which have all been recognized as risk factors in its development as

these conditions either directly or indirectly, promote fat accumulation in the liver[4-7]. Unfortunately, these conditions are not expected to decrease in the forthcoming decades. NAFLD and its related liver complications (NASH, cirrhosis and HCC) are the leading cause of chronic liver disease and the major cause of liver transplantation in the United States[8,9]. NAFLD is not only associated with liver related morbidity and mortality, clinical evidence also suggests its associations with other important extra-hepatic diseases such as cardiovascular diseases ranging from cardiomyopathy, coronary heart disease, cardiac arrhythmias to hypertension and kidney diseases such as chronic kidney disease[10-12]. These cardiovascular manifestations are recognized to be the leading cause of death in patients with NAFLD [13,14]. No wonder a tailored multistep approach involving lifestyle changes, anti-diabetic drugs and lipid lowering medications are have in been put in place for the management of NAFLD to reduce incidence of cardiovascular complication and also concomitantly treat existing comorbid conditions.

Nephrotic syndrome (NS) is a kidney disorder characterized by excessive proteinuria (urinary loss of ≥ 3 g of proteins per 24 h or, on a single spot urine sample, the presence of ≥ 2 g of protein per gram of urinary creatinine) resulting in hypoalbuminemia, dyslipidemia and oedema[15]. Dyslipidemia is known to cause premature atherosclerosis increasing the risk for acute coronary syndrome and stroke. Furthermore, there is increased risk of thrombosis in patients with nephrotic syndrome, not only from increased urinary loss of antithrombotic factors but also atherosclerosis induced platelet hyperreactivity [16]. Nutritional optimization as well as pharmacological interventions involving use of, Ace inhibitors, albumin, corticosteroid, antibiotic, anticoagulation therapy have all been proposed as measures to reduce mortality from NS.

Given their association with dyslipidemia, NAFLD and NS might have similarities in their pathophysiology. Both disease processes are associated with elevated levels of circulating free fatty acid [17-20]. In NAFLD, patients have underlying insulin resistance causing decreased inhibitory effect of insulin on peripheral lipolysis leading to increased pool of circulating free fatty acid and glycerol. As fat and triglycerides in the form of VLDL accumulates in the liver, it eventually leads to excessive production of ROS by Kupffer cells and alteration in mitochondrial DNA occurs. This demonstrates the slowed progression of hepatic steatosis to NASH, hepatocellular necroinflammation and fibrosis and lastly carcinoma[21-24]. Interestingly, patients with NS also exhibit dysregulated fatty acid metabolism with or without the presence of chronic kidney disease. In these patients, injury to podocytes stems from elevated plasma concentrations of major lipoproteins. This alteration in lipid metabolism stems from downregulation of lipoprotein lipase in peripheral tissues, suppression of hepatic lipase and increased activity of acetyl-CoA carboxylase and fatty acid synthase[17-20].

Our hypothesis is that the excess synthesized and circulating lipids in patients with NS affect fat metabolism in the liver, increasing the risk of NAFLD. It has been proven that NS might lead to chronic kidney disease (CKD), and there have been studies suggesting increased prevalence of NAFLD in patients with CKD[4,25-27]. However, there have been few studies, if any, correlating prevalence of NAFLD in patients with NS. Given the increasing prevalence of NAFLD and associated morbidity and mortality, identification of at-risk patients is essential for targeted monitoring and treatment. Since NAFLD and NASH often do not cause any symptoms, surveillance strategies for at-risk patients might aid in early diagnosis and help prevent adverse outcomes. Since both NAFLD and NS are associated with elevated circulating lipids, patients with NS might be at risk for NAFLD, especially if they have other risk factors for NAFLD such as diabetes mellitus, obesity, or steroid use. It is essential to know if NS itself can be a risk factor for NAFLD, since only then can cost-effectiveness and usefulness of any surveillance and preemptive strategies be commented on. Furthermore, if patients with NS are at increased risk of NAFLD, more aggressive approach towards controlling other NAFLD risk factors and reducing use of certain medications such as steroids might be warranted. Therefore, we conducted a study with the aim of assessing the prevalence as well as risk factors of NAFLD in patients with NS.

MATERIALS AND METHODS

Our cohort's data were obtained using a validated, multicenter and daily-updated database called Explorys (Explorys Inc, Cleveland, OH, United States) developed by IBM Corporation, Watson Health [IBM corporation]. Explorys consists of electronic health records of 26 different healthcare systems with a total of about 360 hospitals and more than 70 million patients across the United States. Explorys utilizes Systematized Nomenclature of Medicine-Clinical Terms (SNOMED-CT) for the definition of the diseases. The diagnosis is made by individual health care providers and the collected data is then uploaded into the database in the form of SNOMED-CT codes. The database pools large outpatient as well as inpatient deidentified data that can be formulated into numerous cohorts according to the clinical element being studied. Explorys does not record individual patient data such as laboratory or imaging results. Since the data is pooled from multiple organizations, different organizations, and by extension health care providers, may differ in method of diagnoses of various medical conditions. The way the database is established, assessment of the method of diagnoses is not feasible, and thus the database is largely dependent on individual organizations providing accurate data. The approval of Institutional Review Board is not required since Explorys is a Health Insurance Portability and Account-

ability Act (HIPAA)-compliant platform. Use of this database has been validated in multiple fields including cardiology, hematology and gastroenterology.

Patient selection

A cohort of patients with a SNOMED-CT diagnosis of "Non-Alcoholic Fatty Liver Disease" and "Nephrotic syndrome" between 1999 and May 2022 was identified. Inclusion criteria were age ≥ 18 years, presence of NAFLD, presence of NS. There were no specific exclusion criteria.

Covariates

We collected age > 65 years, gender and Caucasian race as variables. Confounding factors associated with NAFLD and NS were also identified and collected if SNOMED-CT diagnoses were available. These were obesity, diabetes mellitus type 2, metabolic syndrome, dyslipidemia, chronic kidney disease and hypothyroidism.

Statistical analysis

To account for confounding from the covariates listed above, we conducted 1024 searches to explore every probability, with NS as one of the variables. A univariate analysis was conducted initially for all the variables, followed by multivariate analysis. Statistical analysis was performed using R and RStudio (version 1.4.1717), and for all analyses, a 2-sided *P* value of < 0.05 was considered statistically significant. Multivariate analysis was performed to adjust for multiple factors including age ≥ 65 years, gender, caucasian race, obesity, diabetes mellitus type 2, metabolic syndrome, dyslipidemia, chronic kidney disease and hypothyroidism. The study was reviewed by our expert biostatistician Antoine Boustany, MD, MPH, MEM.

RESULTS

Among the 78734750 individuals screened in this database, there were a total of 49700 subjects with NAFLD. Most subjects with NAFLD were between the age of 18-65 years, with female affected more than males. Interestingly, while majority of subjects were Caucasians, 5% were African Americans. About half the patients with NAFLD had BMI ≥ 30, with the prevalence of NAFLD rising with the increase in BMI (Table 1). In univariate analysis, the odds of having NAFLD with age ≥ 65 years was 2.18 [95% confidence interval (95%CI) 2.15-2.22], while it was also high in females [odds ratio (OR) 1.18, 95%CI 1.16-1.20], Caucasians (OR 3.62, 95%CI 3.55–3.69), subjects with NS (OR 14.84, 95%CI 13.67-16.10), type 2 diabetes mellitus (OR 17.05, 95%CI 16.78-17.32), hypothyroidism (OR 6.99, 95%CI 6.87-7.11), dyslipidemia (OR 13.61, 95%CI 13.38-13.84), obesity (OR 19.19, 95%CI 18.89-7.11), metabolic syndrome (OR 29.09, 95%CI 28.26-29.95) and CKD (OR 9.05, 95%CI 8.88-9.22). In multivariate analysis, the odds of having NAFLD amongst patients with nephrotic syndrome was 1.85 (95% CI 1.70-2.02), while the odds also remained high in patients that have type 2 diabetes mellitus (OR 3.84), hypothyroidism (OR 1.57), obesity (OR 5.10), hyperlipidemia (OR 3.09), metabolic syndrome (OR 3.42) and CKD (OR 1.33) (Figure 1).

DISCUSSION

With the high prevalence of NAFLD and its associated complications, there is worldwide interest in learning more about the disease and its associations with other systemic illnesses. To date despite extensive research, we were unable to find another study reporting the prevalence of NAFLD in patients with NS. Two prospective studies conducted by Targher *et al*[28,29], one in patients with type 1 diabetes mellitus (T1DM) and the other in T2DM, to assess the development of CKD in patients with NAFLD did not report development of NS in any patient over a follow-up period of 5.2 years and 6.5 years, respectively. In our study, patients with NS were frequently found to have NAFLD. One explanation is that impairment in lipid metabolism in NS promotes development of NAFLD. However, further studies are needed to explore this possibility.

In contrast, there have been several studies assessing renal impairment in patients with NAFLD. The results of these studies have been contradictory. Musso *et al*[10] conducted a systematic review and meta-analysis of articles published through 1980 -2014 and showed that NAFLD was associated with increase in prevalence as well as incidence of CKD [odds ratio (OR) 2.12, 95%CI 1.69-2.66; and hazard ratio (HR) 1.79, 95%CI 1.65-1.95, respectively]. Furthermore, NASH was associated with a higher prevalence and incidence of CKD (OR 2.53, 95%CI 1.58-4.05; and HR 2.12, 95%CI 1.42-3.17, respectively) than simple steatosis[10]. Our study had similar results, with increased odds of having CKD in patients with NAFLD, which remained significant on multivariate analysis.

In comparison, two studies by Targher *et al*[29], one conducted in patients with type 2 diabetes mellitus and the other in type 1 diabetes mellitus, showed that patients with NAFLD had lower

Table 1 Baseline characteristics of patients with non-alcoholic fatty liver disease

Parameters	NAFLD, *n* (%)	No NAFLD, *n* (%)	*P* value
Age, yr			< 0.00001
Adults 18-65	30980 (62.33)	56486180 (71.79)	
Seniors > 65	18720 (37.67)	22198870 (28.21)	
Gender			< 0.00001
Male	20640 (41.53)	35921730 (45.65)	
Female	29060 (58.47)	42763320 (54.35)	
Race			< 0.00001
Caucasian	39420 (79.32)	40569460 (51.56)	
African American	2550 (5.13)	7765730 (9.87)	
Hispanic/Latino	790 (1.59)	1037520 (1.32)	
Other	6940 (13.96)	29312340 (37.25)	
BMI			< 0.00001
< 18.5	1180 (2.38)	3610880 (4.59)	
18.5-24.9	7860 (15.81)	13727720 (17.45)	
25.0-29.9	16810 (33.82)	13117450 (16.67)	
> 30.0	23850 (47.99)	48229000 (61.29)	
Type 2 diabetes mellitus	24830 (49.95)	4526510 (5.75)	< 0.00001
Metabolic syndrome	3640 (7.32)	205830 (0.26)	< 0.00001
Hyperlipidemia	33130 (66.65)	10,152,960 (12.90)	< 0.00001
Nephrotic syndrome	100 (0.14)	17300 (0.02)	< 0.00001
Hypothyroidism	11930 (24.00)	3472880 (4.41)	< 0.00001
Chronic kidney disease	13485 (27.13)	2347230 (2.98)	< 0.00001
Total	49700	78685050	

NAFLD: Non-alcoholic fatty liver disease.

estimated glomerular filtration rate and increased incidence of CKD as compared to patients without NAFLD In contrast, a study by Sirota *et al*[30] conducted on the National Health And Nutrition Examination Survey III (NHANES III) data showed increased prevalence of NAFLD in patients with CKD, which was not significant after adjusting for certain risk factors. One possible explanation for these discrepancies is that the prevalence of NAFLD in CKD may be driven by race, which was adjusted for in the latter study but not the former one. In our study, the prevalence of NAFLD remained significant in patients with CKD, even on multivariate analysis and adjusting for Caucasian race. The reason for this discrepancy is unclear, although a larger sample size in our cohort might have played a role.

With regards to factors associated with NAFLD, our study concluded that patients with type 2 DM, obesity, hypothyroidism, metabolic syndrome and hyperlipidemia have higher prevalence of NAFLD, even on multivariate analysis, which is similar to studies done elsewhere[6,28,29,31]. One interesting finding was that 5% of patients with NAFLD in our cohort identified as African American, which is consistent with low prevalence of NAFLD in this population as reported in the literature[32]. In our study, the prevalence of NAFLD increased as BMI rose, with a prevalence of 48% in subjects with BMI ≥ 30 as compared to 33.82%, 15.81%, and 2.38% in patients with BMI 25.0-29.9, 18.5-24.9, and < 18.5, respectively. Similar results have been observed in literature, with one study by Loomis *et al*[6], demonstrating a strong and striking near-linear relationship between BMI and future risk of recorded NAFLD.

Our study has several strengths. To the best of our knowledge, this is the first study to assess the prevalence of NAFLD in patients with NS. Being a multicenter study with a large sample size derived from the United States population, our results are reliable and generalizable. We assessed several common risk factors, and our study showed that these factors were independently associated with increased prevalence of NAFLD, which have been well documented in the literature.

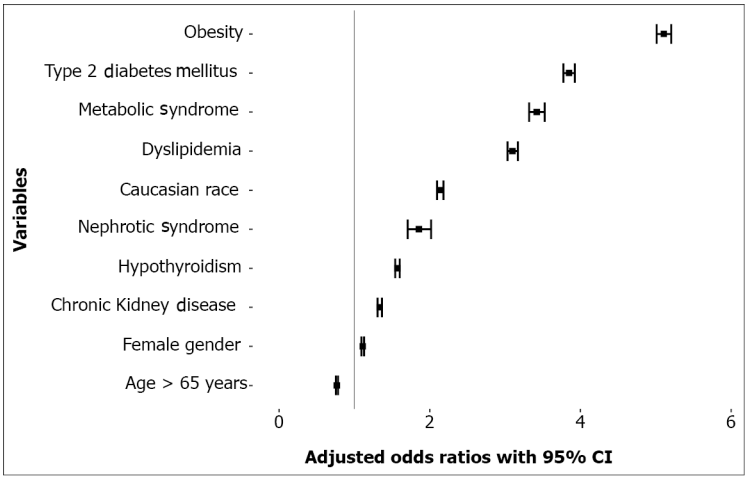

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Figure 1 Multivariate analysis assessing the risk of non-alcoholic fatty liver disease. 95%CI: 95% confidence interval.

LIMITS OF THE STUDY

Limitation to our study includes its retrospective nature and inability to establish causality. Being a database study, there is always a concern regarding selection bias. Furthermore, given that this database is HIPAA-compliant and anonymous, it is not possible to verify the accuracy of the diagnoses made. Hence, further in-depth analysis is not feasible. Also, certain NAFLD risk factors such as presence of elevated uric acid levels and pharmacological interventions such as corticosteroid use, hormonal therapy, certain chemotherapeutic agents, *etc.* could not be assessed.

CONCLUSION

Our study demonstrates that patients with NS are frequently found to have NAFLD, even when adjusting for common risk factors including CKD. Females and subjects with age 18-65 years were most commonly affected with NAFLD, with most subjects being Caucasians and only 5% were African American. The American Association for the Study of Liver Disease still recommends against routine screening for NAFLD in any population[1]. Further studies are needed to assess the relationship between NS and NAFLD. While lipid metabolism is abnormal in both these diseases, whether these diseases develop independently of each other or through a common pathway needs to be further explored. Clinicians should be aware of the increased prevalence of NAFLD in this patient population.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of chronic liver disease worldwide, with hyperlipidemia as one of its risk factors. Nephrotic syndrome (NS) is known to cause hyperlipidemia. Since both NAFLD and NS patients are known to have abnormalities in lipid metabolism, patients with NS might be at increased risk of developing NAFLD.

Research motivation

Given the increasing prevalence of NAFLD and associated morbidity and mortality, assessment of risk factors for targeted surveillance is warranted. This might help in early diagnosis of NAFLD and improve outcomes. We hypothesized that the excess synthesized and circulating lipids in patients with NS affect fat metabolism in the liver, increasing the risk of NAFLD.

Research objectives

To conduct a cross-sectional population-based study to assess the prevalence of NAFLD in patients with NS while adjusting for common risk factors.

Research methods

A large multicenter database (Explorys Inc., Cleveland, OH, United States) was utilized for this study. A cohort of patients with a diagnosis of "Non-Alcoholic fatty liver disease" was identified. Inclusion criteria were age ≥ 18 years, presence of NAFLD, presence of NS. There were no specific exclusion criteria. Univariate and multivariate analyses were performed to adjust for multiple risk factors including age, gender, Caucasian race, nephrotic syndrome, type II diabetes mellitus, hypothyroidism, dyslipidemia, obesity, metabolic syndrome and chronic kidney disease. Statistical analysis was conducted using R, and for all analyses, a 2-sided *P* value of < 0.05 was considered statistically significant.

Research results

In multivariate analysis, the odds of having NAFLD amongst patients with NS was 1.85 (95%Cl 1.70-2.02), while the odds also remained high in patients that have type 2 diabetes mellitus (OR 3.84), hypothyroidism (OR 1.57), obesity (OR 5.10), hyperlipidemia (OR 3.09), metabolic syndrome (OR 3.42) and chronic kidney disease (CKD) (OR 1.33).

Research conclusions

Our study demonstrates that patients with NS are frequently found to have NAFLD, even when adjusting for common risk factors including CKD. Further studies are required to confirm these findings, investigate causality and assess the utility of surveillance strategies for NAFLD in patients with NS.

Research perspectives

Studies assessing associations of NAFLD with other diseases can help identify at-risk populations that may benefit from routine screening. While patients with NS seem to have higher prevalence of NAFLD, further research is required to assess if routine surveillance of patients with NS is cost-effective and improves outcomes.

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FOOTNOTES

Author contributions: Onwuzo S designed the research study; Hitawala A and Boustany A performed the biostatistical analysis; Boustany A and Kumar P carried out the data collection; Onwuzo S, Hitawala A, Onwuzo C, Almomani A, Monteiro J, and Asaad I contributed to the manuscript writing, editing and scientific review; All authors have read and agree to the submitted version of the manuscript.

Institutional review board statement: Our cohort's data were obtained using a validated, multicentered and daily-updated database called Explorys (Explorys Inc, Cleveland, OH, United States). Explorys does not record individual patient data such as name, laboratory or imaging results. Patient's informed consent and approval of Institutional Review Board are not required since Explorys is a Health Insurance Portability and Accountability Act (HIPAA)-compliant platform.

Informed consent statement: Consent was not obtained but the presented data are anonymized without any risk of identification.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at onwuzos@ccf.org. Consent was not obtained but the presented data are anonymized without any risk of identification.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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ORIGINAL ARTICLE

Retrospective Study

Diabetes mellitus is not associated with worse short term outcome in patients older than 65 years old post-liver transplantation

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Abstract

BACKGROUND
Non-alcoholic fatty liver disease is a global health care challenge and a leading indication of liver transplantation (LT). Hence, more patients with diabetes mellitus (DM) are undergoing LT, especially, above the age of 65.

AIM
To evaluate the impact of DM on short-term outcomes post-LT in patients over the age of 65.

METHODS
We collected data of patients who underwent LT from January 2001 until December 2019 using our electronic medical record. We assessed the impact of DM on short-term outcomes, one-year, post-LT based on the following variables: Survival at one year; acute cellular rejection (ACR) rates; intensive care unit (ICU) and hospital length of stay; and readmissions.

RESULTS
Total of 148 patients who are 65 year or older underwent LT during the study

period. The mean age is 68.5 ± 3.3 years and 67.6% were male. The median Model for End-stage Liver Disease score at time of transplantation was 22 (6-39), 39% of patients had hepatocellular carcinoma and 77.7% underwent living donor LT. The one-year survival was similar between DM patients and others, 91%. ACR occurred in 13.5% of patients (P = 0.902). The median ICU stay is 4.5-day P = 0.023. The rates of ICU and 90-d readmission were similar (P = 0.821) and (P = 0.194), respectively.

CONCLUSION

The short-term outcome of elderly diabetic patients undergoing LT is similar to others. The presence of DM in elderly LT candidates should not discourage physicians from transplant consideration in this cohort of patients.

Key Words: Acute cellular rejection; Diabetes mellitus; Elderly; Graft survival; Liver transplantation

Core Tip: Diabetes mellitus (DM) is very common in elderly patients who are candidates for liver transplant. In a single center experience, DM did not affect the short term outcome in elderly patients who underwent liver transplantation (LT). Hepatitis C virus and non-alcoholic steatohepatitis were the leading indications for LT. Majority of patients in this study had living liver donors.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is increasingly becoming a global healthcare challenge with an estimated worldwide prevalence of 24%[1,2]. The leading causes behind the increase are obesity, diabetes mellitus (DM) and dyslipidemia[3]. In recent years, the term metabolic dysfunction-associated fatty liver disease has been put forward as a more inclusive name for NAFLD, however this has not been universally accepted as of yet. Similarly, NAFLD, previously considered a disease of exclusion, is widely accepted as a disease of inclusion and can co-exist with additional chronic liver diseases[3]. It is linked to insulin resistance and fat metabolism dysregulation[4], and it can progress to non-alcoholic steatohepatitis (NASH) and advanced cirrhosis in 25% of patients[5]. Therefore, after the advent of effective direct antiviral therapy for hepatitis C (HCV), NAFLD is now becoming a leading indication for liver transplantation (LT) worldwide[6] and expected to surpass other indications[5].

Currently, the prevalence of NAFLD in Saudi Arabia is 25%, one of the highest rates in the world[7]. Studies have shown a progressive rise in obesity and diabetes in Arab countries and Saudi Arabia[8]. It is not surprising, therefore, that an estimated 30% of the Saudi population could have NAFLD by 2030 [7]. In addition, the median age of the population is also increasing[9]. Hence, an increasing number of LTs will be performed on older patients with DM or obesity.

More people above the age of 65 have become candidates for LT[10]. Studies demonstrate that age alone should not disqualify patients from LT if they have no other major contraindications. Functional status and comorbidities are particularly important considerations regarding transplantation in this cohort of patients. Older people often have multiple comorbidities, such as coronary artery disease and DM, that contribute to worse short- and long-term outcomes which vary across transplant centers[10]. Therefore, LT candidates undergo extensive cardiopulmonary evaluation prior to transplantation.

DM is associated with increased mortality among patients with liver cirrhosis[11]. Studies demonstrate that long-term outcomes after LT on both patient and graft survivals, particularly in older populations, are poor; while studies of short-term outcomes are limited[12]. The impact of diabetes on short-term outcomes such as intensive care unit (ICU) stay, length of hospital stay, and acute cellular rejection (ACR) is unknown with regard to Saudi Arabia. Knowledge of these outcomes can inform guidelines for recipient suitability, pre-operative assessment, and immediate post-operative management.

This study aims to evaluate the role of DM as an independent predictor of short-term outcomes in LT recipients aged 65 and over.

MATERIALS AND METHODS

Using our electronic medical record system, we retrospectively collected data of patients who underwent LT from January 2001 until December 2019 at King Faisal Specialist Hospital & Research Center in Riyadh. We included all patients who were 65 years or older at the time of transplantation.

We collected basic demographic data (age, gender), body mass index (BMI), indication for transplantation, presence of co-morbidities (DM, hypertension, dyslipidemia, coronary heart disease), and outcomes. We assessed the impact of DM on short-term outcomes, one year, post-LT based on the following variables: Survival at one year; ACR rates; ICU and hospital length of stay (LOS); and readmissions. The diagnoses of DM, hypertension, dyslipidemia, and coronary artery disease were based on the international classification of diseases, 10th revision. ACR must have been biopsy proven with histological changes consistent with ACR.

The Institutional Research Board at King Faisal Specialist Hospital and Research Center approved the study. The consent was waived given the retrospective nature of the study.

Transplantation evaluation and follow up

Generally, listing patients for LT and ranking them on the waitlist at our center is based on the Model for End-stage Liver Disease (MELD)[13]. Patients were assigned to one of the following rankings: (1) Status 1A for acute liver failure; (2) The calculated MELD score; and (3) MELD exception for patients with hepatocellular carcinoma (HCC), hepatopulmonary syndrome, or portopulmonary hypertension. Patients with HCC were discussed in the tumor multidisciplinary board for locoregional therapy options while completing the workup or waiting for LT. The MELD score was assessed and updated regularly. All patients were seen in the outpatient clinics regularly and within three months prior to their LT.

The standard immunosuppression protocol in our institution includes calcineurin inhibitors and mycophenolate mofetil during the first 6-12 mo after transplantation and oral prednisone for the first 3 mo. The doses of immunosuppressive medications were adjusted according to their serum levels and were modified in patients with renal impairment. We aim to minimize immunosuppression post liver transplantation in patients with HCC.

Statistical analysis

We used SPSS software (version 21.0; SPSS, Inc., Chicago, IL, United States) for statistical analyses. Data are described in counts and percentages, medians and ranges, and means and standard deviations. Fisher exact or chi-square tests were used to compare categorical variables. Mann-Whitney and *t* tests were used for continuous nonparametric and parametric variables, respectively. Kaplan-Meier curves were used to estimate 1-year patient survival rates, and log-rank test was used to compare survival between the groups. A significance level of alpha = 0.05 was set.

RESULTS

A total of 148 patients aged 65 years or older underwent LT during the study period. Living donor LT was performed on the majority of the patients (115, 77.7%). The baseline characteristics are summarized in Table 1. Patients were predominantly male (100, 67.6%) with a mean age of 68.5 ± 3.3 years. The median MELD score was 22 (6–39) just prior to transplantation, and hepatocellular carcinoma was present in 58 (39.2%) of patients, with or without other liver diseases.

Risk factors-namely hyperlipidemia, essential hypertension, cardiac ischemia, and renal impairment-were similar for both diabetic and non-diabetic patients (Table 1). Nondiabetic patients (non-DM) had a higher BMI (28.6 ± 6.1) than patients with diabetes (DM), P = 0.048. The main indication for LT was HCV (52, 35.1%) followed by NASH (51, 34.5%).

There was a similar median follow-up of 33.5 mo for both diabetic and non-diabetic groups, with a one-year survival rate of 89% (Figure 1). ACR arose in 20 (13.5%) of the total study population (DM = 13, 13.3% and non-DM = 7, 14%; P = 0.902). With regard to ICU readmission, the DM rate was 11 (11.2%) while non-DM was 5 (10%; P = 0.821). Although hospital LOS was comparable (DM = 23 d and non-DM = 22 d; P = 0.717), the median ICU stay was shorter in days for DM patients, DM = 4 (1-70) compared to non-DM = 5 (2-185), P = 0.023. The 90-d readmission rate was likewise largely similar (DM = 38.8% and non-DM = 28%; P = 0.194). The presence of HCC did not affect survival outcomes within the first year after transplantation (Figure 2).

In the first-year post-transplantation, 31.5% of patients experienced at least one infectious event. DM patients had a higher rate of infections (40.8% *vs* 24%, P = 0.043). However, there has been no statistically significant difference regarding the site of infection (Figure 3). Intrabdominal infections are the most commonly seen infectious source 22.4% followed by pneumonia 14.3%.

Table 1 Baseline characteristics of the studied sample

Variables		All, *n* = 148	DM, *n* = 98	No DM, *n* = 50	*P* value
Age (years)[1]		68.5 ± 3.3	68.4 ± 3.1	68.5 ± 3.9	0.578
Gender (Male)[2]		100 (67.6%)	69 (70.4%)	31 (62.0%)	0.301
Living Donor[2]		115 (77.7%)	79 (80.6%)	37 (74.0%)	0.355
Cause of liver disease[2]	HCV	52 (35.1%)	32 (32.7%)	20 (40%)	0.341
	HBV	24 (16.2%)	14 (14.3%)	10 (20%)	
	NASH	51 (34.5%)	35 (35.7%)	16 (32%)	
	Others	21 (14.2%)	17 (17.3%)	4 (8%)	
HCC[2]		58 (39.2%)	40 (40.8%)	18 (36.0%)	0.570
MELD[2]		22 (6-39)	22 (6-39)	21 (8-35)	0.833
BMI[1] (kg/m^2)		26.7 ± 5.1	26.2 ± 4.6	28.6 ± 6.1	0.048[a]
HTN[2]		52 (35.1%)	42 (42.9%)	10 (20.0%)	0.006[a]
Hyperlipidemia[2]		10 (6.8%)	9 (9.2%)	1 (2.0%)	0.100
CAD[2]		4 (2.7%)	3 (3.1%)	1 (2.0%)	0.692
CKD[2]		40 (26.4%)	28 (28.6%)	12 (24.0%)	0.554
On insulin[2]		60 (41.2%)	60 (60.2%)	0	
On OHA[2]		46 (31.1%)	46 (46.9%)	0	
HbA1c[1]		5.9 ± 1.7	6.5 ± 1.7	4.6 ± 0.8	0.000[a]
Length of stay (days)[3]		24 (2-275)	23 (2-275)	22 (4-149)	0.717

[a]$P < 0.05$.
[1]Results in mean ± SD.
[2]Results in counts (percentage).
[3]Results in median (range).
DM: Diabetes mellitus; BMI: Body mass index; CKD: Chronic kidney disease; HbA1c: Hemoglobin A1c; CAD: Coronary arterial disease; HBV: Hepatitis B; HCC: Hepatocellular carcinoma; HCV: Hepatitis C; HTN: Hypertension; MELD: Model for end-stage liver disease; NASH: Non-alcoholic steatohepatitis; OHA: Oral hypoglycemic agent.

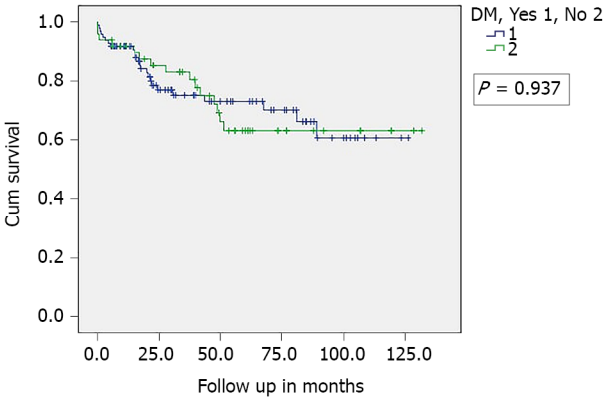

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Figure 1 One-year survival in our sample. DM: Diabetes mellitus.

DISCUSSION

Data from recent literature suggests that diabetics who are candidates for, or are in the post-operative context of, LT might have severe negative impact on the long-term outcome of these patients. Therefore, adequately controlling diabetes is crucial to increasing candidacy for LT and improving long-term outcomes[14]. The short-term outcome of diabetes among older patients undergoing LT is unknown. Furthermore, the data are extremely limited in this subgroup of patients who have undergone living

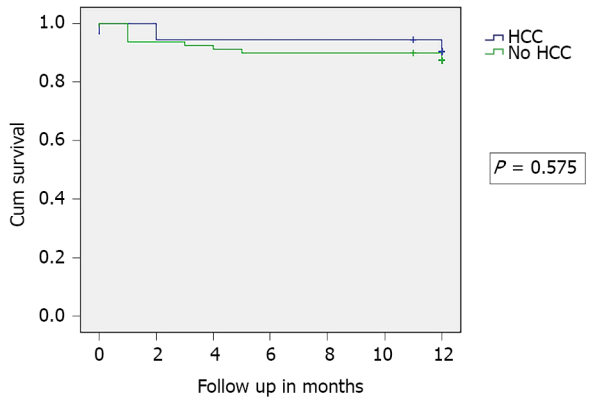

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Figure 2 Survival and hepatocellular carcinoma in our sample. HCC: Hepatocellular carcinoma.

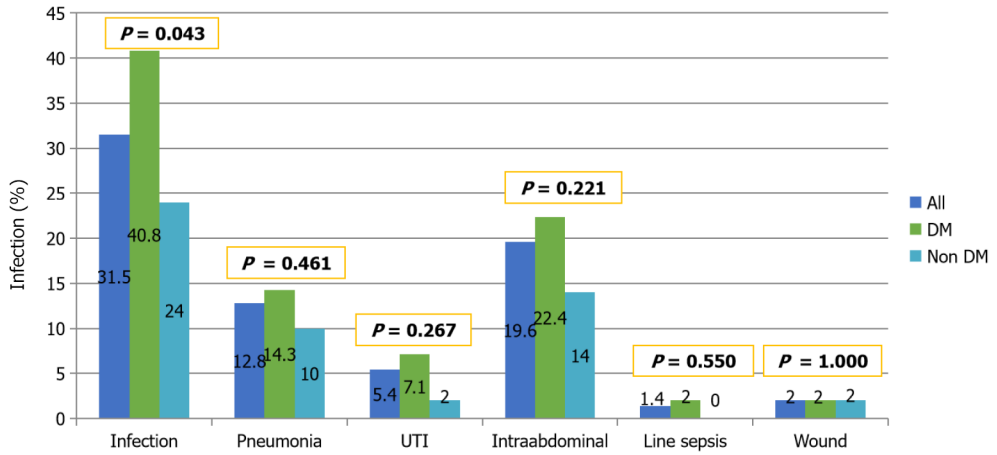

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Figure 3 Infection rates in our sample. DM: Diabetes mellitus; UTI: Urinary tract infection.

donor LT. Our results showed an excellent one-year survival rate of 89%, which is comparable with the survival rate among highly performing LT centers[15,16]. We also found that the survival rate was similar between deceased-donor and living-donor LT patients in this cohort. The presence of DM before LT did not have a negative impact on short-term survival. Aravinthan *et al*[12] showed that neither pretransplant nor posttransplant DM affect the survival post-LT[12]. However, an association was found between chronic renal failure, major cardio-vascular diseases and pretransplant DM. In contrast to our study, they included younger patients as well, with a median age of 54. Other larger studies have shown that DM has a statistically significant negative effect on patient and graft survival[17].

Both patients with diabetes and those without experienced ACR at a similar rate. Several reports illustrated an increased risk of ACR and graft loss among patients with pretransplant DM. Most ACR occurs within the first year after transplantation. A study by Lieber *et al*[18] demonstrated an increased risk of ACR among patients with posttransplant DM[18], although a smaller study did not detect any effect of either pre-transplant DM or post-transplant DM on ACR[19]. In general, however, patients with pre-transplant DM experience worse graft survival rates[17,20]. As expected, more diabetic patients had infections in their first-year post transplantation. The infection specific site was similar between both groups. Despite increased infectious complications in the DM patients, the survival rate is similar as outlined above.

Both groups had similar ICU and hospital stays, and the rate of readmission was also similar. A large study of 3772 patients from the United Kingdom with a 20% prevalence of diabetes showed that DM did not have any effect on LOS[21]. However, a study of 12442 patients from the United States of America with a 24% prevalence of diabetes found that diabetic recipients perform worse with regard to LOS and readmissions[16]. The differences, though, are small and may not be clinically relevant. Rather, individual patient factors are more important. A study by Washburn *et al*[22] showed that MELD score and increasing age are independent predictors of hospital LOS. The overall median LOS was higher than what has been reported in the literature by other centers. This is primarily because of the nature of the health-care system in Saudi Arabia, which has few available acute rehabilitation centers and primary

care physician networks. Therefore, patients remain in the hospital until they are fully mobile and independent before discharge.

Overall, the principle limiting factors of this study are its' retrospectivity and the single-center experience. Nevertheless the data can be considered representative of our region since over half of LTs in Saudi Arabia are performed at our center[23]. Furthermore over the last decades, advances have been made in the medical management of LT patients resulting in improved early outcomes, though not significantly improved long-term survival[24]. Another potential limiting factor is the low number of deceased donors in our cohort. One last limitation for this study is that we did not use the random forest survival analysis, an analysis representing the rapid rise of artificial intelligence in medicine, which surpasses traditional statistical approaches in terms of accuracy and explainable utility.

CONCLUSION

The short-term outcome of elderly diabetic patients undergoing LT is similar to patients without diabetes. The presence of DM in elderly liver transplant candidates should not discourage physicians when considering patients for LT.

ARTICLE HIGHLIGHTS

Research background

More patients older than 65 undergo liver transplantation (LT) nowadays. Significant number of those patients have diabetes mellitus (DM).

Research motivation

To address the impact of DM on short term outcome post liver transplant in patients older than 65. There is limited data in the literature particularly for patients undergoing living donor LT.

Research objectives

To determine the short term impact of DM in older patients post LT.

Research methods

This is a retrospective study of previously collected data from a high volume single transplant center. We included all patients who are 65 years old or older at the time of transplantation and assessed important short term outcomes such as one year survival, intensive care unit length of stay and acute cellular rejection.

Research results

One-year survival was comparable between diabetic and nondiabetic elderly patients undergoing LT. Acute cellular rejection rates were comparable between diabetic and nondiabetic elderly patients undergoing LT. Intensive care unit, hospital length of stay, and readmissions were comparable between diabetic and nondiabetic elderly patients undergoing LT.

Research conclusions

Diabetes was not found to affect the short-term outcomes in elderly patients undergoing LT.

Research perspectives

The presence of DM in elderly liver transplant candidates should not discourage physicians when considering patients for LT.

FOOTNOTES

Author contributions: Alghamdi S, Bzeizi KI, Alabbad S, Alqahtani SA, Broering D and Al-Hamoudi W contributed equally to this work; Alghamdi S, Alamro S, Alobaid D and Soliman E designed the research study; Alghamdi S, Alamro S, Alobaid D, Soliman E, Albenmousa A, Bzeizi KI and Al-Hamoudi W analyzed the data and wrote the manuscript; Alghamdi S, Albenmousa A, Bzeizi KI, Alqahtani SA and Al-Hamoudi W performed the research; Alghamdi S, Bzeizi KI, and Al-Hamoudi W contributed new reagents and analytic tools; All authors have read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the King Faisal Specialist Hospital and Research Center Institutional Review Board.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

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

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at mdisaad@kfshrc.edu.sa.

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ORIGINAL ARTICLE

Retrospective Study

Hospitalizations for alcoholic liver disease during the COVID-19 pandemic increased more for women, especially young women, compared to men

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Abstract

BACKGROUND

Alcoholic liver disease (ALD) remains one of the major indications for liver transplantation in the United States and continues to place a burden on the national healthcare system. There is evidence of increased alcohol consumption during the coronavirus disease 2019 (COVID-19) pandemic, and the effect of this on the already burdened health systems remains unknown.

AIM

To assess the trends for ALD admissions during the COVID-19 pandemic, and compare it to a similar pre-pandemic period.

METHODS

This retrospective study analyzed all admissions at a tertiary health care system, which includes four regional hospitals. ALD admissions were identified by querying a multi-hospital health system's electronic database using ICD-10 codes. ALD admissions were compared for two one-year periods; pre-COVID-19 from

April 2019 to March 2020, and during-COVID-19 from April 2020 to March 2021. Data were analyzed using a Poisson regression model and admission rates were compared using the annual quarterly average for the two time periods, with stratification by age and gender. Percent increase or decrease in admissions from the Poisson regression model were reported as incident rate ratios.

RESULTS

One thousand three hundred and seventy-eight admissions for ALD were included. 80.7% were Caucasian, and 34.3% were female. An increase in the number of admissions for ALD during the COVID-19 pandemic was detected. Among women, a sharp rise (33%) was noted in those below the age of 50 years, and an increase of 22% in those above 50 years. Among men, an increase of 24% was seen for those below 50 years, and a 24% decrease in those above 50 years.

CONCLUSION

The COVID-19 pandemic has had widespread implications, and an increase in ALD admissions is just one of them. However, given that women are often prone to rapid progression of ALD, this finding has important preventive health implications.

Key Words: Alcoholic liver disease; COVID-19; Alcoholic hepatitis; Alcoholic liver cirrhosis; Alcoholism; Pandemic; Young women; Alcohol-related disorders

Core Tip: An increase in alcoholic liver disease admissions was observed in the first year of the pandemic compared to the year prior to the pandemic with various "lock-downs" in place. This trend was most pronounced in the cohort of women below the age of 50.

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INTRODUCTION

The spectrum of alcoholic liver disease (ALD) includes an array of pathologies, from reversible fatty liver, to alcoholic hepatitis and advanced cirrhosis with portal hypertension[1]. Although women have lower and less active alcohol dehydrogenase in the GI tract and liver compared to men, ALD has traditionally been a disease primarily of middle-aged and older men[2]. A study by Shirazi *et al*[3], analyzing the National Inpatient Sample from 2007-2014 showed significantly higher hospitalization rates in males *vs* females, for alcohol associated hepatitis and cirrhosis.

The coronavirus disease 2019 (COVID-19) pandemic has had deep and far reaching consequences on people across the globe, affecting individuals at personal, economic, and social levels. It is alleged to have provoked more significant financial and emotional hardships on women compared to men[4,5]. During the initial phase of the pandemic, liquor stores in the United States were considered essential businesses, and alcohol sales increased by more than 34%[6]. The current study was designed to evaluate whether increased alcohol consumption during the COVID-19 pandemic was associated with an increase in ALD admissions, particularly in women.

MATERIALS AND METHODS

An IRB exception was obtained for this study, prior to any data acquisition or analysis. Admissions to a multi-hospital health system for ALD were compared for two one-year periods [April 2019-March 2020 (pre-COVID-19, "PC") and April 2020-March 2021 (during-COVID-19, "COV")]. One thousand three hundred and seventy-eight admissions from the four regional hospitals for ALD were identified by querying an electronic database using the International Classification of Diseases (ICD-10) codes (K70 and its sub-categories representing the spectrum of alcoholic fatty liver, hepatitis, cirrhosis, fibrosis). Patients with more than one or overlapping diagnoses were only counted once. Data were analyzed using a Poisson regression model and admission rates were compared using the annual quarterly

average for the two time periods, with stratification by age and gender. Percent increase or decrease in admissions from the Poisson regression model were reported as incident rate ratios. Continuous variables were compared using Student's *t*-test, while categorical variables were compared using chi-square of Fisher's exact test. All data were analyzed using SAS v9.4 software (Cary, NC).

RESULTS

Comparing admissions for ALD pre-COVID-19 (PC) and during-COVID-19 (COV) periods, an average quarterly increase of 33% was identified in women below 50 years (75 PC *vs* 104 COV, $P = 0.031$), and an increase of 22% in women above 50 years (131 PC *vs* 163 COV, $P = 0.063$). During the same two periods, ALD admissions for men below 50 years increased 24% (131 PC *vs* 166 COV, $P = 0.043$) (Figure 1).

Interestingly, a 24% decrease in admissions for ALD was observed in males above 50 years (341 PC *vs* 267 COV, $P = 0.003$). Although this group had the greatest number of hospitalizations, a significant proportional decline was observed among them, compared to the other groups.

The total number of admissions for ALD in men and women only increased from 678 pre-COVID-19 to 709 during-COVID-19 (Table 1). Strikingly, the proportion of women increased from 30.4% ($n = 206$) to 38.1% ($n = 267$), demonstrating an increase of 29% ($P = 0.005$). Total admissions for males decreased from 69.6% ($n = 472$) to 61.9% ($n = 433$), a 9% decrease ($P = 0.195$).

No significant racial/ethnic difference was identified, with the majority of the patients being Caucasian (80.7%). Approximately 35% of patients in both groups were decompensated with ascites. For patients with ALD, the length of stay during the pandemic was higher than pre-pandemic (110 h *vs* 96 h, $P = 0.014$). Interestingly, during the COVID-19 period, more patients left the hospital prematurely, against medical advice (4.3% *vs* 2.5%, $P = 0.03$). A higher proportion of patients were discharged to a rehabilitation facility during the pandemic as well (2.6% *vs* 1.5%, $P = 0.03$).

DISCUSSION

The current study, comparing the pre-COVID and during-COVID periods, detected a significant increase in the number of ALD hospital admissions for both women and men below the age of 50 years (33% and 24% increase respectively). With the onset of stressors including those brought by prolonged social isolation and socio-economic instability associated with the pandemic, an increase in substance abuse, not only among the high-risk groups, but also in the general population is not surprising and has been described[7].

Importantly, a significant increase in the number of younger women requiring admission for ALD was identified. To a lesser extent, this trend was observed in women over 50 years of age as well. The contextual, environmental, and social influences impacting alcohol consumption during the COVID-19 pandemic have not been comprehensively evaluated. The current study identified multiple significant trends that are likely related to psychosocial factors and social processes the study was not designed to evaluate. Previous studies have suggested anxiety and depression may be more prevalent in women than men, and the uncertainties during the pandemic may have compounded this[8]. A national survey reported a significant impact of the pandemic on the mental health of women, with 1 in 5 women respondents reporting an increase in alcohol or drug use after the onset of the COVID-19 outbreak[9]. One can speculate the increased consumption of alcohol could be related to stresses in the home added to existing marital responsibilities including: Stresses associated with both partners working remotely, the added stress of having children in the home, and the responsibilities associated with coordinating education for remote learning. The traditional gender gap in alcohol use also tends to be narrowing. A study by Williams *et al*[10] examining heavy drinking trajectories demonstrated an increase in heavy drinking frequency among younger women. These trends are concerning, given that women are not only at a higher risk of developing liver disease with alcohol intake, but are also at increased risk for progression of ALD with increasing alcohol intake compared to men[11]. Differences in gastric alcohol dehydrogenase levels and body fat are thought to be the reason behind this gender difference. A recent study by Bertha *et al*[11], analyzing a national inpatient database, reported that although ALD is seen predominantly in men, there has been a disproportionate increase in ALD mortality among women. Specifically, mortality in women below 34 years has progressed at a significantly high rate[1].

Another trend the current study identified was a decrease in ALD hospitalizations for older males during the COVID-19 pandemic. During the pandemic many individuals made every attempt to avoid hospital visits. Men are especially prone to denial of symptoms and avoidance of healthcare. It is also plausible that the overall consumption of alcoholic beverages by men decreased during the pandemic while increasing in women. Davies *et al*[12] found that drinking in the home with a partner, compared to drinking outside the home, is associated with lower consumption of alcohol. With the temporary closing and restricted capacities of establishments serving alcohol (bars, pubs, and restaurants), males may have consumed less alcohol due to limited access. In addition, social cues may have limited alcohol consumption in the home. Although liquor store sales increased during the pandemic, it is plausible

Table 1 Trends for alcoholic liver disease admissions pre-COVID and during COVID-19, *n* (%)

Variable	Total (*n* = 1378)	COVID-19 period (*n* = 700)	Pre-COVID-19 period (*n* = 678)	*P* value
Age (yr)	53.9 ± 13.1	53.1 ± 12.9	54.7 ± 13.2	0.025
Gender				0.002
Female	473 (34.3%)	267 (38.1%)	206 (30.4%)	
Male	905 (65.7%)	433 (61.9%)	472 (69.6%)	
Race				0.628
Black or African American	168 (12.2%)	91 (13.0%)	77 (11.4%)	
White or Caucasian	1112 (80.7%)	559 (79.9%)	553 (81.6%)	
Diagnosis name				0.274
Alcoholic cirrhosis of liver with ascites	491 (35.6%)	246 (35.1%)	245 (36.1%)	
Alcoholic cirrhosis of liver without ascites	336 (24.4%)	164 (23.4%)	172 (25.4%)	
Alcoholic fatty liver	43 (3.1%)	20 (2.9%)	23 (3.4%)	
Alcoholic fibrosis and sclerosis of liver	1 (0.1%)	0 (0.0%)	1 (0.1%)	
Alcoholic hepatic failure with coma	4 (0.3%)	3 (0.4%)	1 (0.1%)	
Alcoholic hepatic failure without coma	141 (10.2%)	79 (11.3%)	62 (9.1%)	
Alcoholic hepatitis with ascites	85 (6.2%)	52 (7.4%)	33 (4.9%)	
Alcoholic hepatitis without ascites	205 (14.9%)	105 (15.0%)	100 (14.7%)	
Alcoholic liver disease, unspecified	72 (5.2%)	31 (4.4%)	41 (6.0%)	
Discharge disposition				0.034
Expired	57 (4.2%)	34 (4.9%)	23 (3.4%)	
Home or self-care	832 (60.6%)	412 (59.1%)	420 (62.1%)	
Home-health care service	154 (11.2%)	86 (12.3%)	68 (10.1%)	
Hospice/home	30 (2.2%)	16 (2.3%)	14 (2.1%)	
Hospice/medical facility	38 (2.8%)	14 (2.0%)	24 (3.6%)	
Left against medical advice	47 (3.4%)	30 (4.3%)	17 (2.5%)	
Rehab facility	29 (2.1%)	18 (2.6%)	11 (1.6%)	
Short term hospital	58 (4.2%)	34 (4.9%)	24 (3.6%)	
Skilled nursing facility	76 (5.5%)	35 (5.0%)	41 (6.1%)	

COVID: Coronavirus disease.

overall consumption by men decreased due to limited access to previously visited establishments.

Recent studies mirror these findings. Deutsch-Link *et al*[13] reviewed the Centers for Disease Control and Prevention data, and discovered that mortality from ALD rose from 2017 to 2020 in the United States, with females and younger adults having the highest relative increase. Gonzalez *et al*[14] documented an increase in the proportion of female ALD admissions during the pandemic, in their study of 337 patients in the Detroit area. They also found an increase in Black/African American admissions. Sohal *et al*[15] also reported an increase in alcohol-related hepatitis requiring inpatient management, especially in patients under the age of 40 and in women during the pandemic with 329 patients studied in three community hospitals in Fresno, California, United States.

The strength of the current study lies in its longitudinal population-based evaluation of temporal trends for ALD in a large multi-hospital system, reviewing 1378 admissions for ALD. Limitations of the current study include its retrospective design and limited geographical reach. This study definitely underestimates the prevalence of ALD during the COVID-19 era, as only patients with significant ALD would have been admitted. Patient hesitancy to present to hospitals during the pandemic, as well as cessation of elective admissions may have also contributed to underestimating the prevalence of ALD during the pandemic. Further studies are indicated to determine whether these increasing trends of ALD hospitalizations persist, particularly in younger women, and to evaluate the psychosocial factors impacting alcohol consumption during the COVID-19 pandemic.

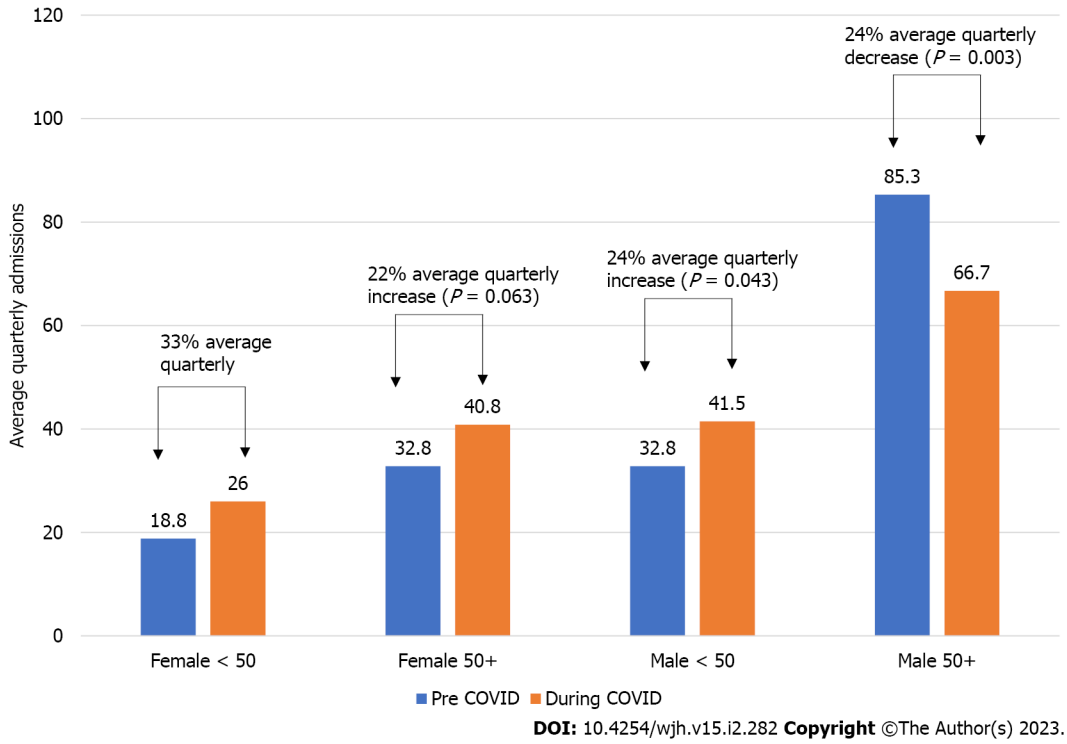

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Figure 1 Alcoholic liver disease admissions in a pre-COVID and during-COVID period by age and gender. COVID: Coronavirus disease.

CONCLUSION

This large multi-hospital analysis demonstrates a concerning gender disparity with women, especially young women, being significantly more likely to be admitted with ALD during the COVID-19 period compared to the twelve months prior to the pandemic. Intensive public health interventions, especially those focused towards women, may help to curb the rising rates of alcoholic liver disease in the United States.

ARTICLE HIGHLIGHTS

Research background

Alcoholic liver disease (ALD) has traditionally been a disease of middle-aged and older men, though recent studies indicate an increasing prevalence of women with ALD.

Research motivation

The coronavirus disease 2019 (COVID-19) pandemic has had widespread consequences affecting many socially and economically. This appears to have resulted in increased alcohol consumption in many individuals.

Research objectives

To assess the trends for ALD admissions during the COVID-19 pandemic and compare it to a similar pre-pandemic period.

Research methods

This was a retrospective analysis of hospitalizations for ALD in a large multi-center hospital system in the United States from April 2019 to March 2021.

Research results

An increase in admissions for ALD in women was noted (33% rise in women< 50 years and 22% rise in women > 50 years). Though ALD admissions for men < 50 years rose 24%, a fall of 24% in those > 50 years was noted.

Research conclusions

This study found a significant increase in younger women requiring hospital admission for ALD.

Research perspectives

It is of significant medical interest to gastroenterologists and hepatologists to determine whether the trend of increased ALD hospitalizations in women persist in future years.

FOOTNOTES

Author contributions: Campbell JP, Jahagirdar V, Muhanna A, Kennedy KF, and Helzberg JH contributed equally to this work; Campbell JP contributed to conceptualization, data curation, writing original draft, review and editing, and project administration; Jahagirdar V contributed to writing original draft, review, and editing; Muhanna A contributed to investigation and methodology; Kennedy KF contributed to formal analysis, data curation, validation, and visualization; Helzberg JH contributed to supervision, writing, editing, and project administration; all authors have read and approved the final manuscript.

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Retrospective Study

Racial and gender-based disparities and trends in common psychiatric conditions in liver cirrhosis hospitalizations: A ten-year United States study

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Abstract

BACKGROUND

Chronic liver disease is associated with various neuropsychiatric conditions. There are currently no large studies assessing and comparing the prevalence of psy-chiatric illnesses based on patient profiles and the etiology of cirrhosis.

AIM

To examine the trends of hospitalizations among psychiatric conditions in cirrhosis.

METHODS

We used the National Inpatient Sample database 2009-2019 for the primary diagnosis of liver cirrhosis. The outcomes included the prevalence, trends, and

associations of psychiatric diagnoses in these hospitalizations. Chi-square for categorical variables and the Wilcoxon rank test for continuous variables were utilized.

RESULTS

The prevalence of generalized anxiety disorder (GAD) in liver cirrhosis hospitalizations increased from 0.17% in 2009 to 0.92% in 2019 ($P < 0.001$). The prevalence of depression increased from 7% in 2009 to 12% in 2019 ($P < 0.001$). Attention deficit hyperactivity disorder (ADHD) prevalence increased from 0.06% to 0.24%. The prevalence of schizophrenia increased from 0.59% to 0.87% ($P < 0.001$). Schizoaffective disorder prevalence increased from 0.10% to 0.35% ($P < 0.001$). Post-traumatic stress disorder (PTSD) prevalence displayed increasing trends from 0.36% in 2009 to 0.93% in 2019 ($P < 0.001$). The prevalence of suicidal ideation increased from 0.23% to 0.56% in 2019. Cirrhosis related to alcoholic liver disease [adjusted odds ratios (aOR) 1.18, 95%CI 1.08-1.29, $P < 0.001$] and non-alcoholic fatty liver disease (NAFLD) (aOR 1.14, 95%CI 1.01-1.28, $P = 0.025$) was associated with depression more than other causes. Alcohol- and NAFLD-associated cirrhosis had a stronger link to psychiatric disorders. Females had a higher association with GAD (aOR 2.56, 95%CI 2.14-3.06, $P < 0.001$), depression (aOR 1.78, 95%CI 1.71-1.84, $P < 0.001$), bipolar disorder (aOR 1.64, 95%CI 1.52-1.77, $P < 0.001$] and chronic fatigue (aOR 2.31, 95%CI 1.31-4.07, $P < 0.001$) when compared to males. Blacks, Hispanics, and Asian/Native Americans had a significantly lower association with GAD, depression, bipolar disorder, PTSD, and ADHD when compared to the white race.

CONCLUSION

The prevalence of psychiatric comorbidities in liver cirrhosis hospitalizations has increased over the last decade. Females had a higher association with psychiatric disorders compared to males. Blacks, Hispanics, and Asian/Native Americans had lower associations with psychiatric comorbidities compared to the white race.

Key Words: Liver cirrhosis hospitalizations; Psychiatric conditions; Racial and gender disparities

Core Tip: Currently, large studies assessing and comparing the prevalence of psychiatric conditions based on patient profiles and the etiology of cirrhosis are lacking in the published literature. In this National Inpatient Sample-based retrospective study, we aimed to assess the trends of hospitalizations among psychiatric conditions in cirrhosis. Our findings highlight the disparities in the diagnoses of certain psychiatric conditions in cirrhotics between gender and race. It is pertinent to recognize these disparities, as doing so may expedite management and improve overall outcomes. Therefore, all patients with cirrhosis should be provided with a referral to a mental health professional at the time of diagnosis.

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INTRODUCTION

Chronic liver disease is associated with a wide variety of neuropsychiatric conditions, ranging from depression and sleep disturbances to coma. The etiology of these illnesses can be either medical or psychiatric, and at times it can be difficult to distinguish. Neuropsychiatric symptoms in chronic liver disease can be partially explained by aberrations in the liver's critical role in filtering neurotoxins such as ammonia and manganese from the blood[1]. This results in a buildup of these neurotoxins, which are implicated in mental status changes and alterations in consciousness. Additionally, liver disease has been shown to increase circulating inflammatory cytokines such as tumor necrosis factor, interleukin-1β, and interleukin-6, which can lead to neuroinflammation[1]. The combination of these factors is thought to lead to the development of hepatic encephalopathy (HE) in patients with cirrhosis.

HE is a relatively common neuropsychiatric manifestation of cirrhosis, and as such, it would not be overlooked by a hepatologist or gastroenterologist. Although the neuropsychiatric signs and symptoms of confusion, asterixis, and sleep disturbances seen in HE can be directly attributed to the cirrhosis itself

via the aforementioned mechanisms, other psychiatric symptoms such as apathy, psychomotor retardation, and low energy are nonspecific. These manifestations of psychiatric disorders may be missed due, in part, to the stigma surrounding mental illness and addiction, along with the lack of systematic screening in specialist offices. Left untreated, mental illness can interfere with treatment compliance for other medical conditions, increase disease burden, and lower quality of life[2,3]. While there is significant data in regard to the pathophysiology and management of HE as well as psychiatric conditions in cirrhosis, the disparities among these conditions are not well studied.

The psychological stress that patients with cirrhosis experience plays a negative role in their mental health. Previous research has shown a correlation between psychiatric conditions and liver disease, in particular anxiety and depression[4-6]. Prior studies have also focused on liver disease associated with substance use disorders, such as alcohol-related liver disease and viral hepatitis related to intravenous drug use, but data on psychiatric conditions in other etiologies of chronic liver disease has only recently gained preeminence. Among other etiologies of liver disease, nonalcoholic fatty liver disease (NAFLD) and autoimmune liver disease comprise a significant component. NAFLD and metabolic syndrome have been associated with increased rates of psychiatric illnesses[7-9]. It is also well understood that patients with autoimmune diseases of any etiology suffer more commonly from psychiatric conditions[10-13]. However, the data on psychiatric conditions in autoimmune liver disease is not as robust[14,15]. There are currently no large studies assessing and comparing the prevalence of psychiatric conditions based on patient profiles and the etiology of cirrhosis. We aim to examine the trends of hospitalizations among common psychiatric conditions in cirrhosis based on gender, race, and the etiology of liver disease over an 11-year period in the United States.

MATERIALS AND METHODS

Design and data source

The National Inpatient Sample (NIS), designed by the Agency for Healthcare Research and Quality (AHRQ), was used. The design of this particular database is to approximate a 20% stratified sample of hospitals along with sampling weights to calculate national estimates[16]. Data in NIS is provided using the International Classification of Diseases (ICD) 9 (before September 2015) and 10 (after October 2015) coding systems. The present study utilized the NIS database to identify patients with a primary diagnosis of liver cirrhosis from January 2009 to December 2019[16]. All patients below the age of 18 were excluded. Additionally, patients with primary biliary cirrhosis were excluded, as these are misnomers. Based on the etiology, cirrhosis was divided into NAFLD cirrhosis, alcoholic cirrhosis, and other causes (viral, autoimmune, or non-specified). The exact codes utilized in this study for each variable can be found in Supplementary Table 1. Additional information on the design and sampling methods of the NIS is available at: https://www.hcup-us.ahrq.gov.

Outcome measures

Primary outcomes included the prevalence of common psychiatric conditions that included GAD, major depressive disorder (MDD), bipolar disorder (BD), attention deficit/hyperactivity disorder (ADHD), schizophrenia, schizoaffective disorder, post-traumatic stress disorder (PTSD), chronic fatigue, and suicidal ideation (SI). Secondary outcomes included associations of gender and race among liver cirrhosis hospitalizations with the aforementioned psychiatric conditions. We also reported trends in liver cirrhosis hospitalizations over the study period with demographics. A trend analysis for the respective outcomes was also reported in order to identify any time-based shifts.

Statistical analysis

Multivariate logistic regression was conducted to assess the relationship between gender, race, and psychiatric conditions among liver cirrhosis hospitalizations; outcomes were reported as adjusted odds ratios (aOR) with 95% confidence intervals (CI) and a *P* value. The analysis used 0.05 as the threshold for statistical significance, and all P values were 2-sided. Bivariate analysis was conducted using a chi-square test for categorical variables and an independent-samples *t*-test or Wilcoxon rank test for continuous variables. Categorical variables were presented as frequency (N) and percentage (%), and continuous variables were reported as mean with standard deviation (SD) as appropriate. For outcomes like the length of stay (LOS) and mean inpatient charges (MIC) given in Supplementary Table 2, a hierarchical multivariate linear regression analysis was conducted to adjust the patient- or hospital-level factors as in prior studies[17-19]. For prevalence, the trend over time was evaluated using the score test with the "tabodds" command; for this, 2009 was used as the reference category. The score test compares the odds of cases occurring consecutively every year[20,21]. Microsoft Excel (Microsoft Corporation, Redmond, WA) was used to generate figures[22,23]. Statistical Software for Data Science (STATA) version 16.0 software (StataCorp LLC, Station, TX, United States) was used for statistical analysis.

Ethical considerations

The NIS contains de-identified information, protecting the privacy of patients, physicians, and hospitals. Therefore, it was deemed exempt from the institutional review board (IRB). As each hospitalization was stripped of any patient identifiers, patient consent was waived.

RESULTS

Demographic characteristics of the study sample

There was a total of 724612 hospitalizations with a primary diagnosis of liver cirrhosis for the study period. Of these hospitalizations, 14.04% were due to NAFLD cirrhosis, 42.89% were following alcoholic cirrhosis, and 43.0% were secondary to other causes (viral, autoimmune, or non-specified). Total liver cirrhosis hospitalizations decreased from 78728 (208 per 100,000 total NIS hospitalizations) in 2009 to 52139 (147 per 100000 total NIS hospitalizations) in 2019 ($P < 0.001$) (Supplementary Figure 1). Liver cirrhosis hospitalizations were more common in males compared to females (62% *vs* 38%) ($P < 0.001$). Most patients belonged to the age group 50-64 years (49%), followed by 65-79 years (21%) ($P < 0.001$). There was white race predominance (70%), followed by Hispanics (20%) and blacks (9%) without significance ($P = 0.16$). Most patients had a Charlson comorbidity index (CCI) score of CCI ≥ 3 (77%) ($P < 0.001$). Urban teaching hospitals had the highest frequency of liver cirrhosis hospitalizations (61%), followed by Urban non-teaching (32%) and rural (7%) hospitals ($P < 0.001$). Medicare remained the primary payer for 39% of hospitalizations for liver cirrhosis, followed by Medicaid (27%), and private insurers (24%; $P < 0.001$). Inpatient mortality significantly decreased from 7% in 2009 to 4% in 2019 ($P < 0.001$). Additional demographic characteristics over the study period are described in Supplementary Table 2. Adjusted linear regression revealed a declining trend in LOS for liver cirrhosis patients from 6.10 ± 0.22 d in 2009 to 5.18 ± 0.08 d in 2019 ($P < 0.001$); and an increasing trend in MIC from $59266 ± 4111 in 2009 to $69882 ± 23608 in 2019 ($P = 0.001$) (Supplementary Figure 2). The associations of common psychiatric conditions with liver cirrhosis hospitalizations are also described in Supplementary Table 3.

Prevalence and trends of common psychiatric conditions in liver cirrhosis population

The prevalence of GAD in liver cirrhosis hospitalizations increased from 0.17% in 2009 (1.76 per 1000 hospitalizations) to 0.92% in 2019 (9.21 per 1000 hospitalizations) ($P < 0.001$). The prevalence of MDD increased from 7% in 2009 (71.7 per 1000 hospitalizations) to 12% in 2019 (120.1 per 1000 hospitalizations) ($P < 0.001$). ADHD prevalence increased from 0.06% in 2009 (0.61 per 1000 hospitalizations) to 0.24% in 2019 (2.49 per 1000 hospitalizations). The prevalence of schizophrenia increased from 0.59% in 2009 (5.93 per 1000 hospitalizations) to 0.87% in 2019 (8.72 per 1000 hospitalizations) ($P < 0.001$). Schizoaffective disorder prevalence increased from 0.10% in 2009 (1.90 per 1000 hospitalizations) to 0.35% in 2019 (3.54 per 1000 hospitalizations) ($P < 0.001$). PTSD prevalence displayed increasing trends from 0.36% in 2009 (3.69 per 1000 hospitalizations) to 0.93% in 2019 (9.39 per 1000 hospitalizations) ($P < 0.001$). The prevalence of SI increased from 0.23% in 2009 (2.38 per 1000 hospitalizations) to 0.56% in 2019 (5.65 per 1000 hospitalizations) ($P < 0.001$) (Table 1) (Figure 1).

Associations of common psychiatric conditions based on liver cirrhosis etiology

The associations based on etiologies were compared against other causes of liver cirrhosis (viral, autoimmune, or unspecified) as they had the highest weights to ensure the best statistical accuracy. Patients with alcoholic liver cirrhosis had a higher association with GAD compared to other causes (aOR 1.79, 95%CI 1.29-2.47, $P < 0.001$). At the same time, no difference existed between NAFLD cirrhosis and other causes ($P = 0.69$). Both alcohol (aOR 1.18, 95%CI 1.08-1.29, $P < 0.001$) and NAFLD cirrhosis (aOR 1.14, 95%CI 1.01-1.28, $P = 0.025$) had a higher association with MDD compared to other causes. Alcohol cirrhosis (aOR 1.62, 95%CI 1.34-1.96, $P < 0.001$) and NAFLD cirrhosis (aOR 1.37, 95%CI 1.04-1.79, $P = 0.021$) had a stronger association with bipolar disorder than other causes. No difference existed between liver cirrhosis etiologies for association with ADHD, schizophrenia, or schizoaffective disorder. Alcoholic liver cirrhosis had a higher association with PTSD (aOR 1.57, 95%CI 1.15-2.13, $P = 0.004$) and SI (aOR 2.01, 95%CI 1.33-3.04, $P = 0.001$) compared to other causes. There was no difference in PTSD and SI between NAFLD cirrhosis and other causes (Table 2).

Gender-based disparities of common psychiatric conditions in liver cirrhosis population

Among liver cirrhosis hospitalizations, females had a higher association with GAD (aOR 2.56, 95%CI 2.14-3.06, $P < 0.001$), MDD (aOR 1.78, 95%CI 1.71-1.84, $P < 0.001$), bipolar disorder (aOR 1.64, 95%CI 1.52-1.77, $P < 0.001$) and chronic fatigue (aOR 2.31, 95%CI 1.31-4.07, $P < 0.001$), when compared to males. There was no significant association between ADHD, SI, schizophrenia, and schizoaffective disorders among females compared to males in liver cirrhosis hospitalizations (Table 3).

Table 1 Trends of psychiatric comorbidities in patients hospitalized with primary diagnosis of liver cirrhosis in the national inpatient database from 2009 to 2019, *n* (%)

Variables	Years											*P* value
	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	
GAD	139 (0.17)	97 (0.12)	158 (0.20)	295 (0.38)	225 (0.29)	275 (0.34)	270 (0.42)	245 (0.55)	285 (0.60)	360 (0.71)	480 (0.92)	$P < 0.001$
Depression	5652 (7)	5835 (8)	6828 (9)	7770 (10)	8599 (11)	9345 (12)	7395 (12)	4885 (11)	5710 (12)	6165 (12)	6264 (12)	$P < 0.001$
Bipolar disorder	1331(1.69)	1552 (2)	1689 (2)	1630 (2)	1730 (2)	1785 (2)	1575 (3)	1085 (2)	1065 (2)	1210 (2)	1390 (3)	$P < 0.001$
ADHD	48 (0.06)	78 (0.10)	151 (0.19)	165 (0.21)	130 (0.16)	235 (0.29)	210 (0.33)	75 (0.17)	85 (0.17)	110 (0.21)	130 (0.24)	$P < 0.001$
Schizophrenia	467 (0.59)	613 (0.79)	491 (0.64)	465 (0.60)	545 (0.70)	535 (0.66)	465 (0.74)	305 (0.69)	400 (0.84)	445 (0.87)	455 (0.87)	$P < 0.001$
Schizoaffective disorder	150 (0.19)	145 (0.18)	170 (0.22)	205 (0.26)	130 (0.16)	210 (0.26)	215 (0.34)	150 (0.34)	165 (0.34)	200 (0.39)	185 (0.35)	$P < 0.001$
PTSD	291 (0.36)	230 (0.30)	300 (0.39)	350 (0.45)	440 (0.56)	555 (0.68)	525 (0.83)	350 (0.79)	410 (0.86)	485 (0.95)	490 (0.93)	$P < 0.001$
Chronic Fatigue	19 (0.02)	28 (0.03)	18 (0.02)	20 (0.03)	25 (0.032)	25 (0.031)	5 (0.007)	40 (0.091)	5 (0.01)	20 (0.039)	45 (0.08)	$P < 0.001$
Suicidal Ideation	188 (0.23)	235 (0.33)	188 (0.24)	265 (0.34)	235 (0.30)	305 (0.37)	215 (0.34)	105 (0.23)	160 (0.33)	250 (0.48)	295 (0.56)	$P < 0.001$

GAD: Generalized anxiety disorder; PTSD: Post-traumatic stress disorder; ADHD: Attention deficit hyperactivity disorder.

Race-based disparities of common psychiatric conditions in liver cirrhosis population

The Black, Hispanic, and Asian/Native American races had a significantly lower association with GAD, MDD, bipolar disorder, PTSD, and ADHD when compared to the white race among liver cirrhosis hospitalizations. Blacks had a higher association with schizophrenia (aOR 3.10, 95%CI 2.60-3.66, $P < 0.001$) and schizoaffective disorder (aOR 2.03, 95%CI 1.50-2.73, $P < 0.001$) when compared to the white race with liver cirrhosis. The black race also had a higher association with schizoaffective disorder (aOR 2.03, 95%CI 1.50-2.73, $P < 0.001$) compared to the white race with liver cirrhosis. There was no significant difference in the association of other races compared to the white race for schizophrenia or schizoaffective disorder. The black race had a lower association with PTSD than whites (aOR 0.70, 95%CI 0.52-0.94, $P = 0.019$). Blacks (aOR 0.64, 95%CI 0.45-0.92, $P = 0.018$) and Hispanics (aOR 0.72, 95%CI 0.56-0.92, $P = 0.009$) had a lower association with SI than whites (Table 4).

DISCUSSION

Our study revealed a significant increase in the prevalence of GAD, MDD, PTSD, ADHD, schizophrenia, schizoaffective disorder, and SI in hospitalized patients with cirrhosis from 2009 to 2019. According to the World Health Organization (WHO), the worldwide diagnoses of all mental illnesses increased by 13% from 2007 to 2017[24]. While this represents a significant increase, the rise of psychiatric diagnoses in cirrhosis hospitalizations was even more staggering in our study. Over the 11-

Table 2 Associations of common psychiatric conditions based on liver cirrhosis etiology compared against "other" causes of cirrhosis (viral, autoimmune and non-specified)

Variables	Adjusted odds ratio with 95% confidence interval	*P* value
GAD		
Alcoholic liver cirrhosis	1.79 [1.29-2.47]	$P < 0.001$
NAFLD cirrhosis	1.09 [0.69-1.73]	$P = 0.690$
Depression		
Alcoholic liver cirrhosis	1.18 [1.08-1.29]	$P < 0.001$
NAFLD cirrhosis	1.14 [1.01-1.28]	$P = 0.025$
Bipolar disorder		
Alcoholic liver cirrhosis	1.62 [1.34-1.96]	$P < 0.001$
NAFLD cirrhosis	1.37 [1.04-1.79]	$P = 0.021$
ADHD		
Alcoholic liver cirrhosis	1.02 [0.55-1.87]	$P = 0.94$
NAFLD cirrhosis	1.49 [0.65-3.41]	$P = 0.34$
Schizophrenia		
Alcoholic liver cirrhosis	1.09 [0.76-1.57]	$P = 0.60$
NAFLD cirrhosis	0.82 [0.45-1.47]	$P = 0.51$
Schizoaffective disorder		
Alcoholic liver cirrhosis	1.18 [0.71-1.94]	$P = 0.50$
NAFLD cirrhosis	0.41 [0.15-1.13]	$P = 0.08$
PTSD		
Alcoholic liver cirrhosis	1.57 [1.15-2.13]	$P = 0.004$
NAFLD cirrhosis	1.05 [0.65-1.69]	$P = 0.81$
Chronic Fatigue		
Alcoholic liver cirrhosis	0.16 [0.03-0.73]	$P = 0.019$
NAFLD cirrhosis	0.73 [0.24-2.20]	$P = 0.58$
Suicidal ideations		
Alcoholic liver cirrhosis	2.01 [1.33-3.04]	$P = 0.001$
NAFLD cirrhosis	0.51 [0.19-1.32]	$P = 0.16$

NAFLD: Non-alcoholic fatty liver disease; GAD: Generalized anxiety disorder; PTSD: Post-traumatic stress disorder; ADHD: Attention deficit hyperactivity disorder.

year study period, rates of GAD and MDD in hospitalized patients with cirrhosis increased by approximately 400% and 70%, respectively. Additionally, the occurrences of PTSD, ADHD, schizophrenia, schizoaffective disorder, and SI increased at greater rates than the worldwide average. Therefore, our study indicates a significantly increased prevalence of mental illness in patients with cirrhosis. This can have a negative impact on quality of life, increase the burden on the healthcare system, and decrease compliance with medical treatment. The results of our study highlight the importance of evaluating patients with cirrhosis for concomitant psychiatric conditions. It could be argued that all patients with cirrhosis should be referred for evaluation by psychiatry and/or psychology at the time of diagnosis. This could lead to improved psychiatric outcomes and have positive downstream effects for the cirrhotic patients, leading to improved clinical outcomes.

Psychiatric conditions based on etiology of cirrhosis

A number of studies have assessed the prevalence of psychiatric disorders in alcohol-related liver disease. However, there are no large studies assessing rates of comorbid psychiatric conditions in cirrhotics based on the etiology of liver disease. Our study compared associated psychiatric diseases by dividing the etiology of cirrhosis into alcohol, NAFLD and other etiologies (viral, autoimmune, or

Table 3 Gender disparities with common psychiatric conditions in inflammatory bowel disease hospitalizations (Females compared against males)

Variables	Adjusted odds ratio with 95% confidence interval	*P* value
GAD	2.56 [2.14-3.06]	$P < 0.001$
Depression	1.78 [1.71-1.84]	$P < 0.001$
Bipolar disorder	1.64 [1.52-1.77]	$P < 0.001$
ADHD	1.07 [0.82-1.39]	$P = 0.610$
Schizophrenia	0.90 [0.79-1.04]	$P = 0.170$
Schizoaffective disorder	0.90 [0.73-1.13]	$P = 0.390$
PTSD	0.83 [0.72-0.97]	$P = 0.021$
Chronic Fatigue	2.31 [1.31-4.07]	$P = 0.004$
Suicidal ideation	0.86 [0.71-1.04]	$P = 0.120$

GAD: Generalized anxiety disorder; PTSD: Post-traumatic stress disorder; ADHD: Attention deficit hyperactivity disorder.

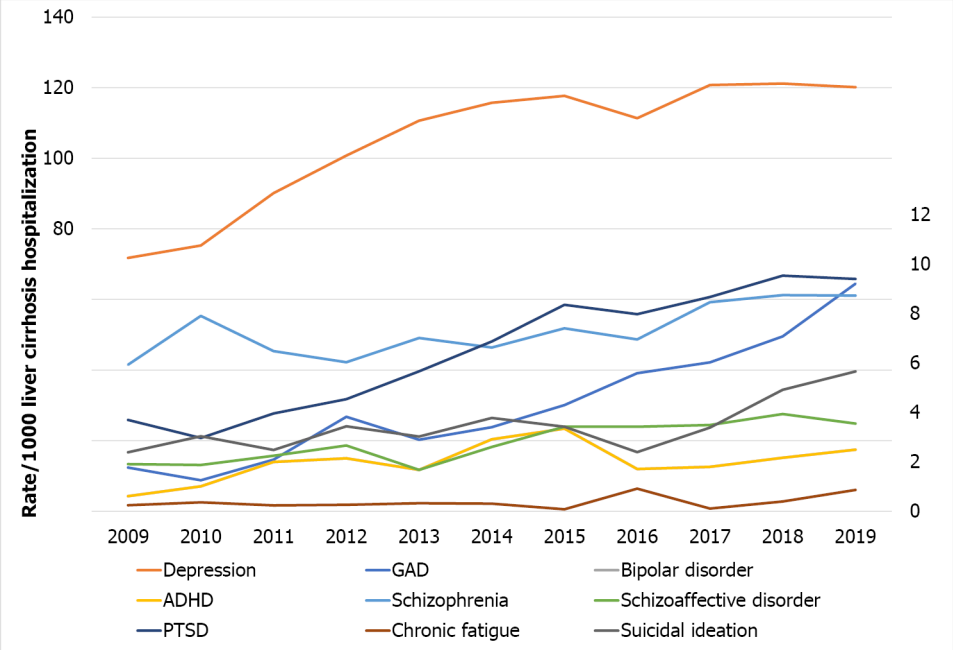

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Figure 1 Rate of common psychiatric conditions in liver cirrhosis hospitalizations. The colored lines represent rates of different psychiatric diagnoses per 1000 liver cirrhosis hospitalizations for the study period (2009-2019). GAD: Generalized anxiety disorder; PTSD: Post-traumatic stress disorder; ADHD: Attention deficit hyperactivity disorder.

unspecified). Our data revealed a significantly higher rate of GAD (aOR 1.79) in alcohol cirrhosis compared to other etiologies of cirrhosis. This association was not statistically significant when comparing the NAFLD cohort to the other etiologies of cirrhosis ($P = 0.69$). As numerous studies have shown, there is a significant association between alcohol use disorder and anxiety[25-28]. It is possible that self-treatment of anxiety with alcohol predisposes these patients to develop alcohol-induced cirrhosis, thus explaining the findings of our study. Interestingly, while Santos *et al*[29] revealed a high prevalence of anxiety in patients with alcoholic cirrhosis listed for transplant, they found patients with cirrhosis related to autoimmune hepatitis to have the most severe anxiety symptoms.

Alcohol use is a commonly associated comorbidity with both PTSD and SI[30-33]. The results of our study mirror these findings, with PTSD (aOR 1.57) and SI (aOR 2.01) significantly associated with alcohol cirrhosis compared to NAFLD and other etiologies of cirrhosis. Disinhibition and executive dysfunction from alcohol intoxication, coupled with stressors from living with a chronic medical condition, potentially play a role in the increased frequency of SI in alcohol cirrhosis. A survey-based study by Le Strat *et al*[34] noted an increased rate of SI among patients with liver disease but did not

Table 4 Race disparities with common psychiatric conditions in inflammatory bowel disease hospitalizations (compared against white race)

Variables	Adjusted odds ratio with 95% confidence interval	*P* value
GAD		
Black	0.33 [0.21-0.53]	$P < 0.001$
Hispanic	0.43 [0.33-0.57]	$P < 0.001$
Asian/Native American	0.08 [0.01-0.60]	$P = 0.014$
Depression		
Black	0.54 [0.50-0.59]	$P < 0.001$
Hispanic	0.58 [0.54-0.61]	$P < 0.001$
Asian/Native American	0.36 [0.30-0.43]	$P < 0.001$
Bipolar disorder		
Black	0.79 [0.69-0.90]	$P = 0.001$
Hispanic	0.48 [0.43-0.55]	$P < 0.001$
Asian/Native American	0.16 [0.08-0.29]	$P < 0.001$
ADHD		
Black	0.14 [0.06-0.34]	$P < 0.001$
Hispanic	0.22 [0.13-0.37]	$P < 0.001$
Asian/Native American	0.16 [0.02-1.14]	$P = 0.068$
Schizophrenia		
Black	3.10 [2.6-3.66]	$P < 0.001$
Hispanic	1.05 [0.88-1.26]	$P = 0.540$
Asian/Native American	0.72 [0.39-1.32]	$P = 0.290$
Schizoaffective disorder		
Black	2.03 [1.50-2.73]	$P < 0.001$
Hispanic	1.05 [0.79-1.39]	$P = 0.710$
Asian/Native American	0.66 [0.24-1.79]	$P = 0.420$
PTSD		
Black	0.70 [0.52-0.94]	$P = 0.019$
Hispanic	0.46 [0.37-0.58]	$P < 0.001$
Asian/Native American	0.69 [0.39-1.22]	$P = 0.210$
Chronic Fatigue		
Black	0.18 [0.02-01.39]	$P = 0.100$
Hispanic	0.50 [0.20-1.19]	$P = 0.110$
Asian/Native American	-	-
Suicidal ideations		
Black	0.64 [0.45-0.92]	$P = 0.018$
Hispanic	0.72 [0.56-0.92]	$P = 0.009$
Asian/Native American	0.43 [0.16-1.16]	$P = 0.097$

NAFLD: Non-alcoholic fatty liver disease; GAD: Generalized anxiety disorder; PTSD: Post-traumatic stress disorder; ADHD: Attention deficit hyperactivity disorder.

differentiate based on the etiology of liver disease. It is possible that the high prevalence of alcohol-related liver disease impacted the findings of their study.

Our study also revealed that the prevalence of MDD was significantly higher in the alcoholic cirrhosis (aOR 1.18) and NAFLD cirrhosis (aOR 1.14) cohorts when compared to other etiologies of cirrhosis. Similarly, bipolar disorder had a higher prevalence in alcoholic cirrhosis (aOR 1.62) and NAFLD cirrhosis (aOR 1.37) when compared to other etiologies of cirrhosis.

There was no statistically significant difference in the rates of schizophrenia, schizoaffective disorder, and ADHD among the different etiologies of cirrhosis in our study. This could be due, in part, to the fact that ADHD is considered a neurodevelopmental disorder per the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), and symptoms of ADHD must be present before the age of 12 years old, which would likely predate the onset of cirrhosis for the vast majority of patients, regardless of the etiology of cirrhosis. Similarly, psychotic disorders such as schizophrenia and schizoaffective disorder are typically diagnosed in late adolescence or early adulthood, again predating a liver cirrhosis diagnosis for most patients. In our results, it does not seem as though having a psychotic disorder would predispose the patient to a certain etiology of liver cirrhosis. It is possible that the neuropsychiatric pathways for the development of these disorders are not as affected by the effects of chronic liver disease when compared to GAD, MDD, SI, and bipolar disorder. Notably, this is a complex pathway that requires greater understanding prior to developing causality related to liver disease.

Psychiatric conditions in cirrhosis based on gender disparities

When comparing the rates of psychiatric conditions between genders, our study revealed a significantly increased rate of GAD (aOR 2.56), MDD (aOR 1.78), bipolar disorder (aOR 1.64) and chronic fatigue (aOR 2.31) among females compared to males with cirrhosis. Similarly, a large United States survey-based study by Vesga-López *et al*[35] revealed a lifetime prevalence of GAD of 5.3% in women and 2.8% in men. Prior studies have shown an approximate 1.6-1.7 fold greater incidence of MDD in females compared to males[36-38]. Our study revealed a slightly higher rate of MDD in female patients with cirrhosis compared to males. These findings are mirrored in studies by Lee *et al*[39] (male gender OR = 0.45, 95%CI: 0.37-0.55) and Rivera-Matos *et al*[40] (12-mo prevalence of MDD: 7% males *vs* 13% females). It is unclear if the higher rates of MDD in females with cirrhosis are significant; however, none of the above-referenced studies included 11 years of data. These findings highlight the importance of screening for MDD, particularly in female patients with cirrhosis.

The data for gender-based differences in bipolar disorder in the general population remains unclear. While some studies suggest an equal distribution of bipolar disorder between males and females, other studies suggest a greater prevalence in females. One large analysis consisting of more than 47000 patients with bipolar disorder revealed that approximately 55%-65% of patients were female[41]. Patel *et al*[42] found that females made up 54.8% of bipolar disorder admissions from 2010 to 2014 using NIS data. On the other hand, a survey-based study involving approximately 13000 patients in New Zealand revealed similar rates of bipolar disorder among males and females[43]. Vega *et al*[44] suggested similar rates of bipolar I disorder between both sexes and a female predominance in bipolar II disorder. Further classification between the two types of bipolar disorder was not available, but females had a greater association with overall bipolar disorder (aOR 1.64) than males among cirrhotics in our study. Pertinently, this is a stronger association compared to the findings of other studies.

It is generally accepted that chronic fatigue syndrome (CFS) is more prevalent in females. A cohort study involving patients with chronic fatigue performed by Faro *et al*[45] revealed an approximate 10:1 ratio between females and males. Another survey-based study from Iceland showed that 78% of respondents with chronic fatigue were female[46]. However, the significant variation between genders with a diagnosis of CFS seen in the general population was not replicated in the cirrhotic patients in our study (F > M, aOR 2.31). This is at least partly explained by the fact that fatigue is a very common somatic symptom in patients with chronic liver disease. Swain *et al*[47] revealed that chronic fatigue may be seen in up to 50% of patients with chronic liver disease. Patients with cirrhosis should be screened for symptoms of fatigue as it may play a role in the development of sarcopenia, which could present a barrier to future liver transplantation[48].

Psychiatric conditions in cirrhosis based on racial disparities

We found that among patients with cirrhosis, Caucasians have a greater association with GAD, MDD, bipolar disorder, PTSD, ADHD, chronic fatigue, and SI when compared to other races. These findings are in line with many previous studies involving the general population, including a 16-year cohort study by Manseau *et al*[49]. Of note, the study by Manseau *et al*[49] did not evaluate rates of psychiatric conditions in Asian and Native Americans. Interestingly, in our study, the Asian/Native American cohort had a significantly lower association with GAD (aOR 0.08), depression (aOR 0.36), and bipolar disorder (aOR 0.16) compared to Caucasians. It is possible that the differences in psychiatric diagnoses among minorities are in part explained by the underutilization of mental health services by minorities. Abe-Kim *et al*[50] showed that Asian Americans had lower rates of utilization of mental health services than the general population. A survey-based study by Dobalian *et al*[51] revealed that African Americans and Hispanics were less likely to have visited a mental health professional than whites. Lipson *et al*[52] found that people of color had more unmet mental health needs than whites in a study

involving over 40000 college students. They also found that Asian Americans had the lowest utilization of mental health services compared to other races[52]. While our study revealed a higher prevalence of mental illnesses among Caucasians, diligence should be taken in assessing mental health conditions in all patients with cirrhosis. Possible explanations for the lower utilization of mental health services by racial minorities may be due to the stigmatization of mental illness, especially in communities of color, the lack of access to psychiatric care, particularly in rural areas, the lack of trust in mental health due to past racist medical practices, and possibly poor rapport due to cultural differences. Normalizing mental illness, developing rapport with the patient, incorporating the patient's own belief system into the treatment plan, and using language interpreters may assist with the proper management of mental health conditions in minority patients with cirrhosis.

According to the DSM-5, schizophrenia falls under the category of psychotic disorders. Other conditions in this category include schizophreniform disorder, schizoaffective disorder, delusional disorder, brief psychotic disorder, psychotic disorder due to another medical condition, substance- or medication-induced psychotic disorder, unspecified schizophrenia spectrum disorder, and other psychotic disorders[53]. Our study revealed a significantly higher rate of schizophrenia (aOR 3.10) and schizoaffective disorder (aOR 2.03) among African Americans compared to other races, including Caucasians. These findings are in accordance with the findings of many previous studies assessing racial differences in the diagnosis of schizophrenia in the general population. Several studies have found that African Americans are approximately 3-5 times more likely to be diagnosed with schizophrenia than Caucasians[54-56]. However, these findings have raised concern about the role of bias in the diagnosis of schizophrenia in African Americans[57]. Additionally, some have postulated that the underdiagnosis of other psychiatric conditions in African Americans has led to an overdiagnosis of schizophrenia[58]. Garb argued that African Americans and Hispanics may receive diagnoses of schizophrenia even when they are not justified using proper diagnostic methods[59]. Cohen *et al*[60] found that African Americans and Hispanics had a higher prevalence of lifetime psychotic symptoms compared to Caucasians and Asians. However, the differences were less significant than in our study. Cirrhosis is a chronic medical condition that not only places psychosocial stress on the patient but also can result in neuropsychiatric symptoms such as HE. As a result, accurate psychiatric diagnoses in these patients are critical. It is plausible that patients with cirrhosis may be misdiagnosed with schizophrenia when their underlying medical condition is contributing to their symptoms. Unbiased examination and optimization of neurologic symptoms such as HE is needed prior to diagnosing psychiatric conditions such as schizophrenia.

LIMITATIONS

While we included a large population of cirrhotics over an 11-year study period, there are a few limitations to our study. The NIS database comprises approximately 20% of the hospitals in the United States. The final data is a national estimate calculated using sampling weights for extrapolation of national numbers. Furthermore, entry into the NIS database represents a single hospitalization. A single patient could potentially have multiple entries in the database through readmissions and hospital transfers. Another limitation is that our study only used ICD coding for psychiatric diagnoses. The DSM-5 classification may be more commonly used among psychiatric professionals, and thus, psychiatric diagnoses may be missed or incorrect. As all psychiatric illnesses are multifactorial, it is impossible to relate them to one specific cause. The exact etiology of the psychiatric illnesses reported here is unknown and likely multifactorial. Therefore, the authors only reported associations (ORs) and not relative risks. Moreover, we only reported prevalence, not incidence, as this is a retrospective database. Finally, data entry for race may have some limitations in that many patients are misclassified or may not have documentation for race during their hospitalization.

CONCLUSION

As mental health conditions continue to become less stigmatized over time, more patients are becoming open to mental health evaluation and treatment. For reasons that are not completely understood, there is a continued rise in the diagnosis of psychiatric conditions in the general population. Our study revealed that the remarkably increasing rate of psychiatric diagnoses in cirrhotics is alarming. Our findings highlight the disparities in the diagnoses of certain psychiatric conditions in cirrhotics between gender as well as race. As a medical professional, it is important to understand and recognize these disparities as they may expedite management and improve overall outcomes. It is not uncommon for neuropsychiatric symptoms in cirrhotics to be ignored or misdiagnosed due to the role that liver disease plays in neurologic function. Although transplant psychiatrists and psychologists play an integral role in the management of all patients evaluated for liver transplantation, this resource is not available for all patients with cirrhosis despite the fact that many patients who are not liver transplant candidates may be suffering from concomitant mental illness. The findings in our study suggest that all cirrhotics should

be provided with a referral to a mental health professional at the time of diagnosis.

ARTICLE HIGHLIGHTS

Research background

Chronic liver disease is associated with various neuropsychiatric conditions, such as generalized anxiety disorder (GAD) and major depression. The psychological stress experienced by patients with cirrhosis can negatively affect their mental health.

Research motivation

There is limited data assessing and comparing the prevalence of psychiatric conditions based on patient profiles and the etiology of cirrhosis.

Research objectives

To examine the trends of hospitalizations among common psychiatric conditions in cirrhosis based on gender, race, and the etiology of liver disease over 11 years in the United States by dividing the etiology of cirrhosis into alcohol, non-alcoholic fatty liver disease, and other causes (viral, autoimmune, or unspecified) using the National Inpatient Sample (NIS) 2009-2019.

Research methods

The present study utilized the NIS database to identify patients with a primary diagnosis of liver cirrhosis from January 2009 to December 2019 and assess the prevalence of common psychiatric conditions that included GAD, major depressive disorder, bipolar disorder, attention-deficit/hyperactivity disorder, schizophrenia, schizoaffective disorder, post-traumatic stress disorder, chronic fatigue, and suicidal ideation.

Research results

Our study showed an uptrend in psychiatric comorbidities over the last decade, with racial and gender disparities.

Research conclusions

The findings of this study revealed a remarkably increasing rate of psychiatric diagnoses in cirrhotics. Therefore, it is imperative for clinicians to understand and recognize associated disparities based on gender and race.

Research perspectives

Our study suggests that all liver cirrhosis patients should be provided a referral to a mental health professional at the time of diagnosis, and more studies are needed to look into the etiology of these diagnoses.

FOOTNOTES

Author contributions: Patel P, Ali H, Inayat F, Pamarthy R, and Giammarino A contributed to conceptualization, methodology, software, data curation, validation, writing, and original draft preparation; Ilyas F and Smith-Martinez LA contributed to writing, reviewing, editing, and supervision; Satapathy SK project administration, supervision, and critical revision of the manuscript; all authors had access to the study data and reviewed and approved the final manuscript.

Institutional review board statement: Patients' data was not acquired by any specific institution but rather open-access United States National Inpatient Sample (NIS) data. The NIS contains de-identified information, protecting the privacy of patients, physicians, and hospitals. Therefore, it was deemed exempt from the institutional review board (IRB).

Informed consent statement: Participants were not required to give informed consent for this retrospective study since the analysis of baseline characteristics used anonymized clinical data.

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ORIGINAL ARTICLE

Observational Study

Outcomes of gout in patients with cirrhosis: A national inpatient sample-based study

Ayham Khrais, Aaron Kahlam, Ali Tahir, Amjad Shaikh, Sushil Ahlawat

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Abstract

BACKGROUND

Hyperuricemia is a prerequisite for the development of gout. Elevated serum uric acid (UA) levels result from either overproduction or decreased excretion. A positive correlation between serum UA levels, cirrhosis-related complications and the incidence of nonalcoholic fatty liver disease has been established, but it is unknown whether hyperuricemia results in worsening cirrhosis outcomes. We hypothesize that patients with cirrhosis will have poorer gout outcomes.

AIM

To explore the link between cirrhosis and the incidence of gout-related complications.

METHODS

This was a cross-sectional study. The national inpatient sample was used to identify patients hospitalized with gout, stratified based on a history of cirrhosis, from 2001 to 2013 *via* the International Classification of Diseases, Ninth Revision, Clinical Modification codes. Primary outcomes were mortality, gout complications and joint interventions. The χ^2 test and independent *t*-test were performed to assess categorical and continuous data, respectively. Multiple logistic regression was used to control for confounding variables.

RESULTS

Patients without cirrhosis were older (70.37 ± 13.53 years *vs* 66.21 ± 12.325 years; $P < 0.05$). Most patients were male (74.63% in the cirrhosis group *vs* 66.83%;

adjusted $P < 0.05$). Patients with cirrhosis had greater rates of mortality (5.49% *vs* 2.03%; adjusted $P < 0.05$), gout flare (2.89% *vs* 2.77%; adjusted $P < 0.05$) and tophi (0.97% *vs* 0.75%; adjusted $P = 0.677$). Patients without cirrhosis had higher rates of arthrocentesis (2.45% *vs* 2.21%; adjusted $P < 0.05$) and joint injections (0.72% *vs* 0.52%; adjusted $P < 0.05$).

CONCLUSION

Gout complications were more common in cirrhosis. Those without cirrhosis had higher rates of interventions, possibly due to hesitancy with performing these interventions given the higher complication risk in cirrhosis.

Key Words: Gout; Cirrhosis; Hyperuricemia; Uric acid; Nonalcoholic fatty liver disease; Arthropathy

Core Tip: Patients with cirrhosis had higher rates of gout-related complications including rates of flares. This could be due to the patients with cirrhosis having higher rates of hyperuricemia, predisposing them to worsening gout. Furthermore, patients with cirrhosis had lower rates of joint interventions, likely due to clinician hesitancy with performing such procedures due to an elevated risk of bleeding in patients with cirrhosis.

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INTRODUCTION

Gout is an inflammatory joint disease present in approximately 3.9% of adults in the United States, with an increasing yearly incidence[1]. Joint inflammation characteristic of the disease process occurs in reaction to deposition of monosodium uric acid (UA) crystals that form due to elevated serum urate levels[2,3]. Deposition occurs in distal joints, where lower temperature and pH decrease urate solubility, thus favoring crystallization. Monosodium UA crystals are processed by immune cells, including neutrophils and macrophages, which release cytokines, reactive oxygen species and prostaglandins that trigger an inflammatory response, resulting in a gout flare[2,4]. If the hyperuricemia of gout is left untreated, chronic granulomatous inflammation occurs resulting in tophi formation[2,4]. While rarely life-threatening, acute gout attacks and their sequelae are a source of significant morbidity. Patients with gout experience severe joint pain, difficulty with ambulation, chronic joint destruction and potentially systemic manifestations, such as nephropathy and urate nephrolithiasis[5].

Gout flares can be triggered by alcohol, fatty foods, dehydration, trauma and medications, including thiazide diuretics, that alter serum urate levels[6]. Serum urate levels are directly relevant to the development and severity of gout. Management focuses on the reduction of serum urate levels *via* lifestyle modifications and pharmacological interventions.

UA is formed from the breakdown of purine amino acids in the liver, and abnormally elevated serum concentrations occur most commonly due to inefficient elimination[7]. Hyperuricemia itself is prevalent in over 21% of adults in the United States[1,7]. Risk factors for the development of elevated serum UA levels are nearly identical to those that predispose individuals to gout, including metabolic syndrome, diet, chronic kidney disease and certain diuretics[7,8]. Hyperuricemia itself has been described as a possible contributing factor to the development of other diseases besides gout, including cardiovascular disease, atrial fibrillation, kidney disease and nonalcoholic fatty liver disease (NAFLD)[9-12].

Multiple studies have shown a positive correlation between serum urate levels and hepatic steatosis and NAFLD[12-14]. Meanwhile, others depict an inverse relationship between liver fibrosis in NAFLD and hyperuricemia, describing a decreased prevalence of hyperuricemia in individuals with significant hepatic fibrosis[15]. While the relationship between NAFLD and gout has been studied, there are few studies exploring the relationship between gout and liver cirrhosis in general (encompassing NAFLD, alcoholic cirrhosis and viral cirrhosis). In this study we analyzed differences in complication rates and mortality between gout patients with and without cirrhosis using data from the national inpatient sample (NIS).

MATERIALS AND METHODS

Data source

Patient information found within the NIS, the largest public all-payer inpatient database containing information on more than 7 million hospital stays in the United States, served as the source of the study population. The NIS was developed by the Agency for Healthcare Research and Quality and contains no patient or hospital identifiers, providing a nationally representative set of data representing 20% of all discharges from hospitals within the United States. Sample weight is applied annually, enabling precise estimates. In this study, the NIS was queried for cases from 2001 to 2013 using International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9 CM) codes to identify patients with cirrhosis who were hospitalized with gout.

Study design

This was a cross-sectional study. Inclusion criteria consisted of patients 18-years-old or older hospitalized in the United States with a diagnosis of gout between 2001 and 2013. These patients were then stratified based on the presence of ICD-9 codes for cirrhosis. Measured outcomes included inpatient mortality, rates of gout flares and complications of gout including flare, tophi, UA nephrolithiasis, nephropathy, septic arthritis as well as rates of arthrocentesis and intra-articular injections. Demographic information such as age, sex at birth and race were analyzed as well.

Statistical analysis

The IBM SPSS Statistics 24 (IBM Corp., Armonk, NY, United States) software was used to conduct statistical analyses. Independent *t*-test and χ^2 test were used to analyze outcomes and demographic data for both groups for continuous and categorical data, respectively. Multiple logistic regression was used to characterize primary and secondary outcomes among both groups while controlling for age, sex at birth, race, alcohol use disorder, cardiac arrhythmias, chronic pulmonary disease, heart failure, diabetes, human immunodeficiency virus, hypertension, peripheral vascular disease and renal failure. Statistical significance was determined with a P value < 0.05. Adjusted odds ratios and associated 95% confidence intervals were calculated.

RESULTS

Of patients hospitalized from 2001 to 2013 with gout, 1491829 did not have a diagnosis of cirrhosis, while 36948 had cirrhosis (Table 1). The majority of both groups were male, but the cirrhosis group had a greater number of males compared to the non-cirrhosis group (74.63% *vs* 66.83%) without statistical significance. Patients without cirrhosis were older (70.37 ± 13.53 years *vs* 66.21 ± 12.325 years; P < 0.05), while those with cirrhosis were younger in age (Table 2). In effect, patients with cirrhosis were younger and had a greater percentage of males than patients without cirrhosis.

Racial distribution was similar in the non-cirrhosis and cirrhosis groups, with Caucasians making up most of the sample size (71.0% *vs* 69.1%, respectively), followed by Blacks (18.6% *vs* 17.4%, respectively), Hispanics (4.4% *vs* 7.4%, respectively), Asians or Pacific Islanders (3.6% for both groups) and Native Americans (0.3% *vs* 0.4%, respectively), all with statistical significance (P < 0.05) (Table 3).

In terms of in-hospital outcomes, patients with cirrhosis with gout had higher rates of mortality (5.49% *vs* 2.03%; adjusted P < 0.05), gout flare (2.89% *vs* 2.77%; adjusted P < 0.05) and tophi (0.97% *vs* 0.75%; adjusted P = 0.677). However, differences in rates of tophi were statistically insignificant. Patients without cirrhosis had higher rates of arthrocentesis (2.45% *vs* 2.21%; adjusted P < 0.05) and joint injections (0.72% *vs* 0.52%; adjusted P < 0.05) (Table 4). Rates of septic arthritis (0.31% in patients without cirrhosis and patients with cirrhosis; adjusted P = 0.977), nephropathy (0.02% in patients without cirrhosis *vs* 0.01% in patients with cirrhosis; adjusted P = 0.19) and UA nephrolithiasis (0.02% in both groups; adjusted P = 0.915) did not differ significantly among both groups.

DISCUSSION

Results from this study demonstrate a significant correlation between gout complications and cirrhosis. The pathophysiologic manifestations of the disease, including rates of gout flare, corresponded positively with the prevalence of cirrhosis, while rates of common diagnostic and therapeutic procedures correlated negatively with rates of cirrhosis. Specifically, the rates of gout flare were higher in patients with cirrhosis. However, the difference in flare rates among both groups was 0.12%. As such, this difference may be statistically significant, but it may be clinically irrelevant. The aforementioned positive correlation may be attributed to the elevated serum UA levels found in patients with cirrhosis.

Table 1 Patient sex at birth for gout with and without a history of cirrhosis

		Patients without cirrhosis, *n* = 1491829		Patients with cirrhosis, *n* = 36948		OR	95%CI	*P* value
		Percentage	*n*	Percentage	*n*			
Sex at birth	Female	33.17	494890	25.37	9372	0.685	0.669-0.701	< 0.05
	Male	66.83	996939	74.63	27576			

95%CI: 95% Confidence interval; OR: Odds ratio.

Table 2 Differences in age distribution among patients hospitalized for gout with and without a history of cirrhosis

	Patients without cirrhosis			Patients with cirrhosis			Mean difference	95%CI	*P* value
	Mean	SD	SE mean	Mean	SD	SE mean			
Age at admission (yr)	70.37	13.53	0.011	66.21	12.33	0.064	4.167 ± 0.071	4.027-4.306	< 0.05

95%CI: 95% Confidence interval; SD: Standard deviation; SE: Standard error.

Table 3 Racial characteristics in patients hospitalized for gout with and without a history of cirrhosis

Race	Patients without cirrhosis		Patients with cirrhosis		*P* value
	%	*n*	%	*n*	
Caucasian	71.0	890040	69.1	22725	< 0.05
Black	18.6	233005	17.4	5723	
Hispanic	4.4	55174	7.4	2426	
Asian or Pacific Islander	3.6	45217	3.6	1184	
Native American	0.3	4289	0.4	122	
Other	2.0	25351	2.1	703	

Table 4 Primary outcomes in gout among hospitalized patients with and without cirrhosis

	Patients without cirrhosis		Patients with cirrhosis		OR	95%CI	*P* value	AOR	ACI	Adjusted *P* value
	Percentage	*n*	Percentage	*n*						
Mortality	2.03	30286	5.49	2029	2.804	2.678-2.937	< 0.050	3.092	2.939-3.252	< 0.05
Gout flare	2.77	41282	2.89	1066	1.044	0.982-1.110	0. 171	0.816	0.765-0.871	< 0.05
Tophi	0.75	11202	0.97	358	1.293	1.164-1.438	< 0.050	1.025	0.914-1.149	0.677
Uric acid nephrolithiasis	0.02	374	0.02	9	0.972	0.502-1.882	0.932	1.037	0.530-2.030	0.915
Nephropathy	0.02	283	0.01	5	0.713	0.295-1.727	0.452	0.548	0.223-1.346	0.19
Arthrocentesis	2.45	36611	2.21	818	0.900	0.839-0.965	< 0.050	0.741	0.686-0.800	< 0.05
Joint injection	0.72	10673	0.52	192	0.725	0.628-0.837	< 0.050	0.713	0.610-0.833	< 0.050
Septic Arthritis	0.31	4637	0.31	114	0.993	0.824-1.196	0.939	0.997	0.821-1.211	0.977

95%CI: 95% Confidence interval; ACI: Adjusted confidence interval; AOR: Adjusted odds ratio; OR: Odds ratio.

Hyperuricemia has a direct impact on cardiovascular mortality, insulin resistance, renal disease and NAFLD[16]. This relationship is thought to be secondary to urate-induced radical oxide species formation, resulting in intracellular oxidative damage[17]. UA has differential functions depending on where it is found in relation to the cell. Extracellular urate acts as an antioxidant within the hydrophilic environment, neutralizing reactive oxygen species and thus protecting the plasma membrane[18]. Antithetically, intracellular urate serves a pro-oxidant function when exposed to the hydrophobic

environment, stimulating the production of inflammatory cytokines and reactive oxygen species-producing enzymes. Within hepatocytes specifically, urate also increases gluconeogenesis *via* AMPK blockade and inflammasome formation and promotes hepatic lipid aggregation[19-21]. Therefore, intrahepatocytic UA accumulation would result in increased radical oxide formation, insulin resistance and lipid accumulation ultimately promoting liver cell damage and steatosis.

Whether serum urate is a risk factor for cirrhosis or vice versa is still in contention. There is evidence that elevated serum urate is an independent risk factor for hepatic steatosis, a harbinger of cirrhosis[18,22]. Furthermore, a reciprocal relationship between the two conditions has been described. Fatty liver disease has been shown to increase serum UA levels[23]. The mechanism of NAFLD-induced hyperuricemia is unclear, yet this interrelationship is strong enough to incentivize clinicians to investigate UA-lowering medications as a potential therapy for patients with fatty liver disease, especially xanthine oxidase inhibitors[24,25]. Other therapies designed to lower intrahepatocyte radical oxide species formation have also been explored, including blockade of chloride ion channels, which would prevent transport of radicals from the extracellular space to within the cell[26]. Hence radical oxide-induced hepatocyte injury plays a significant role in the development of liver disease and reducing levels of these molecules may slow the progression of cirrhosis. Since increased intracellular UA levels promote formation of these radical oxides, urate-lowering therapy may also delay the progression of liver disease.

The negative relationship between rates of cirrhosis and gout-related interventions found in this study may be due to clinician hesitancy with performance of such procedures in the setting of cirrhosis-induced coagulopathy. This hesitancy may be unfounded. While patients with cirrhosis are coagulopathic and at increased risk of bleeding, significant blood loss following minor procedures is rare in the absence of severe thrombocytopenia[27,28]. On the other hand, patients with cirrhosis are generally sicker than the average hospitalized individual and may be too hemodynamically unstable for such procedures.

We also found that patients with cirrhosis had higher rates of mortality than those without cirrhosis. This finding was expected, as cirrhosis has a poor prognosis and patients are at risk for significant complications resulting from end stage liver disease, including bleeding, infection and hemodynamic instability[20,27].

This study was limited by the fact that risk factors for cirrhosis, such as metabolic syndrome and chronic alcohol use, are independently associated with elevated serum UA levels and gout[29,30]. The population of patients with cirrhosis examined in this study encompassed both alcoholic and nonalcoholic etiologies of cirrhosis. Therefore, alcohol use disorder could represent a significant confounding variable. While alcohol use disorder was controlled for as a confounding variable, its relationship to alcoholic cirrhosis could still pose issues when attempting to independently correlate gout with cirrhosis. Another limitation was that the NIS database could not be used to assess whether the interventions designed to diagnose or treat gout led to any bleeding complications. Further studies analyzing clinician decision making regarding interventions in patients with cirrhosis may clarify factors leading to our finding of lower rates in patients with cirrhosis. We did not stratify patients with cirrhosis by subtype of cirrhosis (*i.e.* viral *vs* alcoholic *vs* NAFLD) as there were no specific ICD-9 codes distinguishing viral cirrhosis from NAFLD.

Alternate avenues of research worth exploring include retrospective chart review of patients hospitalized for gout flares with a history of cirrhosis, further stratifying patients into NAFLD or alcoholic cirrhosis. This proposed study would clarify the relationship between gout and cirrhosis, and it would delineate the differences in gout rates in those with alcoholic cirrhosis, who likely have a significant history of alcohol use, which is an independent risk factor for gout development, and those with NAFLD. Another possible future research endeavor includes studying rates of gout complications in patients diagnosed with virus-related cirrhosis, including hepatitis B virus and hepatitis C virus. We established that there are a limited number of studies assessing the relationship between liver disease and gout; there are even fewer studies correlating viral cirrhosis with gout or hyperuricemia. Since the pathophysiology of hepatitis C virus or hepatitis B virus cirrhosis is not connected to that of hyperuricemia (as opposed to metabolic syndrome in NAFLD and alcohol use in alcoholic cirrhosis), isolating cases of gout in those with viral-induced cirrhosis may provide an objective view into the pathophysiology of cirrhosis-induced hyperuricemia and the subsequent effect on gout exacerbations.

CONCLUSION

In summary, patients with cirrhosis may have differential rates of gout exacerbations and potential therapeutic options due to a combination of pathophysiology, cirrhosis-related comorbidities and clinical decision making. As there are few studies connecting both disease states, more investigation is required to further delineate the relationship between liver disease and gout.

ARTICLE HIGHLIGHTS

Research background

Gout is an inflammatory joint disorder with increasing yearly incidence in the United States. It is affected by factors including diet, alcohol use and obesity, all of which are significant contributors to end stage liver disease. Furthermore, studies suggest a correlation between serum uric acid (UA) levels and cirrhosis.

Research motivation

The relationship between gout and cirrhosis and the possible relationship between hyperuricemia and liver disease has not been adequately explored, despite their common risk factors. We aimed to further clarify a possible link between the two disease states.

Research objectives

Our objective was to determine if patients with cirrhosis had differential rates of outcomes regarding hospitalizations for gout, including episodes of gout flares, disease complications and possible invasive interventions.

Research methods

We utilized data from the national inpatient sample, assessing inpatient cases from 2001 to 2013. Specifically, hospitalized individuals with gout were stratified based on the presence of cirrhosis. Outcomes of gout, including flares, tophus formation and joint interventions were explored. Rates of outcomes were compared between patients with and without cirrhosis.

Research results

We found that patients with cirrhosis had greater rates of gout flares, but lower rates of arthrocentesis and joint injections.

Research conclusions

Gout recurrence was more common in patients with cirrhosis, and joint interventions were performed more infrequently in these patients. The increased rate of gout flares could be secondary to elevated serum UA levels, as determined in prior research endeavors, in patients with cirrhosis. The reduced rate of joint interventions could be due to clinician hesitancy to perform these procedures, given the increased risk of bleeding in patients with cirrhosis.

Research perspectives

A link between cirrhosis and gout flares has been established, yet no significant difference was found between cirrhosis and other gout complications. Further prospective endeavors are required to further characterize this relationship.

FOOTNOTES

Author contributions: All authors contributed to the study conception and design; Khrais A contributed to material preparation, data collection and analysis and wrote the first draft of the manuscript; Kahlam A and Tahir A and all authors commented on previous versions of the manuscript; Ahlawat S revised the article critically for important intellectual content; All authors read and approved the final manuscript.

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Informed consent statement: Informed consent was not required.

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Data sharing statement: Statistical code and database is available from the national inpatient sample at https://www.hcup-us.ahrq.gov/db/nation/nis/nisdbdocumentation.jsp. Consent was not obtained, but the presented data are anonymized, and risk of identification is non-existent as data were obtained from a public database.

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CASE REPORT

Autoimmune hepatitis and eosinophilia: A rare case report

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Abstract

BACKGROUND

Autoimmune hepatitis consists of a chronic liver disease whose etiology is unknown. It is comprised of relevant immunological aspects and of immune-mediated liver injury. Eosinophilia may be a considerable feature, particularly happening in male patients.

CASE SUMMARY

We report here a Crohn´s disease patient presenting with de novo hypergammaglobulinemia, circulating autoantibodies and elevated transaminase levels. He also had significant peripheral eosinophilia and elevated immunoglobulin E levels at diagnosis. The pathology findings from liver biopsy were compatible with autoimmune hepatitis with eosinophilic infiltration.

CONCLUSION

This is the first report of autoimmune hepatitis with exuberant eosinophilic infiltration in the liver and bone marrow, described in a patient with Crohn's disease.

Key Words: Autoimmune hepatitis; Eosinophilia; Bone marrow; Crohn's disease; Case report

Core Tip: There are very few reported cases of autoimmune hepatitis presenting with peripheral blood eosinophilia. This is the first report of autoimmune hepatitis with exuberant eosinophilic infiltration in the liver and bone marrow, described in a patient with Crohn's disease.

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INTRODUCTION

Peripheral blood eosinophilia is considered either a primary or a secondary phenomenon[1]. Primary eosinophilia often takes place within hematologic malignancies where cytogenetic or bone marrow histologic evidence regarding the clonal expansion of these cells can be found. On the other hand, causes of secondary eosinophilia include parasitosis, medications, malignancies and inflammatory or allergic conditions. Idiopathic eosinophilia consists of a diagnosis of exclusion, when no primary or secondary causes are detected.

Peripheral blood eosinophilia may be found in several hepatobiliary and gastrointestinal disorders. Indeed, some gastrointestinal diseases are eosinophil-mediated pathologies, such as eosinophilic gastroenteritis, inflammatory bowel disease, *Helicobacter pylori* infection, gastroesophageal reflux disease, collagenous colitis and celiac disease[2]. In addition, hepatic eosinophilia has been presented associated to primary biliary cirrhosis, sclerosing cholangitis, eosinophilic cholangitis and eosinophilic cholecystitis.

Currently, there are very few reported cases of autoimmune hepatitis presenting with peripheral blood eosinophilia, usually associated with other autoimmune conditions[3]. Hereafter we explore a case of autoimmune hepatitis associated with peripheral blood and tissue eosinophilia. This report aims at making physicians aware of that association in order to consider this diagnosis in a patient who presents elevated transaminases in concert with a high eosinophil count.

CASE PRESENTATION

Chief complaints

A 36-year-old Caucasian male with asthma and Crohn's disease (Montreal classification A2L2B1), was under azathioprine until 4 years ago when it was discontinued due to clinical and endoscopic remission. His asthma was under control since childhood. He was not on medication and had no known drug allergies. The patient was asymptomatic.

History of present illness

In routine analysis, it was noticed a new-onset cytocholestasis (aspartate aminotransferase 86 U/L, alanine aminotransferase 240 U/L, gamma-glutamyl transferase 288 U/L, alkaline phosphatase 794/L) without hyperbilirubinemia or coagulopathy.

History of past illness

The patient denied any history that can suggest viral prodrome, sick contacts, recent travel, medication ingestion (comprising herbal or over-the-counter), exposure to well water, exposure to recreational drugs, tattoos, alcohol, high-risk sexual behavior or blood transfusions.

Physical examination

Normal.

Laboratory examinations

Antinuclear antibody was positive (1:100, speckled pattern) as well as anti-smooth muscle. All other liver-related autoantibodies were negative (anti-mitochondrial, anti-liver-kidney microsomal, anti-soluble liver antigen and antineutrophil cytoplasmic). Immunoglobulin G (IgG) levels were elevated (3650 mg/dL). Serology for human immunodeficiency virus, hepatitis A virus, Epstein-Barr virus, cytomegalovirus, and herpes simplex virus type 1 and 2 were negative. Polymerase chain reaction considering hepatitis B, C and E viruses were negative, too. Alpha-1 antitrypsin, ceruloplasmin and iron tests were all normal. The same could be perceived for thyroid function.

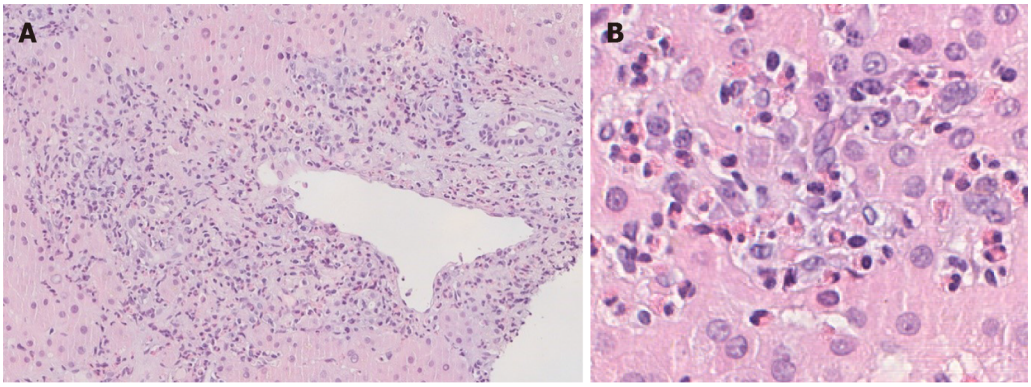

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Figure 1 Liver biopsy. A: Portal tract inflammation with intense lymphoplasmacytic infiltrate and interface hepatitis (HE ×20); B: Intralobular hepatic parenchyma with numerous eosinophils (HE ×400).

Blood tests presented an absolute white blood cell count of 33.46 × 10^9/L. Differential count indicated 89.5% of eosinophilia, as well as an absolute eosinophil count of 30 × 10^9/L. In addition, immunoglobulin E (IgE) levels were elevated (8803 kU/L). Blood cultures and parasitological examination of the stools were negative. FIP1L1-PDGFRA fusion transcript was not detected.

Imaging examinations

The abdominal ultrasound showed a liver with a normal appearance and no intra-or extrahepatic biliary ductal dilation.

Histologic examination

A liver biopsy was then performed, revealing infiltration of the portal tracts and intralobular hepatic parenchyma by numerous eosinophils (Figure 1). Lymphoplasmacytic portal tract inflammatory infiltrate with interface hepatitis was identified as well as small aggregates of plasma cells. The interlobular bile ducts appeared intact and iron and copper stains were negative. Furthermore, bone marrow biopsy showed marked eosinophilia, with normal maturation and absence of blasts (Figure 2).

FINAL DIAGNOSIS

The score regarding simplified diagnostic criteria of the International Autoimmune Hepatitis Group was 7 (likely diagnosis of autoimmune hepatitis)[4]. The score considering the revised original pretreatment scoring system of the International Autoimmune Hepatitis Group was 17 (definite diagnosis of autoimmune hepatitis)[5]. Due to lack of evidence for parasitic infection, the reactive bone marrow and the absence of other systemic conditions or drugs, the patient was diagnosed with autoimmune hepatitis with peripheral blood eosinophilia.

TREATMENT

He started treatment with prednisone at 40 mg/d. Cytocholestasis (Figure 3A) and eosinophilia (Figure 3B) progressively improved. The corticosteroid dose was gradually titrated and azathioprine 2 mg/Kg was then added.

OUTCOME AND FOLLOW-UP

After a three-month treatment, follow-up tests showed a normal eosinophil count, liver IgG levels and function tests. These data supported the definite diagnosis of autoimmune hepatitis.

DISCUSSION

Autoimmune hepatitis is a chronic liver disease which is responsible for up to 20% of chronic hepatitis in Western countries. It has a mean annual incidence of 1.9 per 100.000 individuals and a prevalence of

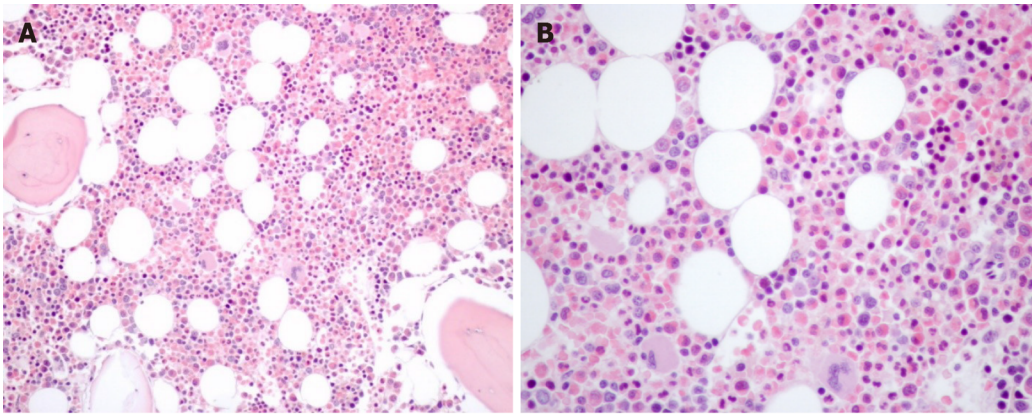

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Figure 2 Bone marrow biopsy. A: Bone marrow slightly hyperplastic, trilinear, with myeloid predominance and eosinophilia (HE ×100); B: Increased number of eosinophils (both precursors and mature forms) (HE, ×400).

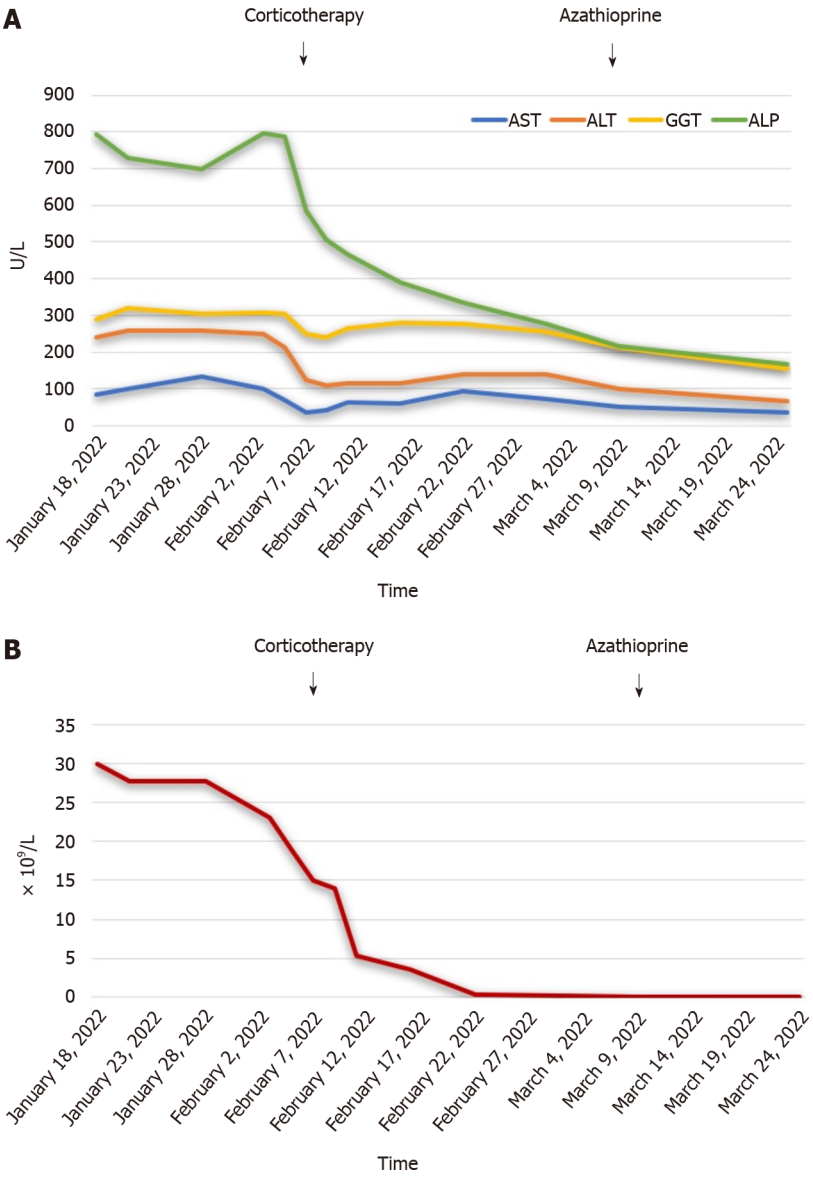

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Figure 3 Follow-up. A: Evolution of liver function tests; B: Evolution of eosinophil count.

Table 1 Reported cases of autoimmune hepatitis associated with peripheral blood eosinophilia

Ref.	Panush *et al* [8]	Kane *et al*[9]	Terrier *et al* [10]	Omata *et al* [13]	Chowdry *et al*[3]	Farani *et al* [11]	Makino *et al*[12]	Present case
Age	14 yr old	41 yr old	16 yr old	49 yr old	18 yr old	41 yr old	7 yr old	36 yr old
Sex	Male	Female	Male	Female	Male	Male	Male	Male
Transaminases	AST 700 U/L; ALT 1560 U/L	AST 200 U/L; ALT --	AST 200 U/L; ALT 320 U/L	AST 1019 U/L; ALT 772 U/L	AST 955 U/L ALT 1194 U/L	AST 70 U/L; ALT 67 U/L	AST 419 U/L; ALT 306 U/L	AST 86 U/L; ALT 240 U/L
Eosinophil count	7.437 × 10^9/L	2.64 × 10^9/L	63.2 × 10^9/L	1.2 × 10^9/L	3.3 × 10^9/L	4.9 × 10^9/L	9 × 10^9/L	30 × 10^9/L
IgG level	2300 mg/dL	2600 mg/dL	--	1930 mg/dL	2760 mg/dL	3180 mg/dL	5234 mg/dL	3650 mg/dL
Positive antibodies	ANA, SMA	SMA	SMA	Negative	ANA, SMA	ANA	ANA	ANA, SMA
Hepatic eosinophilia	No	Yes	No	Yes	Yes	Yes	Yes	Yes
Autoimmune disease associations	Coombs positive hemolytic anemia	Ulcerative colitis, autoimmune thyroid disease	Ulcerative colitis	None	None	Arthritis	None	Crohn's disease

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; IgG: Immunoglobulin G; ANA: Anti-nuclear antibody; SMA: Anti-smooth muscle antibody.

16.9 in northern European population[6]. It is characterized by hypergammaglobulinemia, circulating autoantibodies and elevated transaminase levels[7]. Peripheral blood eosinophilia is present much less frequently. It has been described in a few cases, usually in association with other autoimmune conditions, such as Coombs positive hemolytic anemia, autoimmune thyroid disease, ulcerative colitis and arthritis[8-11]. As far as we know, this report is the first one concerning autoimmune hepatitis with peripheral blood eosinophilia described in a patient with Crohn's disease. There are also some cases of blood eosinophilia associated with isolated autoimmune hepatitis, in the absence of other autoimmune conditions[3,12,13].

The development of hypereosinophilia when there is also ulcerative colitis and autoimmune hepatitis, which are two autoimmune conditions with a Th2 bias, suggests that a Th2-T-cell population is at the crossroads of the pathophysiology underlying these autoimmune diseases[10]. Other authors suggest that the concurrent existence of these processes mirrors related abnormal immunological events [8]. Another mechanism of eosinophilia can be the result of mast cell activation, which may take place in cholestatic liver disease in which mast cell-derived mediators cause activation and eosinophil chemotaxis[14].

It should be noted that, despite the fact that autoimmune hepatitis is most usually found in women (3:1 ratio), our report presents the case of a male patient[7]. Indeed, most cases described in the literature of autoimmune hepatitis with peripheral blood eosinophilia have also occurred in men (Table 1), which makes us ponder whether eosinophilia in autoimmune hepatitis can be considered a characteristic related to males.

Similar to Omata and colleagues, our patient also has a long history of asthma[13]. Nevertheless, the eosinophilia and elevated IgE levels were not associated with exacerbation of asthma but rather with elevated liver function tests. In fact, our patient had asthma under control for many years. In addition, other causes of eosinophilia, particularly immediate hypersensitivity to common allergens and parasitic infection, were excluded.

Liver biopsy is considered a prerequisite for the diagnosis of autoimmune hepatitis[15]. The classic histologic picture of autoimmune hepatitis includes interface hepatitis with dense plasma cell-rich lymphoplasmacytic infiltrates, emperipolesis, hepatocellular rosette formation, pycnotic necrosis and hepatocyte swelling. The discovery of hepatic eosinophils (even though it is not the predominant inflammatory cell type) enables the diagnosis of autoimmune hepatitis. In a study that describes the use of liver biopsy assessment in the discrimination of idiopathic autoimmune hepatitis *vs* drug-induced liver injury, Suzuki and colleagues discovered that intra-acinar eosinophils could be seen in 32.1% of times and portal tract eosinophils could be found in 60.7% of times regarding autoimmune hepatitis cases. Both of them were more usual than in cases of drug-induced liver injury[16].

In our case, the conjunction of plasma cells with interface hepatitis strongly supported the diagnosis of autoimmune hepatitis. However, the most striking aspect of this patient's disease was the exuberant hepatic eosinophilia. Similarly, the bone marrow aspirate showed a marked increase in normal-appearing cells of the eosinophil series. In this case, tissue eosinophilia was marked and blood eosinophilia was significant. In contrast, the other authors reported that none or only a few eosinophils were

present in the liver biopsy[3].

With regard to treatment, it should be noted that in all cases there was an improvement in liver tests as well as in the eosinophil count. Our patient had a favorable response under treatment with corticosteroids and azathioprine. Other authors also used 6-mercaptopurine and mycophenolate mofetil with an equally favorable response[11,13]. There is a need for long-term cautious management so as to prevent the progression into liver failure or hepatic cirrhosis[17].

CONCLUSION

Pathophysiology of autoimmune disorders is incompletely understood. The coexistence of different diseases could suggest common pathogenic mechanisms. Herein we report a case of autoimmune hepatitis associated with peripheral blood eosinophilia and exuberant liver eosinophilia. It is our goal to emphasize this infrequent presentation of autoimmune hepatitis.

FOOTNOTES

Author contributions: Garrido I did literature review and drafted the manuscript; Garrido I, Lopes S, Fonseca E, Carneiro F, and Macedo G have critically revised and finalized the manuscript; All authors have approved the final version of the manuscript.

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LETTER TO THE EDITOR

Glecaprevir/pibrentasvir + sofosbuvir for post-liver transplant recurrent hepatitis C virus treatment

Rishi Arora, Michelle T Martin, Justin Boike, Sonalie Patel

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Abstract

Glecaprevir/pibrentasvir in combination with sofosbuvir may serve as a safe and effective option for treatment of recurrent hepatitis C virus post-liver transplant in patients who previously failed direct-acting antivirals.

Key Words: Hepatitis C virus; Direct-acting antivirals; Liver transplantation; Glecaprevir/pibrentasvir; Sofosbuvir; Ribavirin

Core Tip: In the post-liver transplant population, current national guidance only recommends sofosbuvir/velpatasvir/voxilaprevir, with or without ribavirin, for recurrent hepatitis C virus treatment in direct-acting antiviral-experienced patients. We describe an alternative regimen of glecaprevir/pibrentasvir in combination with sofosbuvir that resulted in sustained virologic response without treatment-related adverse events.

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TO THE EDITOR

For direct-acting antiviral-experienced patients with recurrent hepatitis C virus (HCV), current national guidance recommends treatment with sofosbuvir/velpatasvir/voxilaprevir (SOF/VEL/VOX) or glecaprevir/pibrentasvir (GLE/PIB) and sofosbuvir in combination with ribavirin (RBV) due to their established safety and efficacy profiles. However, for recurrent HCV treatment post-liver transplant, guidance recommends use of SOF/VEL/VOX +/- RBV for 12 wk. This recommendation is based on expert consensus from seven total cases, none of which included patients who failed SOF/VEL/VOX pre-transplant[1-3]. Current guidance does not provide any recommendation for the use of GLE/PIB with SOF +/- RBV post-liver transplant, and we are unaware of published studies describing its use in direct-acting antiviral-experienced patients with recurrent HCV post-liver transplant.

We report outcomes of recurrent HCV in two patients with a history of compensated cirrhosis and hepatocellular carcinoma treated with Y90 radioembolization who underwent 24 wk of GLE/PIB with SOF after orthotopic liver transplantation from HCV-negative donors. RBV was not started in either patient due to hemoglobin < 100 g/L at treatment initiation. At the time of transplant, Model for End-Stage Liver Disease – Sodium scores were 11 and 9 for patient 1 and 2, respectively. Neither patient was co-infected with HIV or hepatitis B virus. Patient 1, a 71-year-old man with genotype 3 HCV, failed two treatments pre-transplant: (1) 12 wk of SOF/VEL; and (2) 12 wk of SOF/VEL/VOX after the patient developed hepatocellular carcinoma. Subsequent resistance testing found no mutations. Patient 2, a 67-year-old man with genotype 1 HCV, failed four regimens pre-transplant: (1) Pegylated interferon + RBV + SOF; (2) 24 wk of ledipasvir/sofosbuvir; (3) 12 wk of GLE/PIB; and (4) 12 wk of SOF/VEL/VOX and RBV. Treatment courses three and four occurred after the patient developed hepatocellular carcinoma. Subsequent resistance testing detected Q30R and Y93N mutations.

Prior to treatment initiation but post-transplantation, HCV RNA resulted as 337 and 667114 IU/mL for patient 1 and 2, respectively. After 4 wk of treatment, HCV RNA levels were undetected and remained undetected throughout treatment. Both patients achieved sustained virologic response at 12 wk after treatment completion. Minor tacrolimus dose reductions were made in the immediate post-transplantation period, but neither patient achieved toxic levels. Neither patient experienced any treatment-related adverse events, transplant complications, acute cellular rejection, or antibody-mediated rejection during and through 12 wk post-treatment completion.

Drug-drug interactions between direct-acting antivirals and immunosuppressants must be carefully considered before use. A 1.5-fold increase in tacrolimus area under the curve can occur with GLE/PIB co-administration; therefore, therapeutic drug monitoring is imperative and tacrolimus dose reductions may be needed during treatment. In those individuals taking cyclosporine, doses should be limited to < 0.1 g/d because higher doses can increase glecaprevir exposure, which may lead to increased risk of adverse events. HCV in the post-transplant setting can cause rapid development of fibrosis and decompensation, leading to higher rates of rejection, graft failure, and mortality[4]. Direct-acting antivirals offer high cure rates, but in patients who fail to achieve sustained virologic response prior to liver transplant, national guidance offers limited recommendations for recurrent HCV treatment post-transplant. Use of GLE/PIB with SOF for 24 wk offered an effective alternative to SOF/VEL/VOX +/- RBV in this small, yet complex cohort of patients and may be considered in patients who failed SOF/VEL/VOX pre-liver transplant.

FOOTNOTES

Author contributions: Arora R and Patel S led and Martin MT and Boike J assisted with the study concept and design; Arora R and Patel S equally contributed to acquisition of data and analysis and interpretation of data; Arora R led initial drafting of the manuscript, Patel S led final drafting of the manuscript, and Martin MT and Boike J edited the manuscript; All authors reviewed the manuscript for important intellectual content, gave final approval of data, and are accountable for the work.

Conflict-of-interest statement: Martin MT and Patel S serve on the speakers' bureau for AbbVie and Gilead. Martin MT has received grant funding from Gilead and Merck, served on the advisory board for AbbVie and Gilead, and is a minor shareholder of AbbVie, Gilead, and Merck stock.

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