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REVIEW

Current therapeutic modalities and chemopreventive role of natural products in liver cancer: Progress and promise

Amit Kumar Singh, Shiv Vardan Singh, Ramesh Kumar, Shashank Kumar, Sabyasachi Senapati, Abhay K Pandey

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Abstract

Liver cancer is a severe concern for public health officials since the clinical cases are increasing each year, with an estimated 5-year survival rate of 30%–35% after diagnosis. Hepatocellular carcinoma (HCC) constitutes a significant subtype of liver cancer (approximate75%) and is considered primary liver cancer. Treatment for liver cancer mainly depends on the stage of its progression, where surgery including, hepatectomy and liver transplantation, and ablation and radiotherapy are the prime choice. For advanced liver cancer, various drugs and immunotherapy are used as first-line treatment, whereas second-line treatment includes chemotherapeutic drugs from natural and synthetic origins. Sorafenib and lenvatinib are first-line therapies, while regorafenib and ramucirumab are second-line therapy. Various metabolic and signaling pathways such as Notch, JAK/STAT, Hippo, TGF-β, and Wnt have played a critical role during HCC progression. Dysbiosis has also been implicated in liver cancer. Drug-induced toxicity is a key obstacle in the treatment of liver cancer, necessitating the development of effective and safe medications, with natural compounds such as resveratrol, curcumin, diallyl sulfide, and others emerging as promising anticancer agents. This review highlights the current status of liver cancer research, signaling pathways, therapeutic targets, current treatment strategies and the chemopreventive role of various natural products in managing liver cancer.

Key Words: Liver cancer; Hepatocellular carcinoma; Signaling pathways; Therapeutic targets; Natural products; Chemopreventive

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Core Tip: Liver cancer is a serious public health concern and its therapy is stage-dependent. Approximately 75% of all liver cancers are hepatocellular carcinoma, which is regarded as primary liver cancer. First and second-line therapies are used to manage the disease but they have their own limitations in terms of toxicity and other severe side effects. Natural products are the prime choice for the future treatment of liver cancer. With advancement in the knowledge about the molecular mechanism of the disease, newer strategies having fewer side effects and greater effectiveness are needed.

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INTRODUCTION

The liver is the human body's largest solid organ, and has a pivotal role in removing various blood toxins and maintaining bioenergetics and cellular metabolism[1,2]. The liver is structured into four lobes that are made up of multiple lobules, each having a flowing duct toward the common hepatic duct, responsible for bile excretion[3]. Changes in lifestyle patterns and excessive use of medicines, alcohol and intake of various unhygienic supplements, impose further stress and finally damage the liver[4,5]. Excess alcohol and viral infections causing hepatitis are critical factors for liver cancer[6,7]. Each year approximately 0.8 million new clinical cases of liver cancer are diagnosed. From this disease, approximately 830180 people died worldwide in 2020 alone, and this figure seems to be increasing daily, according to World Health Organization surveillance reports[8]. Among various liver cancer types, hepatocellular carcinoma (HCC) is the most common type and accounts for approximately 85% of primary liver cancer cases and often occurs in people with chronic liver diseases. It is the most common and second leading cause of cancer-related deaths in Asian and sub-Saharan African countries. It is the sixth most common in western countries due to escalating hepatitis C burden along with nonalcoholic steatohepatitis and obesity[9,10]. In patients with a preclinical history of chronic liver diseases and cirrhosis, the development of HCC is a complex process, including inflammatory damage leading to hepatocyte necrosis, regeneration and fibrotic deposition[11,12].

In recent years, multiple efforts have been made to manage HCC using various chemotherapeutic approaches, of which, targeted tyrosine kinase inhibitors, immunotherapy and anticancer combination therapies are the main ones[13]. However, chemoresistance and initiation and progression of tumors mainly reprogram cellular metabolism, particularly during HCC development[14]. These metabolic alterations are key factors promoting tumor growth, proliferation and requirements of cancer cells, such as increased energy production, macromolecular biosynthesis and maintenance of redox balance. The liver is the main site for contact with a variety of orally ingested therapeutic drugs, alcohol and other xenobiotics after intestinal absorption and this organ is susceptible to various chemicals[15,16]. These chemicals cause serious complications such as acute and chronic hepatitis, granulomatous hepatitis, cholestasis with or without hepatitis, tumors and vascular disorders[17].

Among various factors responsible for HCC, viral hepatitis and resulting cirrhosis cover a significant proportion of clinical cases. Various viral infections cause upregulation of hexosamine and membrane lipid biosynthesis, by modulating glutamine-fructose-6-phosphate transaminase (GFAT)1 and choline kinase A expression[18,19]. These findings have been further validated with some results where GFAT1 is upregulated in HCC patients and its overexpression enhances tumorigenic phenotypes, as observed during *in vitro* studies[20]. Hepatitis B virus (HBV) also alters lipid metabolism, where viral proteins are known for inducing lipid accumulation *via* the upregulation of sterol regulatory element-binding protein (SREBP)1, peroxisome proliferator-activated receptor (PPAR)γ, as well as lipogenic and adipogenic enzymes, which are also reported during HCC progression[21,22]. HCC cells infected with hepatitis C virus (HCV) are also known to exhibit altered glycolysis and gluconeogenesis along with the activation of lipid-metabolism transcription factor PPARγ in human hepatocytes, similar to HCV infection[23]. Some early findings of HCC include the CD36 gene role in free fatty acid uptake and its increased expression during chronic alcohol consumption, thus modulating lipid metabolism, upregulation of SREBP1c and PPARγ, and downregulation of sirtuin 1, collectively leading to impaired fatty acid oxidation[24,25]. Nonalcoholic fatty liver disease also manifests alterations in mitochondrial and

other metabolic pathways reminiscent of HCC metabolism. As mentioned earlier, modifications in the processes are primarily analogous to many contexts observed in HCC. However, there is still a need for a better understanding of various underlying mechanisms governing metabolic changes during HCC.

Surgical resection is the primary choice for treating HCC, where recurrence and metastasis mostly occur, thus limiting proper treatment for HCC. Due to the minimal number of drugs available for the treatment of HCC, chemotherapy has remained insufficient for successful management of HCC[26,27]. Although the first-line and second-line therapies can increase life span for several months, these have serious side effects and resistance problems[28]. Since natural products are promising and cost-effective against various illnesses, it seems reasonable to focus on HCC management using natural products where anticancer drugs are limited[2,4,29]. This review focuses on HCC and its associated pathways, descriptive illustration of various natural products, along with their anticancer properties. This review provides information to investigate further regarding liver cancer, signaling pathways, therapeutic targets, current treatment strategies and the chemopreventive role of various natural products.

LIVER CANCER

Molecular signaling pathways associated with hepatic cancer

The liver is highly exposed to foreign materials, and their continuous processing is required for the body's normal functioning. Alcohol consumption imposes stress on hepatic cells. This condition worsens when combined with a genetic defect in hepatic cells. These factors, either alone or in combination, alter the molecular signaling events responsible for controlled cellular proliferation and differentiation, ultimately leading to hepatic cancer[23]. Targeting these signaling pathways by therapeutic molecules is an important strategy. Inhibition of hepatic cancer-associated signaling pathways ameliorates cancer hallmarks such as increased cellular proliferation, reduced apoptosis, migration, and angiogenesis[24,25]. This section discusses recent advances in molecular signaling pathways associated with the different stages (initiation and development) of liver cancer and their therapeutic target potential. Critical signaling pathways related to HCC include transforming growth factor (TGF)-β, Wnt/B-catenin, Hedgehog, Notch, epidermal growth factor (EGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), Janus kinase (JAK)/STAT3, and Hippo signaling pathways[30].

The human liver possesses regeneration potential, and highly controlled molecular mechanisms regulate its repair and regeneration. The Notch signaling pathway is involved in the repair and regeneration of the liver, but its malfunction (loss or gain of function) is associated with hepatic diseases, including cancer[31]. Notch1 upregulation has been found in most hepatic cancer patients. Molecular profiling studies have revealed Notch target genes such as *Hes1* and *Hey1* in hepatic cancer patients with increased cellular proliferation, reduced apoptosis, increased metastasis and angiogenesis in hepatic cancer cells[32,33]. Notch signaling crosstalk with other molecular pathways (such as hypoxia signaling) is associated with hepatic cancer[34]. Cytokine signaling pathways such as JAK/STAT (Janus kinase/signal transducer and activator of transcription) have been involved in viral escape in virus-induced HCC[35]. Viral invasion and liver injury stimulate hepatocytes and Kupffer cells to secrete sonic hedgehog (SHH) ligands. The ligand triggers Smoothened (Smo) receptor by interacting with the Patched protein, which initiates the Hippo signaling pathway in hepatic cancer cells. The activation of the Hippo signaling pathway results in increased transcription of effector genes (cyclin D, c-Myc, MMP, and CD133, *etc.*), affecting cell proliferation, invasion, and stemness properties of hepatic cancer cells[36-38].

The TGF-β signaling pathway promotes epithelial to mesenchymal transition, angiogenesis, macrophage maturation, cancer stem cell population, and cellular proliferation in HCC. Crosstalk of TGF-β with other pathways (EGF, Wnt, SHH, *etc.*) is associated with liver cancer[39,40]. Increased Wnt ligand expression and/or mutations in the molecular components of the Wnt signaling pathway results in hyperactivation of the pathway in hepatic cancer cells. The binding of Wnt ligand to its receptor, followed by production of free β-catenin and its translocation to the nucleus, activates transcription of target genes (CD44, EpCAM, cyclin D1, c-Myc, *etc.*)[41]. Transcription of target genes ultimately increases cellular proliferation, stemness, angiogenesis and migration potential in hepatic cancer cells. Wnt signaling response to a hypoxic condition in the tumor microenvironment increases stemness potential in hepatic tumor cells. Like other solid tumors, liver cancer cells secrete various growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), HGF, and VEGF. These factors in turn induce angiogenesis to ensure the appropriate supply of nutrients and oxygen. Liver cancer is the result of chronic liver cirrhosis, which ultimately takes the shape of advanced HCC. Available clinical data show that protein mutation increases as the disease progresses from the initiation stage to highly advanced cancer[42]. Mutation in *TERT* gene (catalytic subunit of telomerase reverse transcriptase) is associated with increased cellular proliferation in liver cancer cells. Clinical data revealed that TERT promoter mutation increased up to 10 times in HCC cells compared with low-grade dysplastic nodules[43]. It indicates that mutation plays an important role in the initiation and progression of the pathological stage of HCC. Besides, other mutations are only involved at the later

stage of the disease progression and produce more genetic diversified subtypes[44].

Recent development in therapeutic targets in liver cancer

Sorafenib is a first-line chemotherapeutic agent approved for advanced HCC. It is a multikinase inhibitor targeting Raf, EGFR, VEGF receptor (VEGFR), PDGF receptor (PDGFR), FMS-like tyrosine kinase-3 (FLT3) and c-kit[45,46]. Clinical studies have revealed that sorafenib inhibits hepatic tumor growth and angiogenesis in advanced stage, but its prolonged exposure induces resistance[47-50]. Recently it has been reported that the second-line drugs such as lenvatinib, regorafenib and ipilimumab have a better therapeutic outcome, and increase overall disease-free survival in liver cancer patients[51]. Increased tumor growth and distance metastasis in sorafenib resistance patients and lower overall survival rates in sorafenib-treated liver cancer patients necessitate exploring new and potential therapeutic targets in liver cancer. Exploration of newer therapeutic agents and combinatorial drug regimens may also be explored to target the disease and increase the therapeutic outcome in patients. The current treatment strategy for liver cancer (first- and second-line therapies) is discussed in more detail in the subsequent section of this review.

Luo *et al*[52] identified emerging targets in liver cancer by utilizing comprehensive and integrated multiomics analysis. The study identified potential signaling pathways (Tp53/RB1, Wnt/β-catenin, PI3/Akt/mTOR, JAK/STAT, MAPK and TGF-β) and molecular events (telomere maintenance, cellular differentiation, chromatin remodeling and oxidative stress) in liver cancer. Mutation-mediated protein activation (CCND1, CTNNB1, TERT, PIK3CA, KRAS, KEAP1, NFE2L2, JAK3, FGF4, FGF19, and FGF3) and inactivation (TP53, Rb1, CDKN24, CHN2B, ATM, AXIN1, APC, ZNRF3, HNF1A, APOB, ALB, ARID1A/B, ARID2, SMARC2, BAP1, BRD7, KMT2C, PTEN, TSC1, TSC2, RPS6KA3, and ACVR2A) are associated with the pathophysiology of liver cancer and have emerged as therapeutic targets for hepatic cancer[52]. β2-spectrin (SPTBN1), a cytoskeleton protein is essential for the development of various organs, including the liver. It performs both structural (establishment and maintenance of cellular structure) and functional (apoptosis, cell adhesion, and cell cycle regulation) role[53]. Recently it has been reported that SPTBN1 induces lipogenesis-mediated liver cancer in high-fat diet fed experimental mice. The study proposed SPTBN1 as a potential therapeutic target for liver cancer[54]. Craig *et al*[55] studied the expression profile of cancer testis antigens (CTA) proteins in HCC. CTA was overexpressed in HCC patients and associated with poor overall survival and prognosis. Further experimental evidence of the study showed that melanoma-associated antigens family A (MAGE-A), a member of the CTA family, is responsible for increasing cellular proliferation, and decreased apoptosis and aggressiveness in HCC experimental models. The study revealed that MAGEA3 is involved in the developing hepatic carcinoma and could serve as a potential novel target for the disease[55]. Glypican (GPC)-3, a heparin sulfate proteoglycan, was significantly overexpressed in > 80% of HCC patients and was positively associated with poor diagnosis in the patients[56,57]. Clinical studies showed that targeting GPC-3 by developed antibodies significantly increased disease progression-free survival in patients with overexpressed GPC-3 in comparison with patients with low GPC-3 levels. Combination of chemotherapy and the immunotoxin (antibody + exotoxin) mediated GPC-3 targeting showed better therapeutic outcomes in liver cancer patients[58-60]. These facts indicate the therapeutic potential of GPC-3 proteins in liver cancer. Interaction between HGF and its receptor c-Met is important in liver regeneration. Overexpression and/or mutation in c-kit have been positively associated with liver cancer [61]. Direct or indirect (*via* different signaling pathways) interaction among HGF and c-kit increases the cellular growth, angiogenesis and metastasis in liver cancer cells[62]. Preclinical and clinical studies reported that interrupting the association between HGF and c-kit resulted in a potential therapeutic response in liver cancer[63-65]. Thus HGF and/or c-kit are potential therapeutic targets in liver cancer. Various studies showed that cancer cells rewire their metabolic pathways to fulfil their increased need for nutritional requirement. Liver cancer cells also reprogram their lipid metabolic pathway to combat their increased nutritional requirements, which ultimately help in cellular proliferation, growth and survival. Preclinical studies have shown that biosynthesis of lipids and desaturation process play an important role in liver cancer initiation, progression and survival. Pope *et al*[66]beautifully reviewed aberrant biochemical/molecular players of lipid metabolism as potential therapeutic targets in liver cancer[66,67]. Overexpression of lipid metabolism enzymes such as fatty acid synthetase, ATP citrate lyase, stearoyl-CoA desaturase (SCD)-1, and acetyl CoA carboxylase have been associated with various cancers including liver cancer. Targeting these enzymes with small molecules showed a potential tumor-suppressive nature in experimental models of liver cancer. There is a need to study some enzyme inhibitors in the clinical trial, such as SCD-1 inhibitors[68-70].

miRNAs are short-length noncoding RNAs involved in regulating gene expression and thus controlling the normal physiology and disease pathophysiology by normal and abrupt expression, respectively[71]. Modulating miRNAs by therapeutic molecules, and/or using their respective inhibitors or mimics is an important strategy to target cancer at the gene level[72]. The study showed that aberrant expression of miRNAs (miR34, miR36, miR21, miR203, miR17, miR83, miR93, miR221, *etc.*) in liver cancer cells is associated with the increased cellular proliferation, metastasis, angiogenesis, drug resistance, cell survival and reduced apoptosis[72]. A miRNA-based mouse model of HCC has been developed to study inflammation, tumor initiation, metabolic alteration, and hepatocyte differentiation [73]. The therapeutic potential of miRNAs in liver cancer has been shown by utilizing the miRNA

inhibition/replacement approach. One studyidentified miR-550a, miR-574, miR-424, let-7i, miR-549, miR-518 and miR-512 as being significantly associated with overall survival, using bioinformatics tools that indicated their therapeutic potential. The study proposed that these miRNAs should be studied in detail for their therapeutic potential in liver cancer experimental models[74]. Dai *et al*[75] compared the publically available liver cancer miRNA expression data with the human HCC (hepatitis B positive and negative) data (generated by the study group). The study identified miR-0308-3p as a novel miRNA associated with HBV-positive HCC. miRNA suppresses liver cancer cell proliferation and arrests cells in the G1/S phase by targeting *CDK6* and *cyclin1* genes[75]. These results show that the miR-0308-3p is a novel therapeutic target in liver cancer. Shao *et al*[76]developed personalized miRNA cocktail therapy by combining nanotechnology and gene therapy to treat liver cancer. The research group encapsulated mimics (of miR-199a/b-3p) and inhibitor (of miR-10b) into a polymer-based nanoplatform (PCACP). The *in vitro* and *in vivo* experiments showed the better anticancer potential of the PCACP/miR-cocktail system in comparison with mimic or inhibitor treatment alone in liver cancer experimental models[76]. This study showed a novel potential strategy to treat liver cancer by combining nanotechnology and gene therapy. Wang *et al*[77] studied the relation between LINC01018 (a long noncoding RNA), miR-182-5p and FOXO1 protein in HCC. There was poor expression of the long noncoding RNA and FOXO1, and higher expression of miR-182-5p in the HCC patient samples. Forced expression of LINC01018 in *in vitro* and *in vivo* experimental models showed decreased cellular proliferation and induced apoptosis with increased miR-182-5p levels. The study showed liver cancer therapeutic potential of LINC01018 by miR-182-5p sponge-mediated downregulation of FOXO1 expression[77].

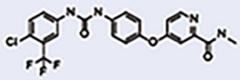
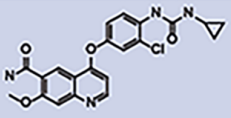
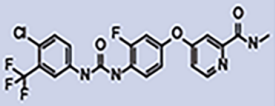
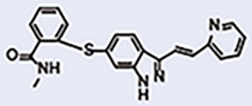
Current treatment strategies for liver cancer

The dysregulated cell cycle, apoptosis, and many other key signaling pathways are linked to HCC pathogenesis. Chemotherapeutic approaches similar to different types of cancer are also reported with a limited number of drugs for the cure of HCC and various side effects. Sorafenib, an oral multitargeted tyrosine kinase inhibitor has been used as first-line treatment for advanced HCC, showing increased survival of approximately 12 mo compared with controls[78]. Various antiangiogenic agents such as bevacizumab (human monoclonal antibody directed against VEGF) and erlotinib (EGF receptor tyrosine kinase inhibitor) have also been studied and shown effective results in early studies[79]. Until 2016, sorafenib was the only FDA-approved first-line treatment for HCC, whereas lenvatinib has also been identified and is in use for advanced HCC[80]. Sorafenib acts as an inhibitor of intracellular tyrosine and serine/threonine protein kinases such as VEGF, VEGFR, PDGFR, c-Raf and b-Raf MAP kinases, which in turn induces autophagy. Due to drug resistance and side effects such as liver fibrosis, clinical usage of sorafenib is limited[63]. Long-term exposure to sorafenib also induces cancer cells with less E-cadherin content making them more invasive. Some second-line treatments are also available for HCC, including regorafenib, ramucirumab and cabozantinib, which are rarely used and are less efficient[28,51,79]. It is reported that chemotherapeutic drugs used for HCC treatments are limited in number and seem to be less effective, considering their efficacy, bioavailability and side effects. Considering the side effects of ongoing therapies, scientific pieces of evidence are also suggestive for the use of natural products for the management of HCC, since they can inhibit viral infection, inflammation, oxidative stress, metabolic disorders, angiogenesis and metastatic activity, which are known as prime contributors in HCC[2,80,81]. Hence, there is strong demand for searching novel plant-based drugs for managing HCC with fewer side effects and less chemotoxicity. Therefore, several drugs are used to treat HCC to target the inhibition of some of these processes (Figure 1). The current therapeutic interventions for patients with HCC are divided into first- and second-line therapies. The pharmacological features of these drugs are discussed in the following section of this review.

First line therapies

Sorafenib: Sorafenib (BAY 43-9006, Nexavar) is the first-ever systemic drug as well as a standard therapeutic agent approved by the US FDA for treatment of liver cancer patients who cannot undergo surgical resection or liver transplantation[79]. Sorafenib was the only first-line treatment in the last 10 years until the FDA approved lenvatinib as a frontline therapy in 2018. It is a tyrosine kinase inhibitor that targets VEGFR1 and VEGFR2 and PDGFR-β. It activates AMP-activated protein kinase (AMPK) that can block the formation of tumor blood vessels and inhibit proliferation of liver cancer cells[82]. For individuals with HCC, sorafenib has a clear advantage in terms of survival. Sorafenib improved overall survival considerably compared with placebo in two phase III clinical randomized controlled trials (10.7 mo *vs* 7.9 mo and 6.5 mo *vs* 4.2 mo). However, the side effects associated with these clinical trials were diarrhea, tiredness, and hand–foot skin response[80,82].

Several factors hinder more people from obtaining benefits after sorafenib treatment. Because of the genetic variability of HCC and other factors, around 40% of people with HCC can benefit from sorafenib. Sorafenib was more beneficial for some patient categories in several trials. The two clinical studies mentioned above featured only a small number of patients; all of whom had good liver function. These individuals were termed Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP)-eligible patients, and only SHARP-eligible individuals benefited from sorafenib treatment[79,82]. Furthermore, the effectiveness of sorafenib is greater in HCV-infected individuals than in others who have always been resistant to sorafenib. Primary resistance is another term for the unclear

Drugs	Chemical structure	Year of FDA approval	Used dose	Family	Target	Ref.
First line therapies						
Sorafenib		2007	400 mg twice daily	Tyrosine kinase receptor family inhibitor	VEGFR 1, VEGFR 2, PDGFR-β, RET, c-Kitand FMS-like tyrosine kinase-3, Ras/MAPK pathway.	[84,87]
Lenvatinib		2018	12 mg(Child-Pugh A), 8 mg (Child-Pugh B) per day		VEGFR 1-4, PDGFR-α, PDGF-β, FGFR 1-4, KIT and RET	[85,90]
Second line therapies						
Regorafenib		2017	160 mg/d	Tyrosine kinase receptor family inhibitor	VEGFR 1-3, PDGFRβ, FGFR-1, KIT, RET, RAF1, and BRAF	[51]
Ramucirumab		2020	8 mg/kg every two weeks	Monodonal antibody	VEGFR-2	[89]

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Figure 1 First- and second-line therapies and their targets. VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet-derived growth factor receptor; FGFR: Fibroblast growth factor receptor.

mechanism of this phenomenon[82,83]. However, some research has uncovered probable explanations. Gene polymorphism may be a crucial factor influencing sorafenib function. Polymorphisms in the ATP binding cassette (ABC) subfamily B member 1 (ABCB1), ATP binding cassette subfamily G member 2 (ABCG2), solute carrier family 15 member 2 (SLC15A2) and endothelial nitric oxide synthase (eNOS) have been linked to the action of sorafenib[83]. This was confirmed by Silvia and co-workers who reported that β-caryophyllene oxide inhibits ABC proteins and causes HCC cells to become chemosensitized to sorafenib[84].

Lenvatinib: Lenvatinib (E7080, Lenvima) is an antitumor drug that belongs to the quinoline carboxiamides. The IUPAC name of lenvatinib is 4-[3-chloro-4-(cyclopropylcarbamoylamino) phenoxy]-7-methoxyquinoline-6-carboxamide. Lenvatinib acts as multikinase inhibitor *via* targeting VEGFR 1-4, PDGFR-α, PDGFR-β, FGFR 1-4, tyrosine kinase receptor (KIT) and rearranged during transfection receptor (RET) that leads to angiogenesis inhibition, and reduced vascular permeability of the tumor microenvironment[28]. Lenvatinib is an effective drug that increases overall survival in patients with advance HCC and whose tumor cannot be removed by surgery. In a phase I clinical trial, lenvatinib (12 and 8 mg) was effective in patients with advanced HCC and Child–Pugh class A or B. The adverse effects observed during 12 mg daily lenvatinib oral treatment were hypertension, decreased body weight, loss of appetite fatigue, and diarrhea[28,70]. A Phase II clinical trial was conducted to evaluate the effectiveness of lenvatinib in advanced unresectable HCC. The trial was conducted on 46 patients who received 12 mg lenvatinib orally once daily for 28 d, and lenvatinib demonstrated high efficacy with a good toxicity profile. However, the efficacy of lenvatinib was influenced by body weight[85,86].

Second-line therapies

Regorafenib: Regorafenib (BAY-73-4506) with the brand name Stivagra is an oral multikinase receptor antagonist developed by Bayer and approved by the US FDA in June 2017 to treat unresectable advanced liver cancers. Despite its structural similarity with sorafenib, regorafenib showed more effect-

iveness in inhibiting the activities of various protein kinases associated with neovascularization (VEGFR 1–3 and tyrosine kinase with immunoglobulin-like loops and epidermal growth factor homology domain-2 (TIE2), oncogenesis (KIT, RET, Raf1 and BRAF) and tumor microenvironment (PDGFR-β, PDGFR-α and FGFR) with better drug tolerance profile[87,88]. HCC patients treated with regorafenib (160 mg/d for 28 d) showed better overall survival, *i.e.*, 10.6 mo compared with 7.8 mo in the placebo group in a randomized, double-blind, placebo-controlled phase III trial. However, the main side effect was hypertension, unlike body weight loss, hepatorenal dysfunction, and fatigue in sorafenib-treated individuals[87,88].

Ramucirumab: Ramucirumab, sold under brand name Cyramza and others, is a recombinant monoclonal antibody (IgG) that targets VEGF2 and blocks its binding to VEGFR ligands. The anticancer activity of ramucirumab as second-line therapy was evaluated in Phase II clinical trials in advanced HCC patients with a high level of α-fetoprotein. These trials found that individuals who received ramucirumab had a better overall survival rate than those who received placebo; the drug was well tolerated and had an acceptable toxicity profile[89].

Future promising therapeutic drugs

Pirfenidone: Pirifenidone (Esbriet®) is an orally administered antifibrotic, antioxidant, and anti-inflammatory drug that has been studied in clinical and preclinical trials to treat hepatic and idiopathic pulmonary fibrosis[86]. Pirifenidone was effective in causing cell cycle arrest at G0/G1, eventually inhibiting cell proliferation in an *in vitro* model. Similarly, it induces apoptosis in HepG2 cells *via* Wnt/β-catenin signaling pathway. Pirifenidone has also been demonstrated to be a potent antifibrotic agent at a dose of 300 mg/kg in a carbon tetrachloride-induced HCC mouse model. However, the cellular mechanisms behind the responses elicited by pirifenidone remain unknown[70,86]. Figure 1 summarizes the pharmacological properties of drugs used in liver cancer[86].

GUT MICROBIOTA AND LIVER CANCER

Multiple lines of scientific evidence have suggested the significant contribution of gut microbes to critical aspects of human health. Even though the gut microbiota offers substantial benefits to the host, particularly in terms of immunity and metabolic activities, there is still growing evidence of the role of gut microbes in several pathological conditions. They promote disease progression not just locally, as in chronic inflammatory bowel syndrome, but also in other parts of the body, such as liver, brain and heart [91]. Similarly, there is mounting evidence that the gut microbiota plays a significant role in carcinogenesis *via* its local and long-distance effects. The liver is intimately connected to the gut through the portal vein. The liver is directly exposed to microbial metabolites and microbe-associated molecular patterns (MAMPs) that can induce inflammatory reactions through pattern-recognition receptors, and receive nutrient-rich blood from the gut. The multilayer epithelial barrier is responsible for minimal hepatic exposure to MAMPs. Although, as in chronic liver diseases, altered gut barrier and microbiota composition increases the incidence of inflammation and progression of liver disorder and thus raises the risk of HCC[92].

According to accumulating scientific evidence, intestinal dysbiosis appears to have a significant role in developing chronic liver disease and HCC. Metagenomic studies have demonstrated significant changes in the gut microbiota composition in a variety of chronic liver diseases as well as in people with cirrhosis[93]. Patients with advanced liver disease and cirrhosis have an increase in potentially harmful bacteria and a decrease in microorganisms with beneficial qualities in their gut microbiomes[94,95].

Toll-like receptor (TLR)4 is found in various liver resident cells such as Kupffer cells, hepatic stellate cells (HSCs), endothelial cells, and hepatocytes. A study conducted by Dapito and colleagues in bone marrow chimeric mice concluded that the presence of TLR4 on these liver-resident cells promotes fibrogenesis and hepatocarcinogenesis[96]. Lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component, is produced through the leaky gut and mainly targets Kupffer cells and HSCs, which appears to increase the incidence of hepatocarcinogenesis. Activation of TLR4 in HSCs causes nuclear factor (NF)-κB-mediated increased expression of epiregulin, a hepatic mitogen belonging to the EGF family, and reported to have strong mitogenic potential in hepatic cells[96,97]. The finding was confirmed when hepatocarcinogenesis decreased in epiregulin-deficient rats treated with *N*-nitrosodiethylamine (DEN)-CCl_4. Another important method through which the LPS–TLR4 axis promotes HCC development is through prevention of NF-κB-mediated hepatocyte apoptosis[96,97].

BIOACTIVE NATURAL PRODUCTS AGAINST LIVER CANCER AND MOLECULAR MECHANISMS INVOLVED

For centuries, bioactive natural products from plants have been extensively used to treat many human

diseases. Recent molecular evidence explains their modes of action, metabolic regulation, and identification of their biological targets. This evidence adds value to their potential use in the chemoprevention of HCC. The promising candidate bioactive natural products are discussed in this section, where their possible role in liver cancer therapy has been reported.

In vitro studies

In the last two decades, growing evidence has suggested an affirmative role of resveratrol (polyphenolic natural product) in the chemoprevention of liver cancer. Its application is limited due to its poor bioavailability. Previously, resveratrol was shown to negatively regulate the cellular proliferation of rat hepatoma and human hepatoblastoma cell line HepG2 at 1–150 μmol/L concentration[98]. Decreased proliferation and invasion of HepG2 cells and AH109A rat ascites hepatoma cells were also reported. In subsequent studies, resveratrol induced apoptosis in *in vitro* studies using HepG2and H4IIE rat hepatoma cells[98]. Notas *et al*[99] showed that even 2 h treatment with resveratrol (10^{-6}–1 μmol/L) interfered with DNA replication and caused cell cycle arrest. Roncoroni *et al*[100] using SK-ChA-1 human cholangiocarcinoma cells in a multicellular tumor spheroid model showed arrest of cell cycle at G1/S phase, at a concentration up to 64 μmol/L resveratrol. Resveratrol limited cellular proliferation and mobility by activating autophagy through p53 and inhibiting phosphoinositide 3-kinase/Akt in MHCC-97H cells. Autophagy thus explained the increased chemopreventive property of resveratrol. A study on HepG2 and Hep3B cells identified that resveratrol regulates the PTEN/Akt signaling pathway through downregulation of membrane-associated RING-CH (MARCH1), which ultimately aggravates apoptosis and inhibits cellular growth[101].

A curcumin analog, CUR3d, inhibited the proliferation of liver cancer cells at 100 μmol/L, which was due to downregulation of PI3K/Akt and inhibition of the NF-κB pathway, which are responsible for cancer cell growth[102]. In another study, supplementation of curcumin (1 g/kg) significantly inhibited the growth and liver metastasis of colorectal cancer cells[103]. Microemulsion formulation improve 1225 times the water solubility of myricetin and enhanced its antiproliferative activity against human liver cancer cells (HepG2)[104].

Extract of immature plum induced extrinsic apoptosis in HepG2 cells as demonstrated by caspase-1, -3 and -8 activation as well as DNA fragmentation[105]. Two natural polyphenolic compounds (epicatechin and gallocatechin gallate) were quantified in the extract and might be responsible for the anticancer potential[106]. The garlic extracts consist of multiple organosulfur components and flavanols that obstruct different stages of the carcinogenic process. Diallyl sulfide is one of the important component of garlic extract and has inhibited diethylnitrosamine (DEN) induced HCC. Another constituent of Allium extracts, S-allyl cysteine, has established antiproliferative and metastatic activity in the management of HCC[107]. 6-Shogaol and 6-gingerol are the most common active constituents in ginger that display anticancer activity against hepatoma cell lines by triggering reactive oxygen species (ROS)-mediated apoptosis and controlling expressionof matrix metalloproteinases (MMP)-9 and tissue inhibitor of metalloproteinase-1[108]. *In vitro* and *in vivo* activities of many natural products are depicted in Table 1.

In vivo studies

Intraperitoneal resveratrol administration (1 mg/kg) for 7 d in male Wistar rats implanted with AH-130 hepatoma cells arrested tumor growth. Liu *et al*[109] showed the immunomodulatory role of resveratrol (500, 1000, 1500 mg/kg for 10 d) in BALB/c mice implanted with H22 hepatoma cells. Rajasekaran *et al*[110] studied the chemopreventive role of resveratrol in a model of DEN-induced HCC in male Wistar rats. It induced apoptosis by PARP cleavage, caspase-3 activation, p53 upregulation and cytochrome c release when given early at a dose of 200 mg/kg. Gao *et al*[111] tested the chemopreventive property of resveratrol in MHCC97-H-inoculated athymic nude mice. The study identified its antitumor activity by downregulating the HGF/c-Met signaling pathway. Resveratrol-gold nanoparticles have shown improved anticancer effects compared with resveratrol alone in HEPG2 cells and xenografted BALC/c nude mice[112].

Lycium polysaccharide portion (LPP) is the most crucial part of *Lycium barbarum* that has abundant biological activities such as antioxidant, neuroprotective, immunoprotective, antitumor, and glucose metabolism regulatory activities. LPP inhibited the propagation of hepatocytes and led to apoptosis of liver hepatocytes, thus indicating its anticancer role. A clinical trial showed that consumption of LPP juice leads to elevation in interleukin (IL-2), IgG, serum antioxidants levels, lymphocyte count and reduced levels of lipid peroxides[113]. Berberine mediates anticancer activity by inhibiting antiapoptotic protein Bcl-2, and activating the caspase cascade and proapoptotic pathway of Egr1-NAG-1 (nonsteroidal anti-inflammatory drug-activated gene). Berberine facilitates phosphorylation of AMPK, thus increasing the concentration of p-AMPK/total AMPK. The AMPK-mediated mitochondrial/caspase pathway by raising the Bax/Bcl-2 ratio may be responsible for the anticancer activity of berberine. Long-lasting polyethylene glycol-based liposomal berberine displayed *in vivo* and *in vitro* anti-HCC activity[114]. Paclitaxel-loaded nanoparticles, followed by galactosamine conjugation on the formed nanoparticles, were effective in reducing the tumor size through apoptosis activation and cell cycle arrest[115]. The efficacy of many natural products against liver cancer is shown in Table 1.

Table 1 Effect of natural products on liver cancer

Natural products	Extract/phytochemicals	Experiment model (*in vitro/in vivo*/clinical trials	Tested concentration	Medicinal effects	Ref.
Broccoli	Sulforaphane	*In vitro* (murine hepatoma Hepa 1c1c7 and human HepG2 cells)	1-20 μmol/L	Sulforaphane showed positive effect on Phase II detoxification enzyme. Sulphoraphane treatment resulted in increased expression of CYP1A1 and quinone reductase	[116]
Grape	Procynidins rich grape crude extract	*In vitro* (HepG2 human liver cancer cells)	0-120 μg/mL	Grape extract in concentrations greater than 20 μg/mL (20.4 μmol/L) was cytotoxic to HepG2 human liver cancer cells, with maximal toxicity of 67.2% and ED50 of 49.6 μg/mL (50.5 μmol/L)	[117]
	Flavan-3-ol rich extract	*In vitro* HepG2 and breast cancers (MCF-7) cells	0-60 μg/mL	Grape extract showed dose dependent cytotoxicity *via* Induction of apoptosis, DNA damage and suppression of oncoprotein Her-2 expression. Treatment also resulted in increased NO production in cancer cell	[118]
Mung bean sprouts	Extract	*In vitro* [Human cervical (HeLa) and hepatocarcinoma cells (HepG2)]	9.37 to 300 mg/mL	Mung bean sprouts was found to be a potent anticancer agent. The cytotoxic effect of Mung bean sprouts extract on HeLa, expressed as IC50, was 13.3 mg/ml 163.97 mg/ml while on HepG2 cells was 14.04 mg/ml. It also increases apoptosis, anti-tumor cytokines (TNF-and IFN-β), IFN-γproduction and subsequently up regulated the cell-mediated immunity	[119]
Cinnamon	Isoobtusilac-tone A	*In vitro* (Hep G2 cells)	100 μmol/L	Induces apoptosis in cancer cell	[120]
Ginger	6-shogaol, 6-gingerol	*In vitro* (Human hepatoma HepG2 and Hep3B cells)	10 μmol/L and 50 μmol/L	The migratory and invasive activity of HepG2 and Hep3B cells were decreased in doses dependent manner post 6-shogaol, 6-gingerol treatment. It suppresses the metastatic activity *via* down regulation of matrix metalloproteinase (MMP)-9 and urokinase type plasminogen and upregulation of tissue inhibitor metalloproteinase protein	[108,121]
Asparagus	asparanin A	*In vitro* (HepG2 cells)	0-30 μmol/L	Treatment with asparanin A resulted in cell cycle arrest at G2/M phase and apoptosis in HepG2 cells. Following treatment of HepG2 cells with asparanin A, cell cycle-related proteins including cyclin A, Cdk1 and Cdk4 were down-regulated, while p21WAF1/Cip1 and p-Cdk1 (Thr14/Tyr15) were up-regulated	[122]
Tomato	Tomatine	*In vitro* (HepG2 cells)	10, 50 and 100 μg/mL	Induces antigen-specific cellular immunity and direct destruction of cancer cell membranes	[123]
	Lycopene	*In vivo* (N-nitrosodiethylamine induced hepatocarcinogenesis in female Balb/c mice)	5 mg/kg bw	Lycopene treatment causes modulation of apoptosis related genes (enhanced expression of caspase 3 and 9 and p53 and decreased expression of Bcl-2). Lycopene exhibits prooxidant activity in tumor that supports observed enhanced apoptosis. This increased apoptosis is a chemopreventive action of lycopene in liver cancer	[124]
Plum	Extract	Benzopyrene-induced hepatocarcinogenesis in rats	2.5 or 5 g/kg bw	Plum extracts may counteract toxic effects of carcinogens and benzopyrene, and therefore have chemopreventive efficacy	[125]
Pomegranate	Emulsion	Diethylnitrosamine (DENA)-induced hepatocarcinogenesis inrat	1 or 10 g/kg bw	Treatment for 18 wk in rats resulted in reduced incidence, number, multiplicity, size and volume of hepatic nodules, precursors of HCC. It showed chemoprevention through potent antioxidant activity as the expression of Nrf-2 is increased in pomegranate treated rats	[126]
Citrus fruit	Auraptine	Diethylnitrosamine (DEN)-induced hepatocarcinogenesis in rat	5 or 10 g/kg bw	It suppresses tumor progression in DEN-challenged rats by negative selection for cancer cells with β-catenin mutation	[127]
Pepper	Glycoprotein	*In vivo* (DEN induced hepatocarcinogenesis in mice)	20 mg/kg bw	Pepper glycoprotein of 24 kDa treatment in rats causes increased activity of natural killer cell and ultimately prevention of DENA induced liver carcinogenesis *via* immunomodulation and	[128]

promotion of apoptosis

CHALLENGES AND WAY FORWARD IN NATURAL-PRODUCT-BASED ANTI-LIVER CANCER THERAPEUTICS

Natural products have become a focus of attention in anticancer drug discovery due to unsolved problems related to current chemotherapy, such as drug resistance and toxicity. It should be noted that from 1940 to 2014, approximately 50% of the small molecules approved for cancer treatment were either natural compounds or their derivatives[129,130]. Natural products for anticancer therapy have had some therapeutic limitations, which affect therapeutic outcome, lower bioavailability, and selected and targeted delivery. This section highlights these issues, recent advances in the field, and future potential. Advancements in computational biology/pharmacology/chemistry and high-throughput *in vitro* screening of natural anticancer drugs have highly accelerated the drug discovery process, resulting in a lead molecule. Most of the time, it is frustrating to obtain unsatisfactory activity of the lead natural molecule in *in vivo* experiments and/or clinical studies, which results in lesser activity and nonselectivity for a given therapeutic target. It has been proposed that delivering natural products to a targeted site using an appropriate delivery system may improve the efficacy by increasing their bioavailability. The process may also decrease the off-target effects and toxicity related issues in a given therapy[131]. Different means of drug delivery or appropriate vehicles have been discussed elsewhere [132]. The use of these tools/vehicles is dependent on their biocompatibility, degradability and functional limitations. However, the concept is promising but has its limitations (rapid elimination from the body, toxicity and inflammation), which still need to be addressed[132,133].

To consider the efficacy of natural products in living systems, it is essential to understand their pharmacokinetics. Absorption, distribution, metabolism and excretion may primarily affect the therapeutic outcome of the natural products. Absorption of a particular drug is influenced by the mode of administration, *i.e.*, whether it is oral, intravenous or inhalation. In each case, the drug shows different kinetic behavior in relation to its therapeutic outcome. Factors such as permeability of barriers, pH of cellular/body compartments, binding affinity with the off-targets and their fat solubility affect the distribution of the natural products in the body. Drug metabolism in the liver or gut introduces alterations in the structure of natural products, as well as irreversible secretion of the drugs through the hepatobiliary system or kidneys, which affects the plasma level of the drug and its efficacy. Few reports are available on the pharmacokinetics of natural products (such as glycyrrhetinic acid, curcumin, ethiodized oil) in liver cancer experimental models or patients[103,104,134]. Most of the lead anti-liver cancer natural products have not yet been studied for the above pharmacokinetic parameters in experimental models. Information on the pharmacokinetic parameters of the particular natural products may shed light on the efforts that should be taken to improve their therapeutic efficacy in *in vivo* experimental models and liver cancer patients.

New approaches have been introduced to improve the natural product delivery and specifically target liver cancer cells. Previously it has been reported that tissue-targeted drug delivery significantly enhances the therapeutic efficacy of anti-liver cancer drugs, confining their bioavailability within the tumor. The concept of tissue targeted drug delivery also minimizes the side effects such as toxicity by reducing systemic bioavailability to other organs of the body. Anti-liver cancer drugs combined with a delivery system providing galactose residues have been utilized to target liver cells (with asialoglycoprotein receptors) specifically[135,136]. Liposomes have been used as carriers for anticancer drugs due to various advantages such as improved drug stability in the body without altering the structural integrity of the drug[136,137]. Li *et al*[138] studied the effect of natural product encapsulated galactosylated liposomes (NPEGLs) to assess their anticancer activity and liver cancer cell selectivity. The study found that anti-liver cancer activity of the NPEGLs was significantly increased compared with normal natural product–liposomes and free natural product treatment in liver cancer cells. Enrichment of NPEGLs with galactosylated stearate significantly increased the uptake of the delivery system by the liver cancer cells compared with gastric and non-small cell lung cancer cells[139].

Toxicity due to the off-target effect of the anticancer therapeutic drug is also an important problem in managing liver cancer at the clinical level. It is challenging to increase bioavailability and decrease off-target effects of anti-liver cancer natural products without compromising therapeutic efficacy. This situation is more difficult when increased effectiveness of the product is required. Nanotechnology-based approaches are promising to provide the solution to this problem. It is possible to deliver the natural products using nanotechnology-based strategies, which not only increase the product's biological activity but also enhance its bioavailability. Targeted delivery using these strategies also lowers toxicity by reducing the systemic circulation of the product. Gera *et al*[140] synthesized a phytocomposite nanoparticle and studied its anticancer efficacy in liver cancer cells. The natural-compound-based nanoparticles produced significantly higher antiproliferative activity in liver cancer cells in comparison with free natural product (non-nanoparticle form). The study suggested that the increased activity of the nano-formulation of the natural product in comparison with its non-nano form might be attributed to its well dispersed, small-sized particles, and thereby increased cellular uptake.

The study also suggested that the attraction of the formulation towards the acidic environment of liver cancer cells enhances the output of targeted therapy with less or no effect on normal cells. Thus, this type of strategy, in combination with other approaches (such as receptor targeting), could be utilized to selectively target liver cancer cells to avoid the off-target effects and increase the drug's bioavailability [141,142].

CONCLUSION

In recent years, liver cancer has emerged as a significant public health concern worldwide. Various factors such as viral infection, alcohol abuse, drug-induced liver injury, or a high fat diet are the leading causes of mortality due to liver diseases. Different signaling pathways, including TGF-β, Wnt/B-catenin, Hedgehog, Notch, EGF, VEGF, JAK and Hippo, are responsible for the progression of liver cancer. First- and second-line treatments produced better therapeutic outcomes than chemotherapy and increased overall disease-free survival in liver cancer patients. Intestinal dysbiosis appears to have a significant role in developing chronic liver diseases. The available modes of treatment include numerous side effects that could be minimized with the use of natural products such as resveratrol, curcumin, diallyl sulfide and many more. However, natural-product-based anticancer therapy also has some limitations, mainly concerning the therapeutic outcome, lower bioavailability, and newer targeted delivery approaches. Targeted drug delivery using NPEGLs and nano-formulations increased the biological activity and bioavailability of the drugs.

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FOOTNOTES

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REVIEW

Therapeutic interventions of acute and chronic liver disorders: A comprehensive review

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Abstract

Liver disorders are one of the most common pathological problems worldwide. It affects more than 1.5 billion worldwide. Many types of hepatic cells have been reported to be involved in the initiation and propagation of both acute and chronic liver diseases, including hepatocytes, Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells (HSCs). In addition, oxidative stress, cytokines, fibrogenic factors, microRNAs, and autophagy are also involved. Understanding the molecular mechanisms of liver diseases leads to discovering new therapeutic interventions that can be used in clinics. Recently, antioxidant, anti-inflammatory, anti-HSCs therapy, gene therapy, cell therapy, gut microbiota, and nanoparticles have great potential for preventing and treating liver diseases. Here, we explored the recent possible molecular mechanisms involved in the pathogenesis of acute and chronic liver diseases. Besides, we overviewed the recent therapeutic interventions that targeted liver diseases and summarized the recent studies concerning liver disorders therapy.

Key Words: Liver disorders; Autophagy; Gene therapy; Anti-hepatic stellate cells; Cell therapy

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Core Tip: Acute and chronic liver diseases are worldwide problems with multifactorial pathogenesis. The exact pathological mechanism of several liver disorders is still unclear. However, many suggested mechanisms are involved, including but not limited to oxidative stress, inflammation, autophagy, and microRNA. The underlying perspective mechanisms are helpful in the discovery of new and effective therapeutic interventions for this annoying problem.

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INTRODUCTION

Chronic liver diseases are a significantly prevalent health problem contributing to the rising burden on countries daily. Specifically, liver cirrhosis - a result of chronic liver damage - is considered one of the well-known causes of morbidity and mortality all over the globe. According to Cheemerla and Balakrishnan[1], liver cirrhosis was responsible for the worldwide death of approximately 1.32 million patients in 2017. Not only that liver cirrhosis ranked 11th among the leading causes of mortality, but it has also become a habitual cause of living with a disability[2].

Acute liver injury is characterized by an abrupt decline in hepatocyte function. Unlike liver cirrhosis, acute liver failure (ALF) typically has no underlying liver problem and worsens rapidly in days or weeks. Regarding etiology, hepatitis B viral infection and medication toxicity, particularly from acetaminophen (APAP), are the primary contributors to ALF. However, other types of hepatitis, autoimmune disorders, Wilson's disease, and cardiovascular diseases are less common suspects for ALF [3]. On the contrary, there are two classes of chronic liver injuries: Cholestatic conditions that block the bile flow and persistent hepatotoxicity. Various factors can lead to hepatotoxicity, such as hepatitis B viruses (HBV), hepatitis D viruses, and hepatitis C viruses (HCV), alcohol abuse, or non-alcoholic steatohepatitis (NASH). At the same time, biliary cholangitis, atresia of bile ducts, and primary sclerosing cholangitis can cause cholestatic injuries. Regardless of the causative agent, chronic hepatic inflammation causes liver fibrosis which, if not reversed, progresses to liver cirrhosis and hepatocellular carcinoma (HCC)[4,5].

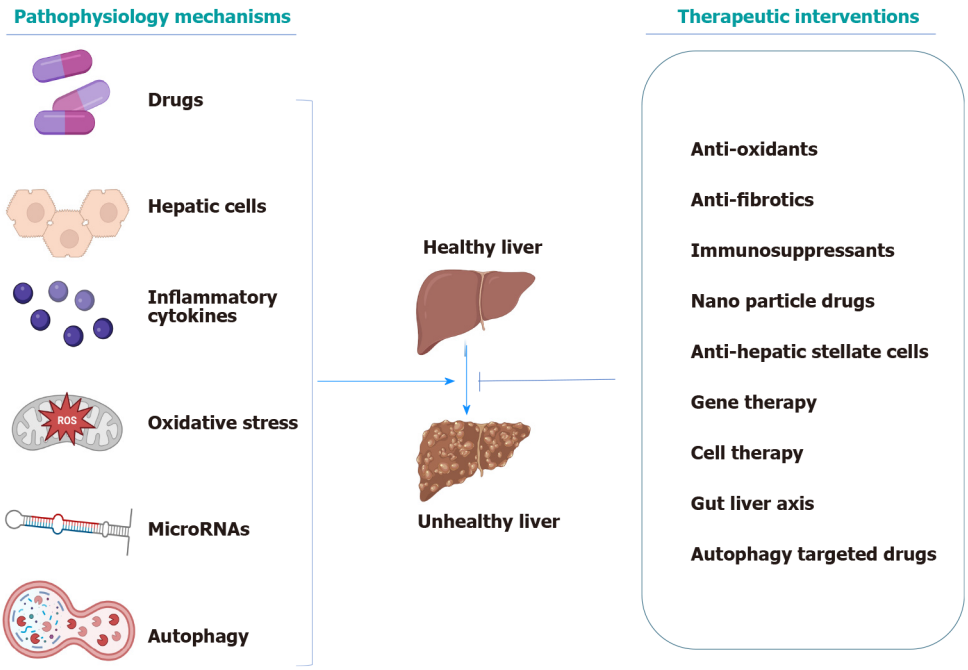
Different physiological mechanisms have been involved in liver injury, including autophagy and their different types, microRNAs (miRNAs) and their crucial effect, inflammation, hepatic cell regulation role, and the main effects of transcription factors and inflammatory cytokines. Considering the therapeutic interventions for liver diseases, there are specific treatments that are basically dependent on the cause of the disease. For instance, alcohol cessation, acetylcysteine for APAP toxicity, antiviral medication for hepatitis viruses, and immunosuppressants for autoimmune hepatitis are considered[3].

Recent studies have discussed various interventions for liver disorders, such as antifibrotic agents, cell-based therapies, gut microbiota, different nanoparticle systems, gene therapy, and much more. Consequently, we aim to discuss the newly characterized pathophysiological mechanisms and the most appropriate and recent therapy discovered to be effective on acute and chronic liver disorders (Figure 1).

DIFFERENT PATHOPHYSIOLOGICAL MECHANISMS INVOLVED IN LIVER INJURY

Both initial liver damage and subsequent multiple organ failure (MOF) can be classified as parts of the pathophysiology of ALF. The mechanism of APAP-induced ALF is the most well-known in terms of the first liver injury. Glucuronidation and sulfation of APAP create harmless chemicals that are eliminated through the urine in nontoxic doses (4 g/d)[5]. The residual APAP is transformed into the hazardous metabolite N-acetyl-p-benzoquinone (NAPQI) by cytochrome P450 enzymes (CYPs), which is then detoxified by bringing it to glutathione (GSH)[6]. Interestingly, after overdosing on APAP, GSH is depleted after its conjugation with NAPQI, and the extra NAPQI binds to hepatocellular proteins causing mitochondrial oxidative stress and necrosis[7]. NAPQI amount is enhanced during the decrease of GSH availability which will exacerbate the toxic effects of an APAP overdose. Antibiotics, antiepileptic medications, and ethanol activate CYPs and increase NAPQI production. Reduced GSH production is a result of fasting and malnutrition[6].

Moreover, the pathogenesis of secondary MOF appears to have several characteristics in common with severe sepsis. The innate immune response is triggered early in the course of a disease. It can be a response to heterotropic viruses' pathogen-specific molecular patterns (PAMPs) or to damage-associated molecular patterns (DAMPs), which include histones, DNA, and high mobility group box



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Figure 1 Graphical abstract. Illustration diagram to explore the pathophysiological mechanisms and possible therapeutic intervenension of acute and chronic liver diseases.

molecules-1 proteins produced from wounded cells following hepatocyte apoptosis as a result of toxic causes[8]. It is well known that the innate response involves a wide range of immune cells, such as monocytes, macrophages, dendritic cells, leukocytes, and natural killer cells. PAMPs and DAMPs are recognized by these cells, which then react and generate proinflammatory mediators like tumor necrosis factor α (TNF-α), interleukin (IL)-1, and IL-6, as well as reactive oxygen species (ROS), which trigger a systemic inflammatory response.

Additionally, IL-17 and IL-10 contribute to the overall inflammatory response[8]. Afterward, MOF is produced, and liver damage is still being brought on by ROS and cytokines. Proinflammatory cytokines entice neutrophils and encourage extravasation into the parenchyma of the liver. They begin to emit ROS and proteases once they are inside the parenchyma, which causes hepatocyte destruction. Promoting neutrophil extravasation into the hepatic parenchyma is greatly aided by mediators released from dying or dead hepatocytes and CXC chemokines. By releasing reactive oxygen intermediates and proteases once they have reached the hepatic parenchyma, neutrophils cause intracellular hepatocyte stress and oncotic necrosis[9]. The vasodilatation of the peripheral microcirculatory leads to inefficient pulmonary oxygen exchange, decreased peripheral tissue oxygen supply, and subsequently, lactic acidosis, which finally causes hypotension. The most severe effects are on cerebral and renovascular tone, which results in hemorrhage, cerebral hyper-perfusion, and functional renal failure[8]. The most common pathological mechanisms of acute and chronic liver disease are summarized in Table 1.

ROLE OF DIFFERENT CELL TYPES IN LIVER DISEASES

The liver is composed of two types of cells; hepatocytes, known as parenchymal cells, which constitute most of the liver and non-parenchymal cells. Around 10% of the liver's mass comprises non-parenchymal cells that include liver sinusoidal endothelial cells (SECs), hepatic stellate cells (HSCs), biliary cells, Kupffer cells (KCs), and immune cells such as neutrophils, natural killer cells, and infiltrating macrophages[10]. Whenever the liver is exposed to a harmful substance, both parenchymal and non-parenchymal hepatic cells take a role in the onset of liver fibrosis and cirrhosis.

Understanding the etiology of chronic liver disorders is essential for their prevention, slowing their progress, and advancing different treatment options. There are various etiologies to chronic liver disease, from alcoholic liver disease (ALD), non-alcoholic fatty liver (NAFLD), steatohepatitis, and chronic viral hepatitis, to other genetic, autoimmune, drugs, or cryptogenic liver diseases. Among the different etiologies of liver disorders, alcohol abuse is the most common cause. As a result of excessive alcohol consumption, the condition of alcoholic liver worsens to fatty liver and chronic steatohepatitis, which in turn triggers liver fibrosis, cirrhosis, or even HCC[11]. The non-ALD shares the same fate as the ALD but is correlated with metabolic syndrome[12]. Also, different types of hepatitis viruses can

Table 1 Summarized the common pathological mechanisms of acute and chronic liver diseases

Disease	Mechanism	Model	Findings	Ref.
ALF	N-acetyl-p-APAP	Mice model	The hazardous metabolite N-acetyl-p-benzoquinone depleted GSH and caused mitochondrial oxidative stress and necrosis	[6]
	Innate immunity, apoptosis, and cytokine release	Bio-samples from roughly 2000 patients with ALF	Generated pro-inflammatory mediators and oxidative stress, vasodilatation of the peripheral microcirculatory, hypoxia, lactic acidosis, and hypotension	[8]
	MiR-122 and miR-192	APAP in mice	Increased miR-122 and miR-192 levels after acute hepatic poisoning with acetaminophen in mice before transaminases	[82]
	MiRNAs	ALF in mice	Up-regulated miR-155, miR-146a, miR-125a, miR-15b, and miR-16	[83]
			Down-regulated miR-1187	
Acute liver injury	MiRNAs	Acetaminophen or carbon tetrachloride in male rats	Down-regulated miR-29c_AS, miR298, miR327, miR342, miR370, miR376c, miR494, and miR503	[66]
			Upregulated miR-153, miR-302b AS, miR-337, miR-363, miR-409-5p, and miR-542-3p	
	MiR-122	I/R mouse model	Elevated miR-122 level	[67]
	MiR-192	APAP induced liver injury in mouse	Dose- and exposure-dependent elevation of miR-192 level	[79]
HBV	MiRNAs	Pooled sera obtained from HBV patients	Up-regulated miR-122 level. miR-122 could inhibit HBV replication in Huh7 and HepG2 cells	[84]
	MiR-155	Human hepatoma cells	MiR-155 enhances innate antiviral immunity by promoting JAK/STAT signaling pathway by targeting SOCS1	[86]
HCV	MiR-122	Human hepatoma Huh-7.5 cells	MiR-122 is the predominant miRNA in the liver tissue. 2′-O-methyl antisense oligonucleotide depletion of miR-122 also inhibits HCV genotype 2a replication and infectious virus production	[89]
	MiRNAs	Human hepatoma cells	MiR-24, miR-149, miR-638, and miR-1181 were identified to be involved in HCV entry, replication, and propagation	[90]
Alcoholic steatohepatitis	MiRNAs	*In vitro* (RAW 264.7 macrophage) and *in vivo* (Kupffer cells of alcohol-fed mice)	Up-regulated miR-155 expression both *in vitro* and *in vivo*	[94]
			Increased TNF alpha production in response to miR-155 induction	
			Increased expression of miR-155 and miR-132 in the total liver	
	MiRNAs	Bile duct ligation rat model	Down-regulated miR-150 and miR-194 expression	[98]
	MiRNAs	Human stellate cell line	Up-regulated miR-199 and miR-200 led to higher expression of fibrosis-related genes in an HSC cell line	[97]
NAFLD and alcoholic liver disease	Autophagy	*In-vivo*	Activation of macroautophagy and CMA eliminated damaged mitochondria, lessens oxidative stress, and promotes regeneration	[136]
Liver cancer	Autophagy	Oncogene-driven cancer models	Protein kinase C promotes autophagy and oxidative phosphorylation	
			ROS generation, which through Nrf2 drives HCC through cell-autonomous and non-autonomous mechanisms	
Liver cirrhosis	Hepatocyte	*In-vivo*	Activation of hepatic stellate cells by damaged hepatocytes	[18]
	Hepatic stellate cell	*In-vivo*	The activated hepatic stellate cells produce endothelin-1, TGF-β, and cytoglobin that share in the process of fibrogenesis	[24]
	Sinusoidal endothelial cells SECs	Co-culture with freshly isolated SECs	Differentiated SECs prevent HSC activation and promote reversion of activated HSCs to quiescence through VEGF-stimulated NO production	[32]

Kupffer cells	Mouse model	Enhanced death ligand expression Inhibition of hepatocyte apoptosis with a caspase inhibitor prevented Kupffer cell activation Hepatic stellate cell activation	[35]

ALF: Acute liver failure; APAP: Aminophenol; HBV: Hepatitis B virus; HCV: Hepatitis C virus; SECs: Sinusoidal endothelial cells; VEGF: Vascular endothelial growth factor; HSCs: Hepatic stellate cells; TGF-β: Tumor growth factor-beta; CMA: Chaperone-mediated autophagy; HCC: Hepatocellular carcinoma; Nrf2: Nuclear factor erythroid 2-related factor 2; NAFLD: Non-alcoholic fatty liver; TNF: Tumor necrosis factor; GSH: Glutathione; ROS: Reactive oxygen species; miRNA: MicroRNA.

result in chronic liver disease, especially hepatitis B and C; hence, they are considered a major concern for cirrhosis and liver cancer[13]. Concerning the less common causes of liver disorders, genetic factors such as hemochromatosis, alpha-1 antitrypsin deficiency, Wilson's disease, and autoimmune hepatitis can all contribute to irreversible cirrhosis[14]. Additionally, hepatotoxic drugs, primarily APAP, followed by idiosyncratic drugs inducing liver injuries, such as antibiotics, nonsteroidal anti-inflammatory drugs, herbal remedies, and statins, can cause the liver to progress to liver fibrosis and cirrhosis [15]. When the liver is exposed to any of the above-mentioned destructive agents, liver cells undergo a remodeling process to compensate for the damage.

Hepatocytes play a complex role in the progression of cirrhosis since they are the main constituent of the liver and are particularly susceptible to harm from hepatotoxic substances[16]. Hepatocytes produce most of the matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases, which regulate the extracellular matrix deposition and thus participate in the process of liver cirrhosis[17]. Damaged hepatocytes activate HSCs, increase the ability of myofibroblasts to synthesize fibrous tissue, and produce ROS and other fibrogenic mediators[18]. The persistence of fibrosis induces hepatocytes to become hypoxic and produce large amounts of tumor growth factor-beta (TGF-β), a powerful stimulator of fibrogenesis[19]. Additionally, recent studies showed that hepatocyte telomere shortening and aging is a possible factor contributing to fibrosis and is thus implicated in the pathogenesis of cirrhosis[20].

HSCs are primarily in charge of regulating and storing vitamin A or retinol, and they are found in the subendothelial space between hepatocytes and SECs. ROS, cytokines, and growth factors, such as TNF-α and TGF-β, respectively, can activate these quiescent cells, causing them to synthesize a lot of extracellular matrixes, which can form a scar in the space of the disease[21-23]. In addition, the activated HSCs produce endothelin-1, TGF-β, and cytoglobin that share in the process of fibrogenesis[24-26]. However, recent studies showed that the delivery of berberine nanoparticles could inhibit the proliferation of HSCs and reverse the damage resulting from fibrosis[27].

SECs are an important type of liver cells, surrounded by the bloodstream from one side and hepatocytes from the other side[28]. Morphologically, liver SECs are characterized by transcellular pores known as fenestrae, which are essential for transporting nutrients and other components from the blood to the hepatocytes and vice versa. Fenestrae is important for normal liver function and plays a great role in maintaining liver homeostasis and regeneration[29]. In pathological conditions, SECs lose their fenestrae and become capillarized, impairing proper liver function[30]. Furthermore, they encourage fibrogenesis by activating HSCs by releasing IL-33[31]. In contrast, several studies have documented that differentiated liver SECs can encourage the reversion of activated HSCs to the quiescent form and thus stop the progress of fibrosis *via* modulating vascular endothelial growth factor (VEGF)-stimulated NO release[32].

KCs are liver macrophage cells that comprise an average of 85% of body macrophages and are present in hepatic sinusoids. KCs are necessary for innate and adaptive immunity as they deal with detrimental pathogens entering the liver from the portal vein[33]. As a result of liver injury, KCs get activated and respond by producing various cytokines, ILs, and chemokines[16]. Additionally, NO produced by KCs, together with TNF-α, TGF-β, and platelet-derived growth factors (PDGFs), activate HSCs, causing an excess of extracellular matrix to be produced[34]. Although KCs produce death ligands and contribute to liver fibrogenesis and fibrosis[35], they are not a suitable target for therapeutic interventions due to their crucial host defense function.

ROLE OF CYTOKINES, TRANSCRIPTION FACTORS, AND ROS IN HEPATIC INJURY

Cytokines are bioactive molecules made by several types of liver cells that are essential in the progression of liver cirrhosis[36]. They consist of TNF-α, PDGF, interferons (IFNs), ILs, TGF-β, chemokines, and adipokines. Several important biological processes, such as hematopoiesis, immunology, inflammation, and body development, are mediated by cytokines. However, they are also linked to several illnesses, including liver disorders, rheumatoid arthritis, and atherosclerosis[37]. A significant coordinated program of cellular and molecular alterations in liver cirrhosis results in a potent fibrotic response. Cytokines are involved in the combative signaling pathways that regulate the

activation of HSCs and fibrogenesis[38].

PDGF is the most powerful HSCs activator concerning all polypeptide growth factors. According to the degree of fibrosis, it seems to be overexpressed, enhancing its receptors and their activity in fibrous tissue[39]. Mainly in reaction to diverse stimuli, including viruses, chemicals, or mechanical injury, KCs manufacture and release PDGF[40]. When PDGF is released, it attaches to a particular receptor on the HSCs' membrane, activating transcription factors and matching signal molecules involved in the process[41]. This causes the activation of its target genes, which are downstream of the receptor, as well as the activation of HSCs. It has been shown that PDGF (P38-MAPK) increases the activity of C-Jun N-terminal kinase, extracellular signal-regulated kinase (ERK) 1/2, MMP, TIMP, protein kinase B/AKT pathways, and P38 mitogen-activated protein kinase[39].

Transforming growth factor-beta is the strongest known fibrogenic inducer during liver cirrhosis[42]. It is released by all types of hepatic cells in response to unpleasant stimuli and is essential for developing and spreading cirrhosis and liver fibrosis. In fibrotic diseases, TGF-β is abundantly expressed and reaches its peak in cirrhosis[43]. TGF-β pro-fibrogenic impact is carried out by boosting the production of HSCs and ECM while inhibiting MMPs, which results in an excessive buildup of collagen fibers and aids in the progression of liver fibrosis[44]. Additionally, it has been demonstrated that TGF-β causes hepatocyte death and inhibits DNA synthesis[38].

TNF-α is a pro-inflammatory cytokine generated during inflammation and oversees various cell signaling processes. HSCs, KCs, monocytes, and macrophages secrete it[45]. According to a study showing that TNF-α is a mediator of hepatotoxicity and inflammation in many liver diseases, hepatocellular injury followed by inflammation and activation of the innate immune system leads to early-stage liver fibrosis, which in turn causes HSC activation and ECM deposition[46]. In addition, TNF-α contributes to ECM deposition by enhancing the expression of TIMP-1 in HSCs[47]. TNF-α has complex and sometimes conflicting effects on HSCs and fibrosis. TNF-α, on the other hand, has also been demonstrated to have an anti-fibrogenic impact in rat's HSCs by lowering GSH and decreasing pro-collagen 1 expression. TNF's function in fibrogenesis is debatable, and it is unknown exactly how TNF receptors contribute to the activation of HSCs. Researchers demonstrate that loss of both TNF receptors decreased pro-collagen 1 expression, slowed HSC proliferation, and impaired PDGF-induced pro-mitogenic signaling in HSC from wild-type, TNF-receptor-1 (TNFR1) knockout, TNFR2 knockout, or TNFR1/R2 double knockout (TNFR-DKO) mice. In response to PDGF, TNFR-DKO HSC showed decreased AKT phosphorylation and *in vitro* proliferation. However, these effects were not replicated in TNFR2 knockout HSC. Additionally, in primary mouse HSC, TNF binding to TNFR1 was necessary for MMP-9 expression. Neutralizing antibodies against TNFR1 and TNFR2 confirmed these findings in the human HSC cell line LX2. Additionally, compared to wild-type or TNFR2 knockout mice, TNFR-DKO and TNFR1 knockout animals showed less *in vivo* liver damage and fibrogenesis after bile duct ligation (BDL)[48].

Oxidative stress is frequently described as a general imbalance between oxidizing and reducing substances in the cell. The signaling transduction pathways are governed by these redox states. Numerous human disorders, particularly chronic liver diseases, have been linked to the development of ROS[49]. The production of ROS is crucial in causing liver injury and kicking off hepatic fibrogenesis. Oxidative stress alters lipids, proteins, and DNA, causing hepatocytes to necrotize and apoptosis and escalating the inflammatory response[50].

Additionally, ROS directly activates HSCs and encourages the synthesis of profibrogenic mediators from KCs and circulating inflammatory cells, which leads to the beginning of fibrosis[51]. Regardless of their underlying causes, almost all liver illnesses have been found to exhibit oxidative stress[52]. Prooxidants are ROS that can harm liver cells and whose levels may be raised by some medications, infections, environmental exposures, tissue damage, and other factors. Oxidative stress can be caused by increased prooxidant production, a reduction in antioxidant levels, or a shortage of antioxidants. Signaling, regulation, and redox balance of the liver system are biased by molecular redox switches, oxygen detection by the thiol redox proteome, NAD/NADP, and phosphorylation/dephosphorylation systems. ROS rapidly interact with all biological macromolecules due to their reactivity. The phosphodiester bonds that keep the bases in RNA and DNA together are cleaved by ROS, causing RNA and DNA to lose their chain structure. In a process known as lipid peroxidation, polyunsaturated fatty acids are another important target for oxidation by ROS. This process disturbs the normal structure of the membrane and results in necrosis. Additionally, since cysteine is necessary for the function of enzymes, ROS, particularly the hydroxyl radical, oxidizes cysteine residues in proteins to form disulfides, sulfoxides, or sulfonic acids. Additionally, oxidative stress promotes fibrogenesis by raising toxic cytokines such as TNF-α, IL-6, and TGF-β[53].

ROS generated by the NADP/NADPH oxidase system can control the cellular redox environment in hepatocytes and KCs. NADPH oxidase activation is the main ROS source in myofibroblasts and the stimulation of profibrogenic pathways[54]. It is regarded as the main producer of superoxide anion and hydrogen peroxide, the two most damaging ROS contributing to liver damage from oxidative stress [55]. NADPH oxidase inhibition is emerging as a target for antifibrotic treatment since NADPH oxidase activation may constitute a central mechanism in fibrosis[56].

The activities of various antioxidant enzymes, whose expression is controlled by several redox-sensitive transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells [nuclear factor-kappaB (NF-κB)] and nuclear factor erythroid 2-related factor 2 (Nrf2), may have an impact on the generation of ROS[57]. Quiescent HSCs lack NF-κB in contrast to activated HSCs, which suggests that a redox-sensitive activation of NF-B might govern the expression of NF-B-targeted genes and provide a suitable cellular redox threshold for quiescent HSCs to enter the proliferative cycle[58]. In support of this theory, it has been shown that blocking NF-κB activity shields rats from the onset of hepatic fibrosis. The suppression of Nrf-2 may also change the expression of antioxidant enzymes, disrupting the cellular redox environment and impacting HSC proliferation, cell death, and collagen formation, all of which contribute to liver fibrosis[49].

Additionally, ROS-sensitive cytokines help activate HSCs during inflammation by receiving paracrine cues from immune cells. Hepatic fibrosis progresses more quickly due to the activated HSCs' increased receptivity to PDGF and TGF-β[40]. TGF-β boosts the generation of ROS while lowering the level of reduced GSH. The production of the collagen I protein is increased when lipid peroxidation is increased, and anti-oxidant defenses like GSH, catalase, or superoxide dismutase are decreased[59].

ROLE OF MIRNAS IN HEPATIC DISEASES

MiRNAs are a group of tiny, non-coding endogenous RNA molecules with a high degree of chemical stability (22 nucleotides). MiRNAs have been thoroughly investigated since their discovery in 1993[60] because of their function in RNA-induced posttranscriptional gene silencing. One of the most prevalent adult hepatic miRNAs, miR-122, controls several important gene networks, including lipid metabolism, cell differentiation, and the hepatic circadian rhythm[61]. Recently, miR-223 is thought to interfere with the development and homeostasis of the immune system as well as it has an important role in inflammatory disorders and other liver disorders[62]. Moreover, MiR-223 also controls the nucleotide-binding oligomerization domain-like receptor (NLR) inflammasome by targeting the NLR protein 3 (NLRP3) 3′-untranslated regions[63]. Notably, different cell types require NLRP3 inflammasome to start the inflammatory reaction and the production of ILs. Accordingly, overexpression of miR-223 reduces IL-1 production from the inflammasome and prevents NLRP3 protein formation. Additionally, miR-223 may prevent macrophage hyperactivation[64].

Recent evidence showed that numerous liver disorders, including viral hepatitis, alcohol-induced liver damage, drug-induced liver injury, NAFLD, cirrhosis, and HCC, have dysregulated the expression of the miR-223 gene. Markedly, Weseslindtner *et al*[65] revealed that, the elevation of miR-106a, miR-122, and miR-197 levels in patients with severe acute viral hepatitis. Interestingly, Fukushima *et al*[66] made a thorough comparative microarray study and looked at how different miRNAs changed in rats after receiving APAP and CCL4 and discovered that eight miRNAs were downregulated while six miRNAs (miR-153, miR-337, miR-363, miR-302b AS, miR-409-5p, and miR-542-3p) were upregulated in both hepatotoxicity models.

Since miR-122 is very liver-specific and makes up around three-quarters of the entire miRNAs that the liver expresses, it has been extensively studied concerning liver damage[67-69]. It is highly expressed in hepatocytes because of liver-specific transcriptional regulation under the effect of hepatic transcription factors[70]. Further, it seems to be elevated in the majority of liver disorders, including HCV and HBV, in addition to ALD, drug-induced liver damage, NAFLD, and HCC[71-74]. Along with this, loss of miR-122 is seen during hepatocellular carcinogenesis due to hepatic cell dedifferentiation[75, 76]. In acute and chronic liver disorders, miR-122 serum/plasma levels are correlated to hepatic necro-inflammation, elevated aminotransferase levels, liver injury, and cell death[77].

Not only miR-122 but miR-192 as well was elevated in the mice sera after APAP administration compared to controls in a dose-dependent and exposure-dependent manner. In this context, the levels of those miRNAs were enhanced sooner than the levels of serum transferases[78], highlighting that they can be used diagnostically superior to the conventional ALF indicators[79]. Similarly, the serum levels of miR-122 were also elevated in the I/R mice models, and they were connected to both aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels and the hepatic cell death identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. As *in vitro* studies showed that miR-122 levels increase in the supernatant after hepatocyte injury. The presence data imply that miR-122 may replace hepatocyte mortality in liver damage[67]. Additionally, the elevation of miR-122 and miR-192 in the sera of patients with APAP-induced ALF could be confirmed, and these findings concur with results from high-throughput sequencing of patients who had taken too much APAP[69]. Krauskopf *et al*[69] showed that, compared to controls, the serum levels of 36 types of miRNAs were higher in these individuals. Additionally, following APAP overdose, miR-122, miR-192, miR-194, miR-210, and miR-483 were shown to be reinforced in the liver.

In ALF, a considerable downregulation of miR-122 is seen in the injured liver in both acute and chronic liver injury, and it showed an inverse correlation between hepatic damage and ALT levels, suggesting that it may play a role in human ALF. When paraquat was administered to humans, miR-122 was noticeably upregulated, whereas miR-483 and miR-711 were concurrently downregulated. This is

consistent with what was shown in the rats given an APAP overdose[78,80]. Zhang *et al*[81] discovered that blood levels of miR-122 and miR-192 were increased after acute hepatic poisoning with APAP in mice before transaminases, particularly ALT, were raised. However, it was shown that the miRNA levels in liver tissue were lower. Since these miRNAs may be detected before the liver experiences apparent cell death, they may serve as a more accurate indicator of liver failure than liver enzymes[82]. Recent studies showed that miR-15b, miR-16, miR-125a, miR-146a, and miR-155 were considerably up-regulated during ALF in mice, while miR-1187 showed a significant down-regulation[83].

Hepatitis B e antigen (HBeAg) positive patients had much greater blood levels of miRs than those with HBeAg negative, especially miR-122 and miR-194, which showed the greatest differential expression[84]. Additionally, it has been shown that the expression of miR-122, miR-638, miR-572, miR-575, miR-638, and miR-744 was dysregulated in chronic HBV patients; these miRs were significantly more abundant in HBV than AST or ALT. MiR-122, miR-572, miR-575, and miR-638 were more abundant than miR-744[85]. In human hepatoma cells, HepG2, miR-155 has been shown to contribute to antiviral immunity against HBV infection[86]. An initial therapeutic response to IFN (independent relationship with early virologic response) may be predicted in HBV patients using a miR profile of 11 miRs for example, hsa-let-7a, hsa-miR-30a, hsa-miR-106b, hsa-miR-198, hsa-miR-1224-5p, and hsa-miR-1290. It has been demonstrated that certain miRs might play a function in the HBV life cycle[87]. According to studies, specific miRs have been shown to affect HCV infection or be affected by the virus. There is still much to learn about how miR-122 interacts with the HCV genome[88].

However, miR-122 expression is unaffected by viral infection or replication. Recently, Randall *et al*[89] looked at miR-21 and miR-122 expression in the liver biopsy samples from patients infected with HCV and controls. They established that miR-122 levels were inversely linked to the fibrotic stage, ALT, and AST but that miR-21 levels were positively linked. It was suggested that rather than levels of expression, fibrosis might be brought on by dysregulation of miR-21 and miR-122. MiRs 24, 149, 638, and 1182, among others, share in HCV entrance, replication, and spread[90]. The tumor suppressor "deleted in liver cell-1" protein was shown to be highly dependent on miR-141 activation, miR-141-targeted downregulation, and depletion for sustained HCV propagation. According to research on the association between HCV and the levels of miR-29 in both HSC and hepatocytes, HSC stimulation results in miR-29 down-regulation[91]. The overexpression of miR-29 in infected cells reduced HCV replication by 70% and inhibited the growth of HSCs and collagen synthesis. When comparing the livers of HCV with non-SVR, miR-29a, b, and c levels were higher[92], indicating a potential function for these biomarkers in monitoring the effectiveness of anti-HCV therapy.

In alcoholic steatohepatitis, miRs are crucial immune response regulators and activators of the innate immune system[93]. Alcohol-induced gut leakiness, which permits endotoxin to enter the blood and begin liver damage, has been shown to play a critical role in ALD and to increase miR-122 expression. It is shown that inducing miR-155 and -132 causes KCs to release higher TNF-α in response to lipopolysaccharide (LPS)[94]. Hepatic miRs 182, 183, 705, 1224, and 199a-3p are modulated by endotoxemia and alcohol use directly[95]. Alcohol specifically targets and upregulates the miR-155 gene in macrophages, which controls the production of TNF-α[96]. Prolonged alcohol exposure also stimulates the miR-155 gene in KCs and RAW264.7 macrophages. As a result, miR-155 upregulation might be engaged in the oxidative stress and LPS pathways, thus promoting the development of ALD[94].

There is mounting evidence that miRs, namely *via* controlling gene expression in HSCs, are important regulators of hepatic fibrogenesis. The advancement of liver fibrosis has been linked to the miR-199 and miR-200 family's expression. Patients with fibrotic livers had higher levels of the miR-199 and miR-200 families, and upregulation of these miRs led to considerably higher levels of the genes related to fibrosis in a cell line of HSC. In a fibrosis model of BDL in rats, miR-150 and miR-194 levels were significantly lower than in animals with a sham procedure. Furthermore, in a human stellate cell line called LX2, it has been shown that overexpressing miR-150 or miR-194 through the reduction of c-myb and rac1 expression can reverse the activated stellate cells (*i.e.*, expression of collagen and alpha-smooth muscle actin genes). Therefore, miR-150 and miR-194 may represent promising therapeutic targets for fibrosis treatment[97,98].

ROLE OF AUTOPHAGY IN LIVER DISEASES

Autophagy is a self-eating catabolic mechanism in eukaryotic cells that ends in the lysosome[99,100]. In addition to its anti-aging function, autophagy plays a significant role in immune response and organ homeostasis[101,102]. Numerous pathological disorders, such as obesity and type 2 diabetes, inflammatory and viral diseases, neurodegenerative diseases, and cancer, exhibit autophagy dysregulation [103,104]. There are distinct phases of autophagy; induction, phagophore development, autophagosome creation, autolysosome formation, and destruction[105,106]. Atg molecules participate in several complexes crucial for triggering autophagy and creating autophagosomes[107]. The unc-51-like kinase 1 complex (Atg1 in yeast) is activated first, then beclin 1 (Atg6 in yeast), and followed by a series of Atg proteins that result in the production of autophagosomes, with LC3 (Atg8 in yeast), being one of them [108]. Further processing of LC3 results in the formation of LC3-I and LC3-II[109]. As soon as the

autophagosome gets created, a blockade of autophagic flux at later stages will suppress the autophagosome's ability to be cleared, ultimately leading to autophagy-dependent cell death[110]. To date, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) are the three main types of autophagy that have been characterized[111,112].

AUTOPHAGY AND THE IMMUNE SYSTEM

Lately, researchers have investigated the relationship between autophagy and the immune system[113,114]. There have been documented non-canonical macroautophagic processes that create lysosome-fusing autophagosomes[115]. Only a portion of the Atgs equipment is utilized. Due to its significance in immunological modulation, LC3-associated phagocytosis (LAP) has received the most attention[116,117]. LAP draws LC3-II to the phagosomal membrane *via* innate immune receptors, such as toll-like receptors, where macrophages consume it. The crucial part that CMA plays is antigen presentation and aging, which has also garnered attention[118]. Innate immunity's ability to hinder macrophage autophagy is also associated with autophagy. Innate immunity and autophagy interact because IFN-α stimulates autophagy in macrophages[119].

AUTOPHAGY AND CELL DEATH

In some circumstances, autophagy can either serve as a defense mechanism or contribute to cellular death[120,121]. The main way autophagy contributes to cellular death is through its influence on apoptosis. Apoptosis and autophagy are linked, and these two cellular destructing processes influence one another[122,123]. This is crucial in the demise of liver cells[124]. Autophagy generally prevents caspase-dependent apoptosis from being induced, whereas apoptosis-related caspase activation halts the autophagic process.

Along with these results, Ni *et al*[125] documented that necrosis and necroptosis are caused by caspase-independent cell death, which is closely linked to autophagy. Cells harmed by the tumor suppressor gene p53 are removed by the induction of apoptosis[126]. In addition to being engaged in autophagy, the mechanistic target of rapamycin (mTOR)/AKT pathway also inhibits apoptosis. For the destiny of damaged cells, p53 and AKT/mTOR must coexist in equilibrium[127]. Numerous proteins linked to autophagy, including Atgs and BECN1, also played a role in ferroptosis. Additionally, erastin, an activator of ferroptosis, caused the formation of autophagosomes, and activation of autophagy resulted in ferroptotic cell death, maybe because of the ferritin being broken down by ferritinophagy [128].

Autophagy and inflammation

Autophagy and the liver's inflammatory response are tightly related. The same inhibitory mechanisms govern autophagy and inflammasome but are regulated by various input pathways. Procaspase-1 activation results from the activation of the NLRP3 inflammasome, which is often triggered by pathogen- or danger-associated molecular patterns[129], which will further stimulate the synthesis of IL-1 and IL-18 that causes pyroptotic cell death. Moreover, the activation of autophagy by caspase-1 prevents these occurrences. Additionally, autophagy decreases inflammasome activation by destroying inflammasomes in autophagosomes and removing damaged cytoplasmic organelles that, in the absence of autophagy, would otherwise create DAMPS and increase inflammasome activation[130]. On the other hand, when autophagy is diminished, the pro-inflammatory IL-1 is produced more often due to the negative association between inflammasomes and autophagy[131,132]. Although the connection between NLRP3 and autophagy is not entirely understood, recent research has indicated that NF-κB activation can similarly modify NLRP3 and autophagy[133].

Given the preceding, it is not surprising that many autophagy reviews emphasize the contrasting impacts that autophagy may have on the same biological process by using the phrase "double-edged sword"[134]. Cancer[135] and viral infections[101] are prominent fundamental paradigms. The fact that autophagy exhibits Jekyll-like and Hyde-like characteristics depending on the cells involved is another trait exclusive to the liver. Hepatocytes in NAFLD and ALD exhibit protective macroautophagy and CMA (in NAFLD). It eliminates damaged mitochondria, lessens oxidative stress, and promotes regeneration. In macrophages, macroautophagy reduces liver fibrosis and inflammation while promoting fibrosis-activated stellate cells. It is preventative in the early stages of HCC but might be damaging in the later stages[136]. Both the non-parenchymal sinusoidal cells of the liver and the hepatocytes depend on autophagy for proper liver function[137], and autophagy abnormalities are linked to most liver illnesses' pathogenesis[138]. Autophagy disorders are linked to both common conditions like alcoholic and NAFLD or viral hepatitis and uncommon conditions like Wilson disease and alpha 1 antitrypsin deficiency[139,140]. Due to the 6-12 mo half-life of hepatocytes, impaired autophagy also contributes to the accumulation of toxic hepatocyte byproducts. A large number of xenobiotics must also be processed by the liver, and autophagy is a cytoprotective mechanism[141].

Therapeutic interventions for acute and chronic liver diseases

Cirrhosis of the liver, the end stage of liver fibrosis after chronic liver damage, used to be cured by nearly liver transplantation only. That is why researchers used to focus on preventing liver cirrhosis by eradicating the cause and reversion of fibrosis. However, if liver cirrhosis develops, treatment is restricted to preventing the progression of the complications and avoiding the need for liver transplantation[142-144]. Besides removing the cause, various categories of treatments have proven to be beneficial in preventing fibrosis progression or regression, such as antioxidants, and antifibrotic agents, including phyto drugs[144-147]. *Via* understanding the process of fibrogenesis, various mechanisms implicated in this process would be potential for the reversion of fibrosis and cirrhosis. Here in, we discuss several conventional and novel therapeutic interventions that showcased, by recent data, the ability to modulate liver fibrosis and cirrhosis. The recent therapeutic interventions are summarized in Table 2.

Antioxidants

Oxidative stress is well known to play a detrimental role in developing liver cirrhosis. When ROS production exceeds antioxidants level, cellular signaling pathways alterations eventually result in liver damage[148]. For this reason, antioxidants received much attention and extensive study to prevent and treat various liver disorders. Silymarin is an herbal extract that consists mainly of silybin, which is responsible for the activity of silymarin. Free radical scavenging activity and inhibition of lipid peroxidation have been exhibited as reasons for the antioxidant activity of silybin[149]. Selenium is an essential element for the GSH antioxidant system in our bodies that has been extensively studied for its antioxidant activity in various cases of liver damage[150]. Selenium showed the ability to decrease DNA damage and hepatocyte necrosis against cyclophosphamide-induced oxidative stress[151]. In cadmium-induced acute liver injury, selenium nanoparticles decreased liver toxicity by boosting the Nrf2 pathway [152]. In chronic liver injury, selenium is reported to mitigate lipid peroxidation and decrease other oxidative stress biomarkers, especially when combined with the natural antioxidant gum arabic[153]. A study investigating the effect of curcumin, selenium, and silymarin showed that the combination of selenium, curcumin, and silymarin ameliorates the oxidant/antioxidant status in lipopolysaccharide and diclofenac-induced liver damage[154]. Vitamin E is a fat-soluble vitamin and one of the most potent antioxidants. This action is attributed to the ability of the hydroxyl group to scavenge free radicals and restoration of GSH levels and hence the improvement of oxidant/antioxidant status. Accordingly, in addition to other mechanisms, vitamin E effectively reduces inflammation[155] but not fibrosis[156].

Nevertheless, a recent *in vivo* study by Aljuhr *et al*[157] showed that using vitamins E and C loaded on selenium nanoparticles effectively reduces the induced hepatocellular damage, making it a potent combination for preventing and treating HCC. Acute hepatotoxicity induced by APAP overdose is typically countered by N acetyl cysteine *via* its antioxidant activity and increasing the level of GSH in the liver[158]. In cases of APAP-induced acute liver injury, N acetylcysteine is the antidote for hepatotoxicity as it can preserve GSH stores and counteract the toxic metabolite NAPQI[159]. In addition, N acetyl cysteine exerted favorable effects at increasing GSH peroxidase and decreasing oxidative stress in liver fibrosis induced by carbon tetra chloride[160]. Mitoquinone (MitoQ), mitochondrial-targeted coenzyme Q, is a recent advance in antioxidant therapy that delivers coenzyme Q directly to the mitochondria[161]. In carbon tetrachloride-induced liver fibrosis, MitoQ showed a reduction in lipid peroxidation marker, 4-hydroxynonenal, *in vivo* and inhibition of cultured HSC activation[162]. Accordingly, MitoQ seems promising in mitigating liver fibrosis, but further studies are needed to confirm its efficacy.

Anti-fibrotic and anti-inflammatory drugs

Both acute and chronic liver disorders involve a series of cytokine and chemokine production and inflammatory cell infiltration that promote fibrogenesis[163,164]. This emphasizes the importance of using anti-fibrotic and anti-inflammatory drugs to modulate fibrogenesis and reduce the progression of liver fibrosis. Pirfenidone is a pyridone derivative with antifibrotic and anti-inflammatory properties and is mainly used for pulmonary fibrosis[165]. These actions are attributed to the ability of pirfenidone to suppress TGF-β and NF-κB activation and thus decrease inflammatory cell infiltration and excess matrix deposition. Along with the antioxidant activity of pirfenidone, it effectively diminishes liver fibrosis[166], as shown in a two-year *in vivo* study on CHC virus-infected patients[167]. Statins and anti-NADPH oxidases as anti-fibrotic classes, peroxisome proliferator-activated receptor alpha modulators, and timolumab as immunomodulators have been recently investigated and declared promising for decreasing inflammation and fibrosis in cases of primary sclerosing cholangitis[168].

Immunosuppressants

Autoimmune hepatitis is a chronic inflammatory liver disease that occurs when helper T cells and cytotoxic T cells attack the liver causing inflammation that may progress to liver cirrhosis. Autoimmune hepatitis can be acute, fulminant, or chronic and, like other autoimmune disorders, require immunosuppressive therapy to suppress the disease progression. The treatment of autoimmune hepatitis involves using corticosteroids as antifibrotic agents and azathioprine, and when this line of management is

Table 2 Therapeutic interventions implicated in acute and chronic liver disorders

Therapeutic intervention	Drugs	Main findings	Ref.
Antioxidants	Silymarin	Possesses free radical scavenging activity and inhibits lipid peroxidation thus improving chronic liver diseases	[149]
	Selenium	Decrease DNA damage, hepatocyte necrosis, oxidative stress biomarkers, and liver toxicity	
	Vitamin E	Reduces inflammation and protects from hepatocellular damage	[155,157,160]
	N acetylcysteine	Increasing GSH peroxidase and decreasing oxidative stress in liver fibrosis	
	MitoQ	Reduces lipid peroxidation and cultured hepatic stellate cell activation	[162]
Antifibrotic agents	Pirfenidone	Pirfenidone is effective at diminishing liver fibrosis as it suppresses TGF-β1 and NF-κB and decreases inflammatory cell infiltration and excess matrix deposition	[166-168]
	Statins, and anti- NADPH oxidases	PPAR-α modulators might decrease inflammation and fibrosis in cases of primary sclerosing cholangitis	
Immunosuppressants	Corticosteroids, and azathioprine	The first line of treatment for autoimmune hepatitis	[169]
Anti-HSC therapy	Imatinib and sorafenib	Respectively act as PDGF and angiogenesis inhibitors thus they modulate fibrogenesis and fibrosis in autoimmune hepatitis	[173]
	Paclitaxel, ferulic acid and methyl ferulic acid	Can inhibit hepatic stellate cell activation through TGF-β /Smad pathway modulation	[175-177]
	Curcumin	Can interrupt the PDGF-β/ERK pathway and inhibit hepatic stellate cell angiogenesis through activation of PPAR-γ. Curcumin can also activate autophagy and thus inhibit the TGF-β/Smad pathway thus reducing epithelial-mesenchymal transition	[178-180]
Gene therapy	HGF	Decreases the expression of TGF-β1, suppresses hepatocyte apoptosis, and improves fibrosis	[181]
	Matrix metalloproteinase-1	Enhances the proliferation of hepatocytes and diminishes fibrosis	[183]
	siRNA	By silencing CTGF, TGF-β, NF-κB target gene A, galectin-3, and αvβ3 integrin, siRNA effectively stops fibrogenesis by preventing HSCs activation and/or promoting their apoptosis	[184]
Cell therapy	MSCs	Inhibit hepatocyte degeneration, promote liver regeneration, and suppress fibrosis through differentiation into hepatocytes and production of various growth factors	[187]
	BMSCs	Decrease serum markers of liver injury and mRNA expression of TNF-α, IFN-γ, and FasL, and increase IL-10 mRNA expression in acute liver failure	[189]
	Matrix metalloproteinase 2, tissue inhibitor of metalloproteinase 1, and growth arrest-specific 6	Promote hepatocytes regeneration, neovascularization, and extracellular matrix remodeling all contributing to liver regeneration	[191]
Gut liver axis	Baicalin	Modulates FXR and G-protein-coupled bile acid receptor TGR5 thus modulating the levels of TNF-α, NF-kβ, and TGF-β. It also inhibits inflammation, autophagy, and necrosis of parenchymal liver cells	[195-198]
	Probiotics	Modulate gut dysbiosis and bile acid dysregulation thus aiding in the treatment of NAFLD. Probiotics also modulate inflammation and fibrosis in NASH	[199-201]
Nanoparticle drug delivery	Gold	Enhances the antifibrotic activity of silymarin through increasing the expression of protective microRNAs and suppression of inflammatory mediators in the TGF-β 1/smad pathway	[204]
	Phosphatidylserine-decorated nanoparticles	Enhances curcumin efficacy in fibrosis reduction	[205]
	Liposome nanoparticles	Can be specifically delivered to integrins of activated hepatic stellate cells, in addition to facilitating gene therapy	[208]

		using siRNAs and mRNAs to modulate gene expression of hepatocytes	
Autophagy inhibition	Becn1 knockdown	Autophagy suppression and inhibition of T lymphocyte infiltration, HSCs proliferation, as well as production of TNF-α, IFN-γ, and TGF-β1	[209]
	Carvedilol	Increased p62 protein levels and inhibited autophagic flux by increasing lysosomal pH	[210]
	Doxazosin	Inhibited HSC proliferation and migration, blocked autophagic flux and induced HSCs apoptosis	[211]
	Resolvin D1	Modulated AKT/mTOR signaling pathway resulting in the inhibition of autophagy and suppression of hepatic stellate cell activation	[212,213]

GSH: Glutathione; PPAR-α: Peroxisome proliferator-activated receptor alpha; CTGF: Connective tissue growth factor; TGR5: G-protein-coupled bile acid receptor; MitoQ: Mitoquinone; HSCs: Hepatic stellate cells; TGF-β: Tumor growth factor-beta; NF-κB: Nuclear factor-kappaB; PDGF: Platelet-derived growth factor; HGF: Hepatocyte growth factor; ERK: Extracellular signal-regulated kinase; siRNA: Small interfering RNA; mTOR: Mechanistic target of rapamycin; TNF: Tumor necrosis factor; IFN: Interferon; NAFLD: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis; FXR: Farnesoid X receptor; IL: Interleukin; MSC: Mesenchymal stem cell; BMSC: Bone marrow-derived mesenchymal stromal cell.

insufficient, mycophenolate mofetil and calcineurin inhibitors are used[169,170]. In contrast, these drugs require more investigation for their use in other autoimmune liver disorders, such as primary sclerosing cholangitis and primary biliary cirrhosis[171].

Anti-HSCs therapy

One of the most important mitogens in profibrogenic HSC activation following liver damage is PDGF [172]. A recent study using PDGF and angiogenesis inhibitors as imatinib and sorafenib, respectively, concluded that they were able to modulate fibrogenesis and fibrosis in induced autoimmune hepatitis models[173]. Silymarin possesses antioxidant activity and antifibrotic properties through inhibition of KCs activation, decreasing extracellular matrix deposition, and inhibiting the production of IL-1 and IL-8 on HSCs[174]. As TGF-β is a crucial cytokine for HSC fibrogenesis and hence liver fibrosis progression [172], different studies have been conducted to study the effect of various substances to obstruct TGF-β /Smad signals. *In vitro* studies on paclitaxel, ferulic acid, and methyl ferulic acid were encouraging for inhibition of HSC activation *via* TGF-β/Smad pathway modulation[175-177]. Curcumin is a natural antioxidant, anti-inflammatory, and antifibrotic agent that can modulate different apoptotic pathways during tissue injury. Recent studies showed that curcumin could interrupt the PDGF-β/ERK signaling pathway and inhibit HSC angiogenesis by activating PPAR-γ[178,179]. Furthermore, curcumin can activate autophagy and thus inhibit the TGF-β/Smad pathway, which reduces epithelial-mesenchymal transition[180]. Accordingly, curcumin is considered a good candidate for treating liver fibrosis.

Gene therapy

Acute liver injury is usually reversible; however, chronic liver damage is a progressive condition that usually progresses from inflammation and fibrosis to cirrhosis. That is why extensive investigations on gene therapy have been conducted with various genes and delivering vectors to modulate liver fibrosis and cirrhosis. Hepatocyte growth factor (HGF) is an essential antiapoptotic and hepatoprotective factor for hepatocytes and an antifibrogenic agent in liver fibrosis models. HGF gene therapy has been studied for liver cirrhosis in rats and was shown to decrease the expression of TGF-β, suppress hepatocyte apoptosis, and improve fibrosis in dimethyl nitrosamine-induced cirrhosis[181]. Due to the ability of HGF to suppress TGF-β, it exhibits immunomodulatory action that is promising in cases of autoimmune disorders, but further investigations are still required[182]. As HSCs generate abundant amounts of extracellular matrix during fibrogenesis, matrix metalloproteinase-1 delivered by adenovirus to fibrotic livers enhances the proliferation of hepatocytes and diminishes fibrosis[183]. Another mechanism involves the use of small interfering RNA (siRNA) to silence the genes that are essential for the process of fibrosis, such as connective tissue growth factor, TGF-β, NF-κB target gene A, galectin-3, and αvβ3 integrin. Silencing these genes stops fibrogenesis effectively by preventing HSCs activation and promoting their apoptosis[184].

Cell therapy

Stem cells are a category of cells that can replicate and differentiate into numerous types of specialized cells in the body[185]. During the last two decades, stem cell-based therapy has been extensively investigated and appears promising for liver regeneration. Thus, it is a considerable alternative for liver transplantation and overcoming its demerits like the shortage of liver donors, high cost, and surgical complications. Various types of stem cells have been studied in acute and chronic liver disorders, including embryonic stem cells, induced pluripotent, and adult stem cells composed of the liver,

mesenchymal, and hematopoietic stem cells[186]. Mesenchymal stem cells (MSCs) are a suitable alternative for liver transplantation because they inhibit hepatocyte degeneration, promote liver regeneration, suppress fibrosis *via* differentiation into hepatocytes, and produce various growth factors [187].

Moreover, combining MSCs with induced bone marrow-derived macrophages showed stronger antifibrotic activity and hence better improvement of the cirrhotic liver than monotherapy[188]. An *in vivo* study investigating cell therapy in mice used four types of cells; mature hepatocytes, fetal liver cells, bone marrow-derived mesenchymal stromal cells (BMSCs), and induced hepatic stem cells for concanavalin A-induced fulminant hepatitis causing ALF and fumarylacetoacetate hydrolase-deficient induced chronic liver failure. Remission of concanavalin A-induced ALF was only noticed with BMSCs as they decreased serum markers of liver injury and mRNA expression of some inflammatory cytokines, including TNF-α, IFN-γ, and FasL, and increased IL-10 mRNA expression. In the chronic liver failure model, mature hepatocytes in the adult liver were the most effective for liver regeneration compared to other cell types. However, these hepatocytes are not common in clinical applications due to their limited sources[189]. In acute liver injury induced by carbon tetra chloride, using hepatocyte-like cells derived from embryonic stem cells showed the potential for attenuation of liver injury and the remission of induced liver fibrosis[190]. Furthermore, rather than cell transplantation, using trophic factors such as matrix metalloproteinase 2, tissue inhibitor of metalloproteinase 1, and growth arrest-specific 6, released from embryonic-derived hepatocyte-like cells, promoted hepatocytes regeneration, neovascularization, and extracellular matrix remodeling, all of which contribute to liver regeneration[191]. Despite the numerous advantages of stem cell therapy, safety concerns such as ethical approval of embryonic stem cell use, lack of knowledge of appropriate transmission methods, enhancement of tumor growth, and incomplete prediction of tissue response are limiting their use nowadays[192].

Gut-liver axis

The relationship between the gut and the liver involves the delivery of intestinal contents to the liver through the portal vein and the transport of bile acids and immunoglobulins from the liver back to the intestines. Any disruption of the homeostasis of this axis through altering gut microbiota (gut dysbiosis), bile acid composition, or intestinal barrier damage will result in the exposure of the liver to these microbes and their metabolites which is critical in the pathogenesis of the ALD, NAFLD and even liver cirrhosis[193,194]. That is why various experiments and clinical trials targeting the gut-liver axis are being studied to treat liver disorders, including NASH, NAFLD, and chronic hepatitis B and C[195]. The farnesoid X receptor (FXR) is a nuclear receptor highly expressed in the gut-liver axis and regulates bile acid production, detoxification, maintenance of triglyceride homeostasis, and enhancement of the function of the intestinal epithelial barrier[194,196,197]. Baicalin is a natural flavonoid studied on various liver disorders and exhibited favorable effects such as inhibition of inflammation and autophagy and necrosis of parenchymal liver cells, thus decreasing liver injury. One of the pathways involved in baicalin effects is FXR and G-protein-coupled bile acid receptor, as they can modulate TNF-α, NF-kβ, and TGF-β levels[195,198]. As gut dysbiosis and bile acid dysregulation are directly related to NAFLD's pathogenesis, using various probiotics, prebiotics, and synbiotics has been proven to be promising for treating NAFLD[199,200]. In addition to the role of probiotics in NAFLD and their ability to modulate inflammation and fibrosis in NASH, probiotics are an attractive target for gut-liver-related disorders as they are also cost-efficient, with mild adverse effects and nearly no long-term adverse reactions[201].

Nanoparticle drug delivery

Recently, nanomedicine gained much attention as an innovative way for effective drug delivery in various resistant types of diseases. Numerous nanoparticle types are used in liver fibrosis treatment: Inorganic oxides and metals[202] or organic micelles and liposomes[203]. Gold, an inert inorganic widely used material, is formulated in nanoparticle form to deliver silymarin to fibrotic livers induced by carbon tetrachloride. This process enhanced the antifibrotic activity of silymarin, attributed to increased expression of protective miRNAs and suppression of inflammatory mediators in the TGF-β /Smad pathway[204]. As we previously mentioned, the anti-fibrotic action of curcumin, enhancing drug delivery and bioavailability of curcumin using phosphatidylserine-decorated nanoparticles, further enhances curcumin efficacy in fibrosis reduction[205].

Interestingly, nanoparticles can also target different liver cells involved in liver fibrosis. As the expression of c-x-c chemokine receptor 4 (CXCR4) and VEGF is associated with HSCs activation and hence liver fibrosis progression, combining CXCR4 antagonist in nanoparticles with siRNA against VEGF provided significant inhibition of the process of angiogenesis making it auspicious treatment for liver fibrosis[206]. Considering liposome nanoparticles, the use of liposomes to be specifically delivered to integrins of activated HSCs rather than any other type of liver cells has been conducted, making it available to deliver therapeutic drugs to special sites overcoming their complications[207]. A novel advantage of liposome nanoparticles is that they facilitate gene therapy using siRNAs and mRNAs to modulate gene expression of hepatocytes instead of using viruses as carriers[208].

Autophagy

As we have mentioned, the BECN1 protein has been involved in autophagy, resulting in ferroptotic cell death. A study on knocking down BECN1 showed inhibition of autophagy and its consequent inflammation in addition to increasing prostaglandin E2 (PGE2) levels. Modulation of the prostaglandin-endoperoxide synthase 2/PGE2 pathway may cause suppression of HSC proliferation and lymphocyte infiltration, all contributing to MSCs' enhanced antifibrotic activity[209]. That is why inhibition of autophagy is a potential target for liver fibrosis treatment. Carvedilol, a non-selective B-blocker, has been thought to possess antifibrotic activity. Testing this theory *in vitro* revealed that carvedilol can alleviate liver fibrosis by inhibiting the autophagy of HSCs and enhancing their apoptosis[210]. Doxazosin, an alpha-1 adrenergic receptor agonist, has also been studied *in vitro* and *in vivo* and showed similar action to carvedilol on activating apoptosis of HSCs and inhibiting autophagy through the PI3K/Akt/mTOR signaling pathway[211]. Resolvin D1 is a polyunsaturated fatty acid that has been proven effective in various liver disorders, such as acute liver injury and liver fibrosis, due to its antioxidant, anti-inflammatory, and antifibrotic effects. Further investigations on resolvin D1 on CCL4-induced liver fibrosis demonstrated its ability to modulate the AKT/mTOR signaling pathway, resulting in inhibition of autophagy and suppression of HSC activation, which further intensifies resolvin D1 liver protective effect[212,213].

CONCLUSION

Collectively, acute and chronic liver diseases are worldwide problems with multifactorial pathogenesis. The exact pathological mechanism of several liver disorders is still unclear. However, many suggested mechanisms are involved, including but not limited to oxidative stress, inflammation, autophagy, and miRNA. The role of autophagy and miRNA is still unclear and requires more clarification. Besides, it may be a new way to find new therapy for hepatic disorders. Recent therapeutic strategies like gene therapy, stem cell therapy, gut microbiota, and even nanoparticle formulations require more investigations and improvements.

FOOTNOTES

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MINIREVIEWS

Acute-on-chronic liver failure in patients with severe acute respiratory syndrome coronavirus 2 infection

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has had a significant impact on the lives of millions of people, especially those with other concomitant diseases, such as chronic liver diseases. To date, seven coronaviruses have been identified to infect humans. The main site of pathological action of these viruses is lung tissue. However, a substantial number of studies have proven that SARS-CoV-2 shows affinity towards several organs, including the gastrointestinal tract and the liver. The current state of evidence points to several proposed mechanisms of liver injury in patients with COVID-19 and their combination. Liver impairment is considered to be the result of the direct effect of the virus on the hepatic tissue cells, a systemic reaction consisting of inflammation, hypoxia and cytokine storm, drug-induced liver injury, with the possible contribution of a perturbed gut-liver axis. Reactivation of chronic hepatic disease could be another factor for liver impairment in patients with SARS-CoV-2 infection. Acute-on-chronic liver failure (ACLF) is a relatively new syndrome that occurs in 10%–30% of all hospitalized patients with chronic liver disease. It is crucial to recognize high-risk patients due to the increased morbidity and mortality in these cases. Several published studies have reported virus infection as a trigger factor for ACLF. However, to date, there are few relevant studies describing the presence of ACLF in patients with acute SARS-CoV-2 infection. In this minireview we summarize the current state of knowledge regarding the relation between ACLF and acute SARS-CoV-2 infection.

Key Words: SARS-CoV-2; COVID-19; Acute-on-chronic liver failure; ACLF, Liver; Coronavirus

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Core Tip: The main aim of this brief review is to summarize current knowledge on the acute-on-chronic liver failure (ACLF) in patients with coronavirus disease 2019 (COVID-19). We also describe several mechanisms by which severe acute respiratory syndrome coronavirus 2 infection induces liver injury. Although several systematic reviews have already been published regarding liver impairment in COVID-19, there are few studies focusing on ACLF. We believe that this brief review has an informative value for clinicians and could contribute to better understanding of the disease and therefore improved management of this serious condition.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has had a significant impact on the lives of millions of people, especially those with other concomitant diseases such as chronic liver diseases.

This minireview focused on acute-on-chronic liver failure (ACLF) in COVID-19-involved cases. ACLF is a relatively new syndrome that occurs in 10%-30% of all hospitalized patients with chronic liver disease[1]. Patients with ACLF are considered to be high-risk patients when they become infected with SARS-CoV-2 because of the increased morbidity and mortality in these cases. The etiology of chronic liver diseases varies substantially (*e.g.*, viral hepatitis B and C, alcohol liver disease, non-alcohol steatohepatitis, automimune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, Wilsons's disease). Even after more than two years of global pandemic, this is a rather underestimated topic with an uneven ratio of patients with chronic liver disease who have been infected with SARS-CoV-2.

Therefore, understanding the pathophysiology mechanisms of SARS-CoV-2 virus affecting the liver along with improved stratification of patients with chronic liver diseases can ultimately result in better management, and a significant reduction in mortality and morbidity in the case of COVID-19 infection.

CORONAVIRUS DISEASE 2019 AND THE HEPATOBILIARY SYSTEM

Pathophysiology

To date, seven coronaviruses have been identified to infect humans. While human coronaviruses HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1 cause a "common cold", the other three, severe acute respiratory syndrome-related coronavirus (SARS-CoV) (2002-2003), Middle East respiratory syndrome-related coronavirus (MERS-CoV) (2012) and SARS-CoV-2 (from 2019), are highly pathogenic to humans and cause severe acute respiratory syndrome (SARS), with significant morbidity and mortality[2,3]. The main site of pathological action of these viruses is lung tissue. It has been widely hypothesized that the SARS-CoV-2 virus uses the angiotensin-converting enzyme 2 (ACE2) receptor to enter the respiratory tract cells[4]. The ACE2 receptor is expressed not only in the lungs, but also in other organs, such as the heart, intestine (ileum), pancreas, kidneys and endothelium, which may explain the multi-organ effect of virus infection[5]. A huge number of studies have proved that SARS-Co-V-2 shows affinity towards several organs, including those of gastrointestinal tract, such as the liver[6-9].

Evidence that coronaviruses could damage liver cells through the induction of apoptosis by activating caspase has been known for some time[10,11]. Liver impartment was also confirmed in the case of SARS-CoV during the pandemic in the early years of new millennium, and several studies have shown the direct negative impact of SARS-CoV on the liver at the cellular level[12,13]. Liver biopsies in these patients revealed common pathologic findings, such as the presence of acidophilic bodies, the ballooning of hepatocytes and mild to moderate lobular activities[12]. Studies based on autopsies of SARS-CoV victims showed that the virus was detectable in 41% of liver tissue samples with a relatively high viral load[14]. Hepatocellular necrosis, mitoses, cellular infiltration and fatty degeneration were all visible in these biopsies[14]. Interestingly there was no detection of viral particles in liver specimens from patients with MERS[15].

Almost three years after the COVID-19 pandemic broke out, there is undoubtedly a large amount of scientific and clinical evidence that COVID-19 is in many cases directly connected with abnormal liver function to a varying extent. Right from the beginning of the pandemic there were indications of a similar mechanism of influence of SARS-CoV and SARS-CoV-2 on hepatocytes[16], although the strains bear approximately 79% structural similarity[17]. It was pointed out that recipients of liver transplant could be at higher risk for virus transmission through the transplanted organ[18]. The fact that non-alcoholic fatty liver disease (NAFLD) presents with a proinflammatory hypercoagulable state could be associated with a more severe course of the disease and thrombosis in these patients when infected with SARS-CoV-2[19]. The structural hepatic abnormalities could persist even after acute COVID-19, as was shown in a study using multiparametric ultrasound[20]. These changes include increased liver stiffness and increased viscosity and attenuation, which could be indicative of various types of parenchymal impairment, including fibrosis, inflammation and steatosis[20].

The current state of evidence points to several proposed mechanisms of liver injury in patients with COVID-19 (Figure 1). Liver impairment is considered to be the result of the direct effect of the virus on hepatic tissue cells, a systemic reaction consisting of inflammation, hypoxia and cytokine storm, and drug-induced liver injury[21-23] with the possible contribution of a perturbed gut-liver axis[24]. Reactivation of chronic hepatic disease could be another factor for liver impairment in patients with SARS-CoV-2 infection[25].

Moderate microvesicular steatosis and mild inflammation in the lobular and portal area was observed in the liver tissues obtained during autopsies of COVID-19 victims[26]. This is, however, not disease-specific, as it could also be detected in liver tissue samples in patients with sepsis or drug-induced liver injury (DILI)[26].

DIRECT INFLUENCE OF THE VIRUS ON THE LIVER CELLS

There are several proposed mechanisms of SARS-CoV-2 influence on hepatocytes. One of the early histological and ultrastructural studies identified typical coronavirus particles in the hepatocytes' cytoplasm, with mitochondrial swelling, endoplasmic reticulum dilatation and glycogen granule decrease with a general histological picture of massive hepatocyte apoptosis and binuclear hepatocytes [27].

One possible explanation is based on the binding of SARS-CoV-2 to the ACE2 receptors on the cholangiocytes, leading to their dysfunction and induction of a local and systemic inflammatory response, ultimately resulting in liver injury[28]. Although the ACE2 receptor is present on the biliary epithelial cells, it was repeatedly observed that the bilirubin level was normal in most of the cases[29], regardless of severity of the disease itself[30]. Although the effect of the virus is primary on the bile duct epithelial cells, some researchers have proposed that the compensatory hyperplasia of hepatic parenchymal cells induce the up-regulation of ACE2 receptor expression in liver tissue[22]. This could be one of the pathways by which SARS-CoV-2 is responsible for direct liver parenchyma injury.

A study by Zhao *et al*[31] showed a significant increase of viral loads in cholangiocytes 24-h post-infection with a substantial decrease 48 h after infection. Their data also indicated that the virus impairs the bile acid transporting function of cholangiocytes and impairs the luminal barrier by modulating the expression of genes involved in sustaining the tight junctions and transportation of bile acids[31]. The direct viral cytopathogenic effect is predominantly on target cells that express ACE2 and TMPRSS2[31]. ACE2 expression level is higher in cholangiocytes (59.7%) than in hepatocytes (2.6%)[32].

Stebbing *et al*[33] reported massive induction of ACE2 expression in hepatocytes after 16 h of exposure to Interferone-α2 (IFN-α2) and Interferone-β. Exposure to Interferone-γ, tumor necrosis factor-α and interleukins (IL-1, IL-6, IL-10, Il-18) does not have the same effect. They further pointed out that the effect was strongest with Interferone-α2. Therefore, it has been proposed that the increased levels of IFN-α2 predominantly in patients with severe inflammatory response to SARS-CoV-2 infection could lead to significant ACE2 expression in parenchymal liver cells, contributing to virulence and further damaging the cells by the virus[33].

Another study focused on the expression of ACE2, TMPRSS2 and FURIN (paired basic amino acid-cleaving enzyme) levels in various cells within the liver tissue. It was shown that these receptors are expressed across various cell types. ACE2 is mostly expressed in cholangiocytes and hepatocytes, TMPRSS2 in cholangiocytes, hepatocytes, periportal liver sinusoidal endothelial cells, erythroid cells, non-inflammatory macrophages and T cells, and FURIN is expressed through all cell lines within liver tissue[23].

A recent study by Wanner *et al*[9] has provided multilevel evidence of SARS-CoV-2 human liver tropism using a wide range of clinical, histopathological, virological, molecular and bioinformatic approaches. Their data showed strong upregulation of JFN responses, JAK-STAT signaling and liver-specific metabolic modulation. Mismatch of the expression of the ACE2 protein and the location of the SARS-CoV-2 spike protein in Kupffer cells was also observed in this study[9]. Also, the main pro-inflammatory cytokines, such as IL-6, which is responsible for cytokine storm, is regulated by JAK-STAT signaling. Due to this known pathophysiological mechanism, JAK inhibitors such as baricitinib

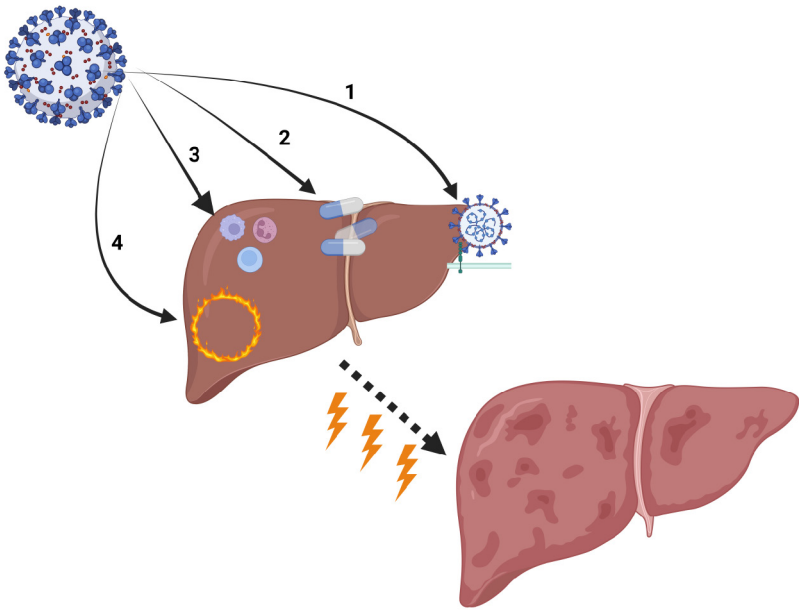


Figure 1 Different mechanisms of liver impairment due to severe acute respiratory syndrome coronavirus 2 infection. 1: Direct effect of the virus on the liver cells; 2: Drug-induced injury; 3: General response of the immune system; 4: Systemic inflammation and tissue hypoxia. The figure was created with BioRender (https://biorender.com).

have been used for treatment and have shown improvement in clinical outcomes in patients infected with SARS-COV-2. On the other hand, considering the potential adverse effects of this drug on the liver, more studies are needed to establish the proper dosage and timing, so the risk/benefit ratio can be determined in patients with high vulnerability for drug-induced liver injury[33,34]. Although several medications were used for treating COVID-19 with different outcomes, the "perfect" compound is still missing. However, results of the studies mentioned herein could facilitate the push of research towards targeting signaling pathways, receptors or even the virus itself.

Another study proposed high-density lipoprotein scavenger receptor class B member 1 (SRB1) as a facilitator for cell entry for the SARS-CoV-2 because of its strong protein expression in human liver cells [35]. This is based on the observation that SRB1 plays a crucial role in hepatitis virus C (HCV) cell entry [36]. SARS-CoV-2 shares some molecular features with HCV in the means of liver tropism[9]. Therefore, it is possible to assume that SRB1 could facilitate SARS-CoV-2 entry into liver cells along the well described ACE2 pathway.

It is interesting to compare the mechanism of action of SARS-CoV-2 with other coronaviruses. An indirect mechanism that resulted in hepatic damage through a complex inflammatory cascade was proposed in case of the SARS-CoV virus infection[13,37]. On the other hand, MERS-CoV requires dipeptidyl peptidase-4 (DPP-4) receptor for cell entry, which is different from SARS-CoV-1 and SARS-CoV-2 adherence mechanisms. Thus, the pathophysiology of the disease is different to some extent. The liver damage observed in MERS-CoV-infected cases was mostly mild. It is difficult to determine whether this is the result of a direct action of the virus or the inflammation-mediated reaction due to a lack of sufficient data[37]. An interesting fact is the comparison with hepatitis viruses, which, from a phylogenetic point of view, have developed a natural affinity for the liver tissue and whose infections are of a stealthy nature. Despite the fact that the mechanism of infection is not fully understood, it is assumed that hepatitis viruses do not have a direct cytopathic effect on hepatocytes but rather to trigger immune mechanisms that result in liver damage[38-40].

DRUG-INDUCED LIVER INJURY

Lopinavir and ritonavir are widely used antiviral drugs that are predominantly metabolized by the liver. These drugs were shown to have a potentially damaging effect on the liver by inducing inflammation and lipid metabolism disorders *via* the endoplasmic reticulum stress pathway and also could cause apoptosis of hepatocytes *via* the caspase system[22].

Integration of drug cytochrome P-450 could contribute to the secondary toxicity of several drugs commonly and widely used in the treatment of COVID-19 such as paracetamol (acetaminophen), lopinavir/ritonavir or azithromycin[41]. The meta-analysis by Yadav *et al*[42] showed that treatment by lopinavir/ritonavir is strongly correlated with liver injury, while other commonly used medications are not significantly connected with hepatic impairment.

Another commonly used drug in COVID-19 treatment is the antiviral drug favipiravir. It was reported that favipiravir used with interferon alpha resulted in liver injury in 2.9% of these patients[22].

RESULTS OF SYSTEMIC INFLAMMATION RESPONSE AND GENERAL HYPOXIA

One of the factors contributing secondarily to the hypoxic damage of hepatocytes could be hepatic congestion due to high positive end respiratory pressure in critically ill, mechanically ventilated patients [32]. Platelet activation is well described in patients with a serious course of COVID-19, and it has been proposed that vascular dysfunction due to endotheliopathy and platelet activation in response to a systemic inflammatory response could contribute to impaired liver function, predominantly in patients with a pre-existing chronic liver disease[43].

Systemic inflammatory response generally leads to cellular ischemia and abnormal coagulation with micro-thrombotic events. Inflammatory response in COVID-19 is characterized by high lymphocyte activation, neutrophilia with significantly elevated levels of serum interleukins, tumor necrosis factor, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon inducible protein 10, monocyte chemotactic protein 1 and macrophage inflammatory protein 1 alpha[44]. Accumulation of T cells in the post-mortem liver histological findings further supports the theory of immune-mediated response related to liver damage[44].

ROLE OF IMMUNITY

A well-functioning immune system is essential in the fight against infections. The liver is known to play an important role in the body's immune response to an infectious stimulus. Many factors are involved in the physiological immune response of the host, such as immune cells, antimicrobial peptides and so-called pattern recognition receptors (PRRs), which can detect dangerous microbial signals through molecular patterns[45]. The liver is the major source for the production of PRRs, which have two main functions: complement activation and opsonization, which is an important step of phagocytosis[46]. An important subgroup of PRRs is the toll-like receptors, which play a crucial role in several liver disorders, such as alcoholic liver disease, non-alcoholic steatohepatitis, viral hepatitis, hepatic fibrosis, autoimmune hepatitis and liver cancer. Thus, the liver plays an important role in the adaptive immunity of the body, which is essential against infections and not only bacterial ones. Liver cirrhosis interferes and damages the proper functioning of adaptive immunity by impairing the synthesis of PRRs and various proteins, which can result not only in immune dysfunction but also in immunodeficiency[47, 48]. The association between SARS-CoV-2 and the activation of the pro-inflammatory cascade results in excessive overproduction of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-alpha, and attenuation of the body's anti-inflammatory response, resulting in the development of the so-called cytokine storm, as it has been repeatedly described in the case of COVID-19 infection. The cytokine storm possibly reflects the severity of the disease[49]. Cirrhotic patients are at a higher risk of developing a systemic inflammatory response syndrome with overproduction of the above-mentioned cytokines, which, together with deregulation of the immune response and ongoing acute infection, may have fatal consequences. ACLF is a relatively novel umbrella term where acute and chronic liver insults exist along with an imbalance between systemic pro-inflammatory and anti-inflammatory responses. All the above-mentioned could then trigger an uncontrolled and complex sequence of events, which may result in ACLF with fatal consequences to patients with acute COVID-19[47,50].

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

A study based on histological findings from COVID-19 victim biopsies showed a 10-fold increase in the number of ACE-2 positive cells in the liver (predominantly in the form of activated hepatic stellar cells) in patients with pre-existing alcohol use disorder compared to patients with normal liver function who died before the pandemic[51]. As chronic alcohol abuse is related to chronic liver damage, these findings may have potential clinical implications. These are further supported by evidence of massive up-regulation of ACE2 (a 97-fold increase in a widespread parenchymal pattern) in cirrhotic liver and NASH induced by high-fat diet [52]. The significant ACE2 upregulation in liver cells was also observed in animal models with high-fat-diet-induced non-alcoholic steatohepatitis, with concomitant treatment with pioglitazone[24]. Therefore, diabetic patients who are treated with PPARγ agonist and present with chronic liver impairment have a higher susceptibility to SARS-CoV-2 infection, and possibly with more severe consequences. There is also evidence that the level of hepatokines is disturbed in patients with COVID-19, and these are associated with disease severity and outcomes[53]. A relationship between hepatokines, liver steatosis and metabolic diseases, such as diabetes mellitus[54], has been suggested. ACE2, as a main receptor for viral entry and a modulator of inflammatory responses, is also

considered a potential target for treatment strategies. There are only a few ACE2-related molecules (*e.g.* DIZE, Ang 1-7) that are tested in humans. Some of these molecules can, for example, reduce tissue ACE2 activity. Many of them have already been tested on animal models; however extensive research in humans is still needed[55].

In patients with viral hepatis B (HBsAg-positive and hepatitis B core antibody positive patients) a higher risk of HBV reactivation with liver injury and fatal course of the COVID-19 was observed[56]. This could be considered a secondary result of SARS-CoV-2 infection on the liver in patients with chronic hepatic disease.

CLINICAL ASPECTS OF ACLF AND COVID-19

ACLF is a relatively new syndrome that occurs in 10%-30% of all hospitalized patients with chronic liver disease[1]. It is crucial to recognize high-risk patients due to the increased morbidity and mortality in these cases. The main hepatological societies (APASL, EASL and AASLD) have proposed their own definitions of ACLF, each of which differs from the others[57]. However, despite several differences, the main criteria are roughly the same. These are dominantly the presence of liver disease, precipitant factors of ACLF and hepatic or extrahepatic failure[58-60]. One definition was proposed by the World Gastroenterology Organization in 2014 to unify and simplify the diagnosis. It defined ACLF as a syndrome with very a high short-term mortality in patients with chronic liver disease with known or unknown cirrhosis characterized by acute hepatic decompensation, resulting in liver failure and at least one extrahepatic failure[61,62].

Activated pathogen-associated molecular patterns and damage-associated molecular patterns as drivers of systemic inflammation are proposed as the main etiopathological factors[63]. Activation of this systemic inflammatory response can be triggered by various conditions. Identification of precipitating factors can predict the course of the disease. The trigger of ACLF depends on the region. While in Asian populations this is usually reactivation of hepatitis B, in Western countries it is usually alcohol hepatitis, gastrointestinal bleeding or another infection[57,62].

Several published studies have reported virus infection as a trigger factor for ACLF. Infection with hepatitis B virus could lead to occurrence of a specific syndrome – hepatitis B virus-related ACLF with a wide variety of disease course[64,65]. Hepatitis A and hepatitis E viruses lead significantly less often to the development of ACLF[66,67]. The ability of the SARS-CoV-2 virus to adhere to ACE2 on the hepatocyte and cholangiocyte membrane is known[68]. However, the data describing the prevalence of ACLF in patients with chronic liver diseases suffering SARS-CoV-2 infection are scarce. Iavarone *et al* [69] carried out a retrospective study on a cohort of 50 cirrhotic patients infected with SARS-CoV-2 with an observed high mortality rate of > 34%. ACLF was present in 28% of patients, and death related to liver impairment was present in 29% of the cases. An independent factor for worse prognosis of COVID-19 in patients with concomitant chronic liver disease is the presence of an alcohol-related liver disease and ongoing drinking[69]. Reports of a predictive role of the CLIF and MELD scores in the setting of ACLF influenced by acute SARS-CoV-2 infection are emerging[70]. Sarin *et al*[71] investigated a population of 228 patients with liver disease (185 patients with chronic liver disease and 43 patients with cirrhosis) and found that 43% of patients with chronic liver disease infected with SARS-CoV-2 also presented with acute liver injury. Almost 12% (11.9%) of cirrhotic patients in this patient group developed ACLF. Complications related to liver function deterioration were present in half of the patients with decompensated cirrhosis, with higher mortality. Obesity was identified as a predictor of worse prognosis. In a multicenter study, Bajaj *et al*[60] reported the incidence of ACLF within a group of cirrhotic patients infected with SARS-CoV-2 as high as 36%. Interestingly there was no significant difference in mortality rate compared to patients with cirrhosis and negative for acute SARS-CoV-2. Another study from Shalimar *et al*[72] recorded the presence of ACLF in 9 of 28 patients from their study cohort. Mortality in these patients reached 100%[72], and mechanical ventilation was associated with poor prognosis. Besides a scarce number of prospective or retrospective cohort studies, there are also several individual case reports describing the occurrence of ACLF in a patient with chronic liver disease[68].

CONCLUSION

SARS-CoV-2 is a virus with multiorgan affinity. A substantial percentage of patients with COVID-19 could be simultaneously diagnosed with liver impairment to a varying degree, with different prognosis and duration. The virus affects the liver *via* different pathways (Table 1). Patients with chronic liver disease are at a higher risk for poor disease outcome when infected with the novel coronavirus. One of the lesser reported and described subgroups of these patients are those who developed ACLF. Patients with chronic liver disease and cirrhosis simultaneously infected with SARS-CoV-2 are at a risk of developing ACLF, with poor prognosis of survival. Available data are heterogenous, and the incidence of ACLF varies from 11.9% to 36%.

Table 1 Summary of the relationship between the pathophysiologic mechanism of a virus and its possible clinical impact in the context of liver damage caused by severe acute respiratory syndrome coronavirus 2

Pathophysiologic mechanism of virus	Clinical impact	Considerations for clinical management
Direct influence of the virus on the liver cells	Significant ACE2 expression in parenchymal liver cells contributing to virulence and further damaging effect of the virus on the cells	Several antiviral agents are approved for treatment of SARS-CoV-2 infection *e.g.* remdesivir and ritonavir-boosted nirmatrelvir, which can inhibit viral replication. Also, monoclonal antibodies reduce the binding ability of SARS-CoV-2 to the ACE2 receptor
Drug-induced liver injury	Drug metabolized by cytochrome P-450 could contribute to secondary toxicity of several drugs (paracetamol, antibiotics)	Following the strict rules for avoidance of hepatotoxic drugs if possible. Standard use of hepatoprotective medications
Results of systemic inflammation response and general hypoxia	The hypoxic damage of hepatocytes, platelet activation, endotheliopathy, immune-mediated response related to liver damage	Administration of corticosteroids and other immunomodulators can reduce or modulate the adverse impact of immune over-response
Role of immunity	Impaired synthesis of PRRs are toll-like receptors, activation of the pro-inflammatory cytokines such as IL-1, IL-6, TNF-alpha	Use of IL-1 and IL-6 inhibitors, such as anakinra or tocilizumab, as well as Janus kinase inhibitors, such as baricitinib, can decrease the excessive effect of pro-inflammatory cytokines

ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IL-6: Interleukin 6.

Although the clinical management of patients with liver diseases who contracted SARS-CoV-2 infection is still evolving, several consensus guidelines have been developed[73-75]. These guidelines were created based on multicenter and international studies, which can provide guidance for better clinical management. Several steps should be followed by clinicians to identify patients with higher risk of liver disease progression according to these recommendations. Thorough history taking and physical examination should be a cornerstone in the diagnosis process. It is also crucial to further investigate the possible presence of underlying chronic liver diseases. For doing this, a serological test for hepatitis viruses, frequent monitoring of liver enzymes or imaging examinations, such as ultrasound, could be used. It is also important to thoroughly review patients' chronic and currently administered medications due to the possibility of liver damage related to specific drugs (*e.g.*, antivirals, antibiotics, anti-inflammatory medications, *etc.*).

To summarize, it is important to consider patients with ACLF as a distinct patient population with a high risk for a severe course of SARS-CoV-2 infection and to manage them appropriately.

FOOTNOTES

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MINIREVIEWS

Liver immunity, autoimmunity, and inborn errors of immunity

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Abstract

The liver is the front line organ of the immune system. The liver contains the largest collection of phagocytic cells in the body that detect both pathogens that enter through the gut and endogenously produced antigens. This is possible by the highly developed differentiation capacity of the liver immune system between self-antigens or non-self-antigens, such as food antigens or pathogens. As an immune active organ, the liver functions as a gatekeeping barrier from the outside world, and it can create a rapid and strong immune response, under unfavorable conditions. However, the liver's assumed immune status is anti-inflammatory or immuno-tolerant. Dynamic interactions between the numerous populations of immune cells in the liver are key for maintaining the delicate balance between immune screening and immune tolerance. The anatomical structure of the liver can facilitate the preparation of lymphocytes, modulate the immune response against hepatotropic pathogens, and contribute to some of its unique immunological properties, particularly its capacity to induce antigen-specific tolerance. Since liver sinusoidal endothelial cell is fenestrated and lacks a basement membrane, circulating lymphocytes can closely contact with antigens, displayed by endothelial cells, Kupffer cells, and dendritic cells while passing through the sinusoids. Loss of immune tolerance, leading to an autoaggressive immune response in the liver, if not controlled, can lead to the induction of autoimmune or autoinflammatory diseases. This review mentions the unique features of liver immunity, and dysregulated immune responses in patients with autoimmune liver diseases who have a close association with inborn errors of immunity have also been the emphases.

Key Words: Liver immunity; Autoimmunity; Immune tolerance; Autoinflamation; Autoimmune liver diseases; Inborn errors of immunity

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Core Tip: The various repertoires of immune cell populations in the liver play a central role in maintaining homeostasis between inflammation and tolerance. Inflammatory and immunoregulatory interactions within the liver are essential for maintaining systemic homeostasis. In this review, we summarize the molecular mechanisms involved in these seemingly contradictory immune processes and how liver immunity functions during normal liver homeostasis and liver pathologies, such as viral hepatitis, autoimmune hepatitis, and hepatocellular cancer.

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INTRODUCTION

The immune system is a complex cellular and molecular network that provides the body with defense against harmful and foreign substances. While the immune system provides defense against pathogens in healthy individuals, it also plays a role in clearing the body's own dead cells and cell remnants to prevent tumoral cell formation. On the other hand, one of the main features of the immune system is "immune tolerance" which ensures that the body does not harm its own tissues and maintains tissue homeostasis while performing the aforementioned active immune screening of tissues and organs.

Conceptually, the elements of the immune system can be divided into two main groups: Innate and acquired (adaptive) immunity, which interact closely with each other. The innate immune system provides a pre-structured first response to a wide range of situations and stimuli, and thus constitutes an initial rapid response against immune insults. However, the adaptive immune system learns to recognize previously encountered stimuli and provides a specific immune response against them. Both types of immunity are mediated by both molecules and cells. The general characteristics of the innate and adaptive immune systems are summarized in Table 1[1].

Immune tolerance implies the inertia of the immune response towards self-antigen. Immune tolerance can occur in two ways. "Central tolerance" is acquired by training lymphocytes about autoantigens during their development in primary immune organs (*e.g.*, thymus), whereas "peripheral tolerance" defines the maintenance of tolerance towards self-antigens by lymphocytes at the target organ, such as the liver, which has previously completed their development and spread around the body. Central tolerance is achieved by apoptosis of self-reacting T lymphocytes in the thymus and by the loss of autoreactive feature of lymphocytes by changing their receptors in the bone marrow. Peripheral tolerance is provided by T regulatory (Treg) cells and co-stimulatory surface molecules that control antigen presentation by dendritic cells (DCs)[2].

Autoimmunity is the formation of a cellular or humoral immune response against the body's own antigens due to defects in immune tolerance mechanisms. Autoimmune diseases are characterized by tissue damage resulting from a dysregulated immune reaction of an organism against its own antigens [3]. The autoimmune reaction can be limited to an organ (*e.g.*, autoimmune hepatitis [AIH]) or systemic reactions involving several organ systems (*e.g.*, systemic lupus erythematosus). Although both the innate and adaptive immune systems contribute to the development of autoimmune disease, it is generally known that adaptive immunity plays a major role. Indeed, recent literature proposed to classify the disease with loss of immune tolerance as "autoinflammatory disease" which is mainly associated with disorders in innate immunity and "autoimmune disease" which is driven by pathological responses of the adaptive immune system[4].

Various factors and mechanisms can trigger autoimmunity. In general, the presence of underlying genetic predisposition factors, environmental factors, infection, inflammation, and apoptotic bodies triggers the development of autoimmunity[5]. Although the pathogenesis of autoimmunity is still not fully understood and studies are ongoing, the currently known mechanisms that are thought to cause autoimmunity are summarized at the cellular and molecular levels in Table 2[6].

The prevalence of autoimmune diseases in the general population is around 3%-5%[7,8]. They include a diverse group of diseases that can affect almost all organs and sometimes multiple systems, such as autoimmune thyroiditis and autoimmune hemolytic anemia, which can be organ-specific whereas systemic lupus erythematosus and vasculitides have systemic involvement. The presence of one autoimmune disease predisposes patients to other autoimmune diseases. In fact, autoimmune liver diseases (AILD) are organ-specific, namely, liver-restricted autoimmune diseases, and are commonly associated with autoimmune diseases of other organs[9].

Table 1 Characteristics of immune system components

	Innate immunity	Adaptive immunity
Cells	Macrophages, dendritic cells, neutrophils, eosinophiles, basophiles, mast cells, NK cells, γδT cells	T lymphocytes, B lymphocytes, NK cells, γδT cells
Molecules	Complement, cytokines, glycoproteins, chemokines, TLR, NLR, IL-1 beta, IL-18	Immunoglobulins, cytokines, chemokines
Response time	Rapid (minute-hour)	Slow (hour-days)
Response type	Response is non-spesific	Pathogen and antigen specific response
Memory	No immunological memory	Immunological memory
Dysregulated disease	Autoinflamatory diseases (*e.g.*, periodic fever syndromes, systemic juvenile idiopatic artritis, adult onset Still disease, gout aritis)	Autoimmune diseases (*e.g.*, mixed connective tissue diseases, systemic lupus erythnatosus, systemic sclerosis, idiopatic inflamatory myopaties, primary sjögren syndrome)

TKR: Toll-like receptors; NLR: Nod-like receptor; IL: Interleukin; NK: Natural killer.

Table 2 Mechanisms of autoimmunity

Item	Description
Exogen	Molecular mimicry
	Superantigen stimulation
	Microbial and tissue damage related adjuvant effect
Endogen	Loss of central and peripheral tolerance
	Autoreactive B and T lymphocytes
	Apoptotic defects and defects in cleaning apoptotic substance
	Disturbances in cytokine balance
	Change in immunoregulation

Dysregulated immune responses not only increase infection risk but also make individuals prone to autoimmune and malignant diseases. Inborn errors of immunity (IEI) were previously named "primary immunodeficiency diseases". IEI is a heterogeneous group of diseases caused by one or more disorders in the innate or adaptive immune system, affecting the development or function of the immune system and increasing susceptibility to infections[10]. Unlike secondary immune deficiencies, which develop due to various drugs and diseases, IEI is a genetic disorder. More than 350 genes involved in the etiology of IEI have been identified. While some IEIs are inherited by a single gene, other is polygenic. Except for selective IgA deficiency, all other forms are rare, occurring in approximately 1:10000 Live births. However, it is estimated that IEI is more common in consanguineous or genetically isolated populations[11]. According to a classification updated in 2019, IEIs were grouped under ten headings as shown in Table 3[12,13]. Most IEIs present with symptoms and are diagnosed in childhood; however, symptoms of some diseases, such as common variable immunodeficiency (CVID), may appear later in life. Diagnosis may be delayed because of the heterogeneous and indolent course of symptoms associated with IEI. The risks in these patients are not limited to susceptibility to bacterial, viral, or opportunistic infections but also include autoimmunity, malignancy, lymphoid proliferation, atopy, and granulomatous disease[12,14,15]. The treatment method varies according to the type of IEI, such as prophylaxis for bacterial, fungal, and/or viral infections; intravenous or subcutaneous immunoglobulin; immunosuppressive or modulatory drugs; and hematopoietic stem cell transplantation.

ROLE OF THE LIVER IN IMMUNITY

The liver has been proposed as an "immunological organ". Beginning with intrauterine life, the liver has several unique immunological features, including a high level of immune tolerance, powerful innate immunity, and over-reactive autoimmunity against a weak adaptive immune response. In addition, the liver has a dual arterial blood supply from the hepatic artery and portal vein; thus, it is a bridge between the two circulatory systems of the body, namely, the caval and portal systems. Oxygen-rich arterial blood enters the liver *via* the hepatic artery, which supplies one-third of the liver's blood flow. The

Table 3 Categories of inborn errors of immunity

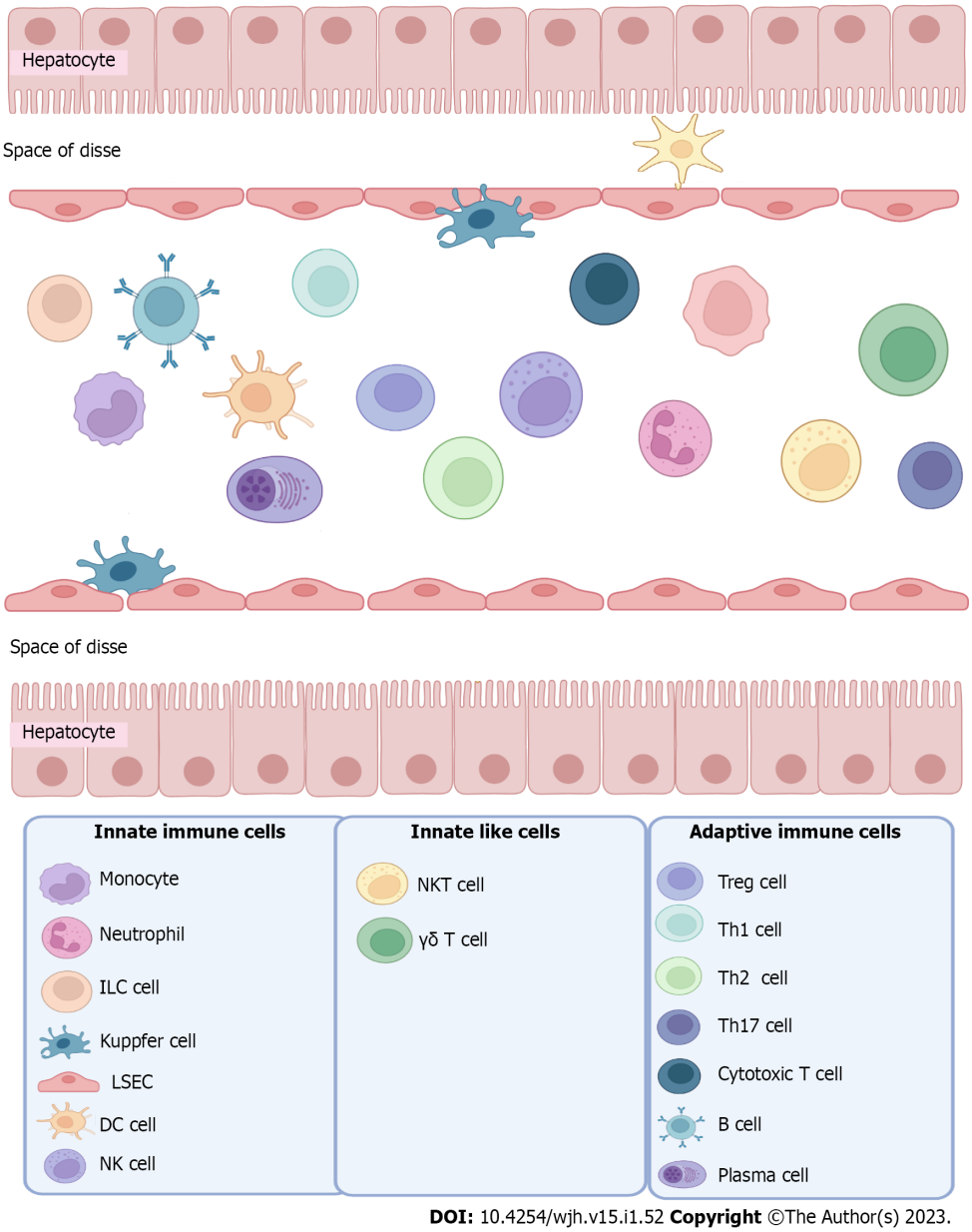
No.	Inborn errors of immunity phenotypical classification
1	Immunodeficiencies affecting cellular and humoral immunity
2	Combined immunodeficiences with associated or syndromic features
3	Predominantly antibody deficiencies
4	Disease of immune dysregulation
5	Congenital defects of phagocyte number and function
6	Defects in intrinsic or innate immunity
7	Autoinflammatory disorders
8	Complement deficiencies
9	Phenocopies of inborn errors of immunity
10	Bone marrow failure

portal vein carries most of the blood to the liver, which is rich in both nutrients and pathogen-derived molecules[16,17]. After passing through a network of liver sinusoids, blood leaves the parenchyma *via* the central hepatic veins. Various antigenic structures and cells from the gut and other organs mix within the liver sinusoids and are cleaned by hepatocytes. Approximately 30% of the total cardiac output passes through the liver every minute, and it carries approximately 10^8 peripheral blood lymphocytes in 24 h[18]. Decreased blood velocity in the feeding vessels of the liver, minimal increases in systemic venous pressure, and disturbances in sinusoidal flow result in stasis. This prolongs the contact time between lymphocytes and antigen-presenting cells (APCs) in the sinusoids and promotes lymphocyte extravasation. The sinusoids are lined with special liver sinusoidal endothelial cells (LSECs) containing multiple fenestrae that allow blood lymphocytes to reach the space of Disse between LSECs and hepatocytes, where they contact the extracellular matrix, stellate cells, and hepatocytes[19].

The liver is considered to be one of the primary organs of the immune system, with its own microanatomy and lymphoid and non-lymphoid cells. Liver parenchymal cells are hepatocytes and cholangiocytes, which constitute 60%-80% of liver tissue (Figure 1) and function as part of the "liver immune system". Non-parenchymal cells, namely, LSECs, hepatic satellite/into cells, Kupffer cells, neutrophils, mononuclear cells, T and B lymphocytes, natural killer (NK) cells, and NKT cells, also have immunological functions[18]. Lymphocytes are scattered throughout the hepatic lobules and portal areas. The liver contains approximately 10^{10} lymphocytes, including conventional and nonconventional lymphocyte subpopulations of the immune system.

Conventional T cells include clusters of differentiation (CD)8^+ and $CD4^+$ T cells. Both groups of T cells exhibit a diverse repertory of T cells that recognize antigens in the context of major histocompatibility complex (MHC) class I and II molecules. $CD4^+$ T cells are less in number than $CD8^+$ T cells in the liver. There are more memory cells in the liver than in blood. Unconventional T cells contain a variety of cell types and are categorized into two main populations based on NK cell marker presentation. Unconventional T cells presenting T cell markers are named NKT cells, and they bridge the gap between the adaptive and innate immune systems. NKT cells have a limited T-cell receptor (TCR) repertoire. They recognize and eliminate tumor and virus-infected cells. Unlike conventional T cells, NKT cells recognize glycolipid antigens that are presented by CD1d. NKT cells are further classified as "classical NKT cells" and "nonclassical NKT cells". Classical NKT cells are divided into two groups: CD4-positive or CD4/CD8-double negative. Nonclassical NKT cells contain TCR αβ and TCR γδ T cells[20]. Classical and non-classical NKT cells are found in higher proportions in the liver than in other organs and may constitute 30% of the intrahepatic lymphocyte population[21].

The liver comprises various types of resident APCs that can capture cell-associated released antigens, either passing through the liver or during the death of pathogen-infected hepatocytes. Resident APCs include Kupffer cells, LSECs, and DCs. Kupffer cells constitute the majority of the macrophage group in the body and constitute approximately 20% of the non-parenchymal cells in the liver[22]. Kupffer cells originate from circulating monocytes and localize in the sinusoidal vascular space of the liver. Here, they settle perfectly to remove endotoxins from the blood and phagocytose residues and microorganisms. Their slow migration through hepatic sinusoids leads to temporary stasis, facilitating close contact with the passing lymphocytes[23]. LSECs constitute the majority of non-parenchymal cells in the liver (50%). Their morphology forms a sieve-like fenestral endothelium. LSECs express molecules containing mannose and scavenger receptors, which facilitate antigen uptake. LSECs also include MHC class I and II and co-stimulatory molecules (CD40, CD80, and CD86) that facilitate antigen presentation [24]. DCs are professional APCs that control immunity and tolerance. Hepatic DCs are derived from the bone marrow and are mostly found around the central veins and portal tracts of the liver[25]. Hepatic



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Figure 1 Cell composition of the healthy liver. ILC: Innate lymphoid cells; DC: Dendritic cells; LSEC: Liver sinusoidal endothelial cells; NK: Natural killer; NKT: Natural killer T.

DCs produce certain cytokines in response to signals from invading microbes and their cellular environment, support the adaptive immune system, and act as a bridge between innate and adaptive responses[26].

IMMUNE SYSTEM ELEMENTS IN THE LIVER

Innate immunity

The innate immune system is the first crucial defense against infections. It quickly reacts to possible pathogenic attacks. The innate immune system contains physical and chemical barriers, humoral factors, phagocytic cells, and lymphocytic cells (NK and NKT cells). Although innate immune responses kill pathogens non-specifically, recent studies suggest that innate immunity can detect specific infections through "pattern recognition receptors (PRRs)". PRPs identify structures reflected by pathogens called pathogen-associated molecular patterns (PAMPs)[27]. Among them, the best-defined PAMPs are lipopolysaccharides and peptidoglycans.

Hepatocytes play an important role in the control of systemic innate immunity by secreting PRRs and complementing plasma. Liver expression of genes encoding these proteins is governed by transcription factors such as hepatocyte nuclear factors (nuclear factor-1) and CCAAT-enhancer-binding protein.

During the acute phase of the systemic inflammatory response, various pro-inflammatory cytokines [such as interleukin (IL)-6, IL-1, tumor necrosis factor α (TNF-α), and interferon-gamma (IFN-γ)] stimulate hepatocytes to produce high levels of complement and PRRs[28].

The complement system comprises plasma and membrane proteins that affect each other to protect against infection. In addition, it contributes to the pathogenesis of various liver disorders including fibrosis, alcoholic liver disease, and ischemic liver injury. There are three different ways to activate the complement system: Classical, lectin, and alternate pathways. After activation, the complement system mediates various biological activities, such as opsonization, and inflammatory and cytotoxic functions. The liver biosynthesizes the main complement components in the plasma, including C1r/s, C2, C4, Cbp, C3, mannan-binding lectin, factor B, mannan-binding lectin-associated serine proteases 1-3, and the terminal components of the complement system C5, C6, C8, and C9. Hepatocytes are also involved in the biosynthesis of certain regulatory proteins in the plasma, such as factor I, factor H, and C1 inhibitors [29,30].

The liver contains membrane-bound PRRs, such as Toll-like receptors (TLRs), which are a family of proteins that recognize PAMPs expressed by microorganisms. Diverged TLRs are expressed by liver cells. They have been shown to participate in liver injury and repair, and contribute to the pathogenesis of various liver diseases. Recently, cytoplasmic PRRs, including nucleotide-binding oligomerization domain-like receptors and retinoic acid-inducible gene (RIG)-like helicases, have been identified. RIG-1 serves as a pathogen receptor that regulates cellular transition to hepatitis C virus (HCV) replication[31].

Many studies have shown that hepatic NK cells play a significant role in innate immune responses against tumors, viruses, intracellular bacteria, and parasites. NK cells also contribute to innate defense against primary liver tumors and liver metastases in patients. This effect is achieved by direct killing of tumor cells and stimulation of tumor-specific immunity[32]. Activation of NK cells is also involved in liver injury, fibrosis, and repair[33]. Liver lymphocytes are enriched in Tγδ cells. Evidence suggests that Tγδ cells play an important role in innate defense against viral and bacterial infections and in tumor formation. The percentage of Tγδ cells is considerably increased in the livers of tumor-bearing mice and patients with viral hepatitis[34].

In addition to host defense against infection, innate immunity can detect signals from damaged hepatocytes during non-infectious liver injury. Acetaminophen hepatotoxicity and ischemic liver injury can cause liver damage by inducing sterile neutrophilic inflammation. Neutrophilic inflammation after partial hepatectomy can promote liver regeneration by triggering a local inflammatory response, leading to hepatocyte proliferation[35]. IL-1 is an important mediator of sterile neutrophilic inflammation in liver injury.

All chronic liver diseases lead to liver fibrosis, which is characterized by the activation of hepatic satellite cells (HSCs) overproducing collagen, and eventually, its accumulation in the liver[36]. HSCs are generally inactive in healthy livers, but become activated during liver injury and differentiate into myofibroblastic cells. Transforming growth factor β (TGF-β) and platelet-derived growth factor induce HSC transformation and proliferation. Evidence suggests that the innate immune system plays a key role in regulating HSC activation and liver fibrosis[37]. The complement system is activated after liver damage. A recent study showed that C5 deficiency caused a decrease in liver fibrosis, whereas overexpression of the C5 gene caused an increase in liver fibrosis[38]. TLRs likely play a significant role in the pathogenesis of liver fibrosis because various TLRs are expressed in liver cells, including HSCs[39]. TLR9-deficient mice have been shown to be resistant to liver fibrosis because HSCs require TLR9 for DNA activation[40]. Kupffer and NK cells have been shown to play significant roles in liver fibrosis[33]. It is thought that Kupffer cells activate HSC by producing cytokines/growth factors such as TGF-β. NK cells have an inhibitory effect on liver fibrogenesis. Activated HSCs are directly killed by NK cells by expressing the NK cell-activated ligand retinoic acid early inducible gene 1 and tumor necrosis factor-related apoptosis-inducing ligand receptors[41,42].

Adaptive immunity

The liver is a front-line filter for pathogens and PAMPs entering the body from the gut *via* the portal vein, and is often one of the first points of contact with other antigens entering the body. Similar to lymphoid organs, the liver is involved in the development and function of the adaptive immune response. Despite the abundance of APCs in the liver and their ability to rapidly recruit diverse immune cell populations, establishing an integrated adaptive immune response in the liver is a complex process. The immune response in the liver must be in delicate balance between tolerance to non-threats and immunity to pathogens.

There is insufficient data on the functions of B cells in the liver. The scarcity of B cells in the healthy liver is the reason for not obtaining the intended information. In adaptive immunity in the liver, these T cell subsets are highly regulated in all stages of diverse disorders. The major T lymphocytes involved in adaptive immunity include $CD4^+$ T cells, $CD8^+$ T cells, and γδ cells. $CD4^+$ T cells have at least five functional subgroups, including helper T (Th), Th2, Th17, follicular helper T (Tfh), and T-regulatory (Treg) cells. The innate and adaptive immune responses in the liver are supported by Tfh cells, which are often suppressed by Treg cells. $CD8^+$ T cells are composed of two subgroups: Cytotoxic T (Tc) cells and $CD8^+$ Treg cells. Tc cells are the main killer cells in adaptive immunity, and CD8 Treg cells suppress immune responses to infection. Tγδ cells participate in both the innate and adaptive immune responses.

Adaptive immunity and viral hepatitis

Although hepatitis B virus (HBV) and HCV are both hepatotropic viruses, hepatocellular necrosis during infection primarily results from an adaptive immune response targeting virus-infected liver cells [43]. Naive T cells specific to viral antigens can be locally activated in the liver. In the initial stage of adaptive immunity, antigen-specific naive T cells are usually prepared by APCs in the lymph nodes, differentiate into effector cells, and then migrate to the target (liver)[44]. However, HBV-specific naïve T cells can exert their anti-HBV effects by directly entering the liver before maturation in lymphoid organs [45]. Th17 cells can exacerbate liver lesions during HBV infection. In patients with HBV infection, the number of Th17 cells increases in the blood and liver, accompanied by high levels of IL-17 and IL-22 in the blood[46]. In contrast, HBV-specific $CD4^+CD25^+foxp3^+$ Treg cells have immunosuppressive effects during HBV infection[47]. Evidence demonstrates that HBV-specific $CD8^+$ T cells play a significant role in viral clearance and in the prognosis of HBV infection. When HBV-specific $CD8^+$ T cells are activated, they produce IFN-γ and TNF-α, which in turn inhibit HBV replication in infected hepatocytes and enable viral clearance. However, studies in mice infected with HBV have shown that HBV components also induce specific immune tolerance through clonal deletion, clonal ignorance, and clonal anergy[48]. It has been reported that there are more $CD11b^+Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSCs) in the liver of patients with chronic hepatitis B. The suppressive role of MDSCs in T cells contributes to the dysfunction of HBV-specific $CD8^+$ T cells. Additionally, γδ-T cells may promote $CD8^+$ T-cell depletion in these patients by recruiting MDSCs to the liver[49].

Adaptive immunity and hepatocellular carcinoma

Most cases of hepatocellular carcinoma (HCC) occur in individuals with a history of HBV or HCV infection, with or without cirrhosis. Two main mechanisms explain the close association between viral infection and HCC: Immunosuppression due to viral infection, and viral gene integration. The occurrence and prognosis of HCC are closely related to T-cell-mediated immunity[50]. It has been known that $CD8^+$ T cells are the essential cells of adaptive immunity that kill tumor cells *via* histocompatibility leukocyte antigen class I molecule limitation on the tumor cells. Several HCC tumor-associated antigen (TAA)-specific CD8 T cells have been identified. Alpha-fetoprotein (AFP) is the most common TAA in HCC patients. AFP has been reported to transform DCs into tolerogenic DCs, which inhibit the induction of tumor-specific $CD8^+$ T cells[51]. Among the $CD4^+$ T-cell subsets in HCC, $CD4^+CD25^+Foxp3^+$ Treg cells play an important immunoregulatory role. As the number of infiltrating Treg cells increased, the number of $CD8^+$ T cells in the liver decreased. When the number of Treg cells is decreased by cyclophosphamide treatment in patients with HCC, the number of $CD4^+$ T cells that secrete IFN-γ increases[52]. Evidence suggests that the number of MDSCs is increased in the peripheral lymphatic tissue and blood of patients with HCC, resulting in suppression of both innate and adaptive immunity. MDSCs suppress NK cells in HCC *via* cell-cell contact. Studies have suggested that MDSCs inhibit $CD8^+$ T cells through indirect pathways by producing inhibitory cytokines such as IL-10[53]. It has been shown that programmed death 1 (PD-1) is highly expressed in T cells that are infiltrating the hepatic tumor, whereas PD-1 Ligand (PD-L1) is overexpressed on tumor cells. IFN-γ secreted by $CD8^+$ T cells with increased PD-1 expression induces high levels of PD-L1 expression in cancer cells. This may lead to the exhaustion of TAA-specific $CD8^+$ T cells in the tumor through tumor cell immune escape. Increased PD-L1 expression in HCC cells is inversely related to HCC prognosis[54].

IMMUNE TOLERANCE AND THE LIVER

Besides being an immunological organ, the liver is also an "immune tolerant" organ. Approximately 1.5 L of blood per minute comes to the liver from both the circulatory systems. This blood contains pathogenic antigens as well as harmless substances such as dietary antigens, intestinal microbiota products, and autoantigens. This necessitates advanced "immune tolerance mechanisms" that prevent untoward immune responses in the liver. The first observations on the immunotolerant effect of the liver are that rejection did not develop in liver transplant patients despite allograft major MHC incompatibility, and also that combined transplant patients (transplantation of other organs together with liver from the same donor) accepted non-hepatic allografts more easily even without immunosuppression[55]. Therefore, considering the antigenic diversity to which the liver is exposed in its normal physiology, it is accepted that the liver is not an "immune reactive" but an "immune tolerogenic" organ [56]. Immunosuppressive agents, including calcineurin inhibitors (cyclosporine and tacrolimus) and corticosteroids, which target the activation, expansion, and cytotoxicity of the recipient's T lymphocytes, have led to advances in transplant surgeries since the 1970s, reducing the rate of acute rejection to less than 15%. However, the long-term use of immunosuppressants is associated with an increased risk of infection and malignancy. It has been observed that hepatic allografts can be accepted by MHC-incompatible individuals for a short period of time without immunosuppressant treatment. Cellular and humoral alloimmune responses contribute to the rejection. It is also important to know that liver transplantation itself can induce inflammatory pathways, such as hepatic ischemia-reperfusion injury. The liver microenvironment is permeated by waves of pro-inflammatory and anti-inflammatory

responses throughout life, and this regenerative profile, as well as the subtypes of secreted cytokines, is closely associated with the restoration of liver function and clinical outcomes after liver transplantation.

Immune cells in the liver have their own mechanisms that make the liver more immune tolerant than other organs. The key factor in ensuring immune tolerance is the anti-inflammatory effect of Treg cells ($CD4^+25^+$ T lymphocytes) on other lymphocytes. Although Tγδ lymphocytes have cytotoxic effects against bacteria and tumors, they also play a role in limiting hepatic inflammation and fibrosis by releasing anti-inflammatory cytokines. Unlike other tissues, antigen-presenting DCs in the liver exhibit an "immature" phenotype that expresses low levels of MHC and costimulatory molecules (CD40, CD80, and CD86). DCs also contribute to immune tolerance by secreting IL-10, which activates Th2 rather than Th1, and by enabling the formation of Treg cells. In response to inflammation, PD-L1 upregulation occurs in hepatocytes and HSCs; thus, inflammation is suppressed. It is interesting that "autoimmunity" can also be seen in the liver, an organ where such different immune-tolerance mechanisms are at the forefront[57,58].

AUTOIMMUNITY AND LIVER DISEASE

AILD is a group of diseases, including AIH, primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), and variant syndromes (AIH with PBC or PSC). Each AILD is heterogeneous in itself, and genetic and environmental factors play roles in the underlying pathogenesis. Although all of them affect the liver, the target cells for autoimmune damage, the pattern of inflammation, presenting clinical findings, and treatment options vary divergently within the AILD spectrum.

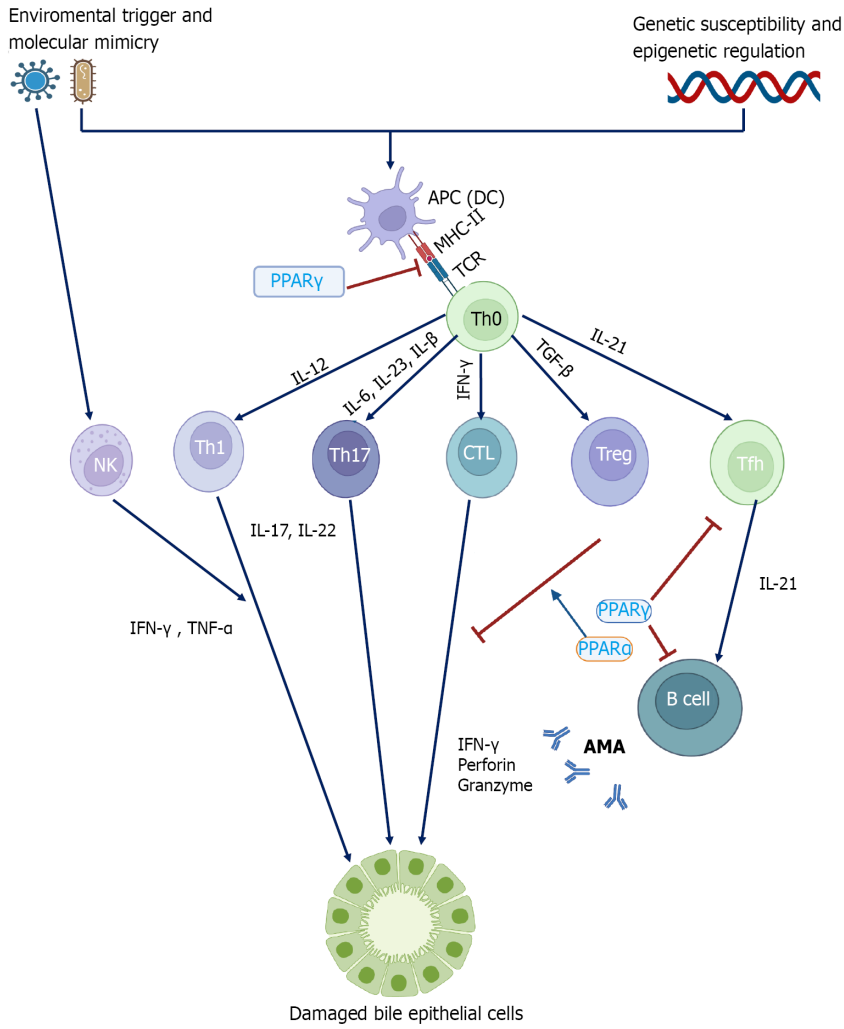
Primary biliary cholangitis

PBC is a typical organ-specific autoimmune disease, in which the biliary tract is the main target of destruction. Patients with PBC experience symptoms ranging from lymphocytic cholangitis associated with cholestasis and biliary fibrosis to progressive ductopenia. The presence of antimitochondrial antibodies (AMA) directed to pyruvate decarboxylase E2 (PDC-E2) is a diagnostic and serological feature of PBC. Anti-PDC-E2 antibodies primarily belong to the IgG3 subclass; however, IgM and IgA autoantibodies targeting this antigen may also be found. Anti-PDC-E2 antibodies have a potential pathogenic role, and immunohistochemical examinations of liver tissues from patients with PBC revealed predominantly CD4 and CD8 T cells of the bile ducts in the portal area[59]. The innate and adaptive immune cell elements and cytokines involved in the PBC pathology are shown in Figure 2.

Adaptive immunity and PBC: Infiltration of mononuclear cells around the small- or medium-sized bile ducts in the hepatic portal area is one of the characteristic histopathological features of PBC. These infiltrating lymphocytes are adjacent to the biliary epithelial cells in the damaged bile ducts. Loss of tolerance to PDC-E2 is the initiating event leading to clinical biliary pathology, and PDC-E2-specific $CD4^+$ and $CD8^+$ T cells are highly enriched in the PBC liver[60]. Among the T cells, $CD8^+$ T cells play a predominant role in the immunopathogenesis of PBC. In patients with PBC, $CD8^+$ T cells highly infiltrate the portal area. PDC-E2-specific $CD8^+$ T cells were detected in the peripheral blood at the early stages of PBC. In experimental models of PBC, liver lesions with extensive $CD8^+$ T-cell infiltration in the portal region, granuloma, and even fibrosis have been detected[61,62]. Different subsets of $CD4^+$ T cells are also involved in the pathogenesis of PBC. In liver samples from patients with PBC, infiltration of CD4 T cells, including PDC-E2-specific $CD4^+$ T cells, is evident during inflammation in the portal areas [63]. An increased number of $CD4^+$ T cells (Th17) have been observed in the portal tracts compared to the peripheral blood in PBC patients. The analysis showed that Th17 cells play a significant role in maintaining PBC immunopathology, which is mediated by Th1 cells at an early stage[64].

IL-12 and IL-23 are pleiotropic cytokines with proinflammatory effects that play an important role in various autoimmune diseases. Additionally, genome-wide association studies identified the important elements of the IL-12/Th1 signaling pathway, IL-12A, IL-12Rβ2, and STAT4, as susceptibility gene loci for PBC[65]. Although there was a low amount of Treg cells in the serum of patients, they were detected in lymphocyte aggregates located in the portal area. Studies have shown that Treg cells from patients with PBC significantly increase IFN-γ secretion in response to low-dose IL-12 stimulation. This effect was achieved by rapid and potent phosphorylation of STAT4 on Treg cells in these patients[66].

Innate immunity and PBC: The role of innate immunity in the immunopathogenesis of PBC has been supported by numerous studies, demonstrating the ability of cholangiocytes to express various TLRs, cellular activators of innate immunity, and other PPRs. Peroxisome proliferator-activated receptor γ (PPARγ) is constitutively expressed in biliary epithelial cells of small intrahepatic bile ducts. PPARγ appears to be downregulated in the bile ducts of PBC patients. PBC is characterized by the upregulation of TLR4 and TLR9 in cholangiocytes, and TLR3 and type I IFN-γ signaling pathways in the portal tracts [67]. Evidence suggests that IL-17-positive cells accumulate around the damaged bile ducts. Biliary epithelial cells can produce Th17-inducible cytokines, such as IL-6 and IL-1β, as a result of the innate immune response. These results suggest that periductal IL-17-secreting cells facilitate the migration of inflammatory cells around the bile ducts in PBC, which may worsen chronic cholangitis[68].



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Figure 2 Model of pathogenic mechanisms in primary biliary cholangitis. APC: Antigen presenting cell; DC: Dendritic cell; NK: Natural killer; IL: Interleukin; TNF: Tumor necrosis factor; TGF-β: Transforming growth factor β; IFN-γ: Interferon-gamma; PPAR: Peroxisome proliferator-activated receptor; AMA: Anti-mitochondrial antibody.

Autoimmune hepatitis

AIH is an autoimmune chronic inflammatory liver disease characterized by the presence of multiple autoantibodies, elevated serum aminotransferase levels, and excessive hepatic lymphoplasmacytic infiltration. However, the exact pathogenesis of AIH remains unclear. Although autoantibody positivity is a *sine qua non* of AIH, T cells rather than B cells are the major mediators of AIH immunopathogenesis. Current evidence suggests that T cells are immune regulators, and multiple autoantibodies are also important participants[69].

The frequency of infiltrating $CD4^+$ T cells is histopathologically higher than that of $CD8^+$ T cells in the early stages of AIH. Spontaneous apoptosis of $CD4^+$ T cells is markedly reduced in AIH[70]. The ratio of liver $CD8^+/CD4^+$ T cells (Tc/Th) increases with disease activity in patients with AIH. CXCR3 and CCR6 are highly expressed in $CD8^+$ T-cells. This shows that the ligands CXCL9 and CCL20 are highly expressed in the inflamed liver, thus facilitating the uptake of $CD8^+$ T cells into the liver[71]. Emperipolesis is defined as the presence of an intact, viable cell (lymphocyte) within the cytoplasm of another cell (hepatocyte), and is one of the histopathological and diagnostic features of AIH. Emperipolesis is predominantly mediated by $CD8^+$ T cells and is correlated with severe necroinflammation and fibrosis [71].

Different subsets of $CD4^+$ T (Th) cells, particularly Treg cells, have been found to exert remarkable effects in AIH. Treg cells in patients with AIH suppress autoimmunity by direct contact with $CD4^+CD25^-$ T cells and secretion of regulatory cytokines, such as IL-4, IL-10, and TGF-β[72]. Treg cells mediate immune suppression through the expression of CD39 and CD73. Treg cells in AIH exhibit reduced NTPDase-1 activity as well as a reduced ability to inhibit IL-17 secretion from Th17 cells in AIH, which contributes to autoimmunity. Circulating and intrahepatic IL-17 Levels were significantly higher in AIH patients than in healthy controls. Hepatic expression of IL-17 is associated with inflammation and

fibrosis in the liver[73]. Studies have shown that the interaction between Gal-9 on Treg cells and Tim-3 on Th cells may be an important mechanism for direct contact suppression mediated by Treg cells. Although some studies have reported a decrease in the number of Treg cells in AIH, others have shown that Treg cells do not decrease in AIH[74,75]. These results suggest that the role of Treg cells in AIH immunopathology remains controversial.

In addition to Treg cells, Thf cells are associated with adaptive cell immunity in AIH. CD8 T cells have been shown to be activated by IL-21, secreted by Tfh cells. Tfh cells are widely recognized as a subset of $CD4^+$ T cells that aid in B-cell development[76]. The number of T γδ cells was increased in patients with AIH. T γδ cells secrete higher levels of IFN-γ and granzyme B than healthy controls, which may contribute to autoimmune damage in AIH patients.

Studies have shown that B cells inhibit $CD4^+$ T cells in animal models of AIH. Its suppressive function is dependent on the expression of CD11b in B cells. IL-10 is mainly secreted by $CD4^+$ T cells and increases CD11b expression. This means that $CD4^+$ T cells and B cells can regulate each other in AIH [77]. The possible immune cells and mediator cytokines involved in the autoimmune hepatitis pathogenic pathway are shown in Figure 3.

AUTOIMMUNITY AND IEI

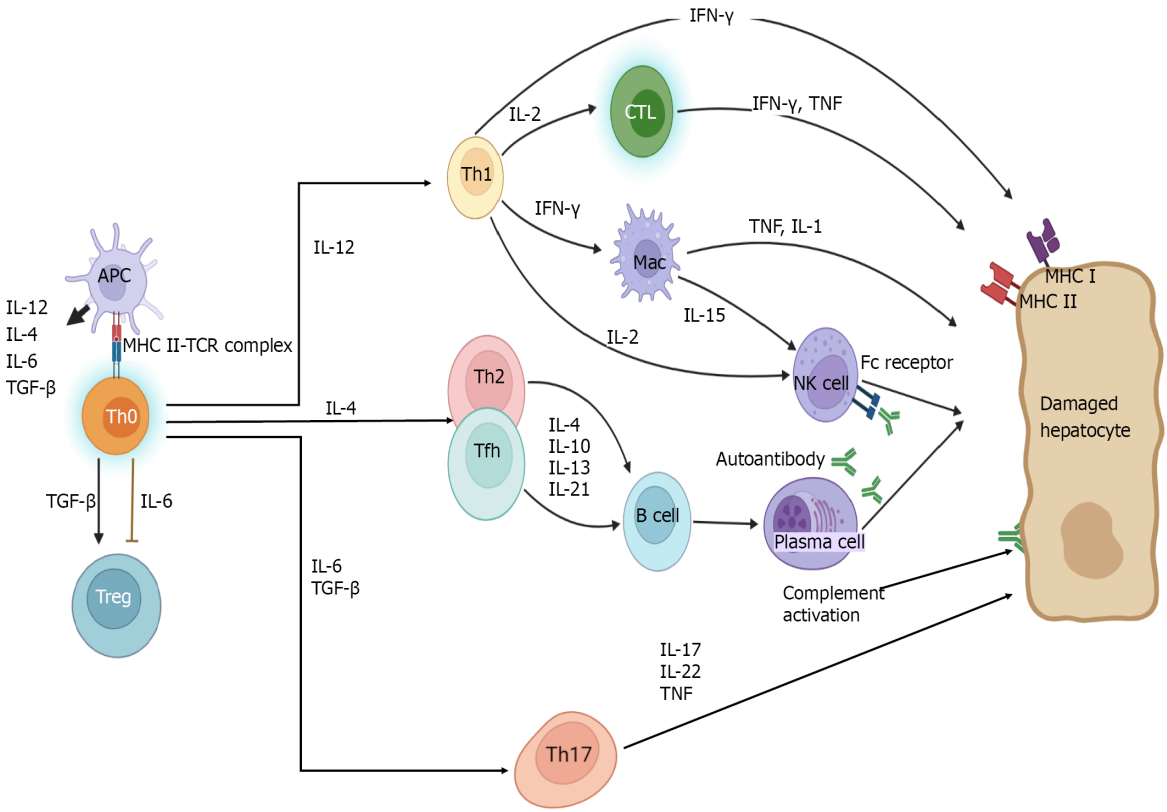
With a simplistic approach, autoimmunity and IEI can be thought of as "over" and "insufficient" functioning of the immune system, respectively. In other words, autoimmunity and IEI might be accepted as opposites in the spectrum of immune system functioning. However, with the accumulation of knowledge and experience in both disease groups, this simple distinction disappeared, and it was revealed that the immune system was "dysregulated" in both groups.

The coexistence of autoimmunity and IEI is a well-known entity[78]. An analysis conducted in France showed that 26.2% of patients with IEI had one or more autoimmune or autoinflammatory symptoms during their lifetime[79]. In a two-center prevalence study in Turkey including 1435 patients with IEI, autoimmunity was reported at a rate of 2.2%[80], although antibody deficiencies take the first place among immunodeficiencies. According to this study, the most common type of immunodeficiency associated with autoimmune diseases is CVID, and the most common accompanying autoimmune diseases include vasculitis, autoimmune hemolytic anemia, and autoimmune thrombocytopenia. In a national data-based study conducted in France, Fischer *et al*[79] found that autoimmunity is mostly associated with T cell-related diseases and CVID. The cumulative incidence graph of lifelong autoimmune development in patients with IEI increased almost linearly after 8-10 years of age, and 40% of patients developed autoimmune disease by the age of 50 years. The most common accompanying autoimmune diseases were cytopenia and gastrointestinal, skin, rheumatological, and endocrine diseases. Therefore, it is important for all physicians dealing with autoimmune diseases or immunodeficiencies to keep in mind that various autoimmune diseases can accompany almost all types of IEI syndrome, either as the first finding or during their course.

Pathophysiology of autoimmunity developing on the background of IEI

It is thought that there are common genetic and pathophysiological mechanisms for IEI and autoimmune diseases based on the frequent occurrence of their association and the increased incidence of autoimmunity in the families of individuals with IEI. The leading cause of autoimmunity in IEI is loss of immune tolerance. In Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) and DiGeorge syndrome, T cell development and function are impaired, resulting in "loss of central tolerance", and the development of autoreactive T cells triggers autoimmunity[81]. The "peripheral tolerance loss" is lost in patients with Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome, hyper immunoglobulin-M (HIGM) syndrome, and CVID, and autoreactive B cells play a role in the emergence of autoimmunity in these patients[82]. Autoimmunity can also occur with a disorder in signaling pathways in the immune system, and one of the best examples is Wiskott-Aldrich Syndrome (WAS). Loss of the WAS protein, a regulatory protein that plays a key role in signaling from TCR to the cytoskeleton in WAS, results in impaired number and function of Treg lymphocytes, which triggers autoimmunity[83]. Autoimmunity may develop as a result of the failure of autoreactive lymphocytes to be cleared by apoptosis in autoimmune lymphoproliferative syndrome (ALPS) and some combined immunodeficiencies[84]. Autoimmunity develops in partial IgA (PIgA) deficiency and complement disorders due to impaired antigen clearance and increased exposure to antigens[85]. X-linked chronic granulomatous disease causes an abnormal immune response against cellular wastes, and this is blamed for the pathogenesis of SLE developing in one-third of female carriers of this disease[86].

Autoimmunity should also be considered as a warning sign in terms of the IEI. On the one hand, the hypogammaglobulinemic state and cellular deficiency affect the results of serology tests and biopsies, creating diagnostic difficulties for autoimmune diseases in patients with IEI. Therefore, the interpretation of diagnostic tests in these patients should be done very carefully, and even weak autoantibody positivity, which is normally ignored, should be taken into account.



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Figure 3 Pathogenic pathways of autoimmune hepatitis. APC: Antigen-presenting cell; CTL: Cytotoxic T lymphocyte; Mac: Macrophage; IL: Interleukin; TNF: Tumor necrosis factor; MHC: Major histocompatibility complex; TGF-β: Transforming growth factor β; IFN-γ: Interferon-gamma.

Association of AILD and IEI

Although AILD can be seen together in IEI, it is difficult to state its prevalence due to the rarity of both groups of diseases. The most well-known type of IEI that accompanies AILD is CVID, which can accompany all AILD types[87]. PBC was detected in three of 248 patients with CVID followed at a center in New York between 1973 and 1986; one of the patients died due to liver-related causes and the other two died due to non-hepatic causes. In the same study, although no definitive diagnosis was made, three patients were considered to have AIH, and all of them died due to liver failure[88]. PIgA deficiency is accompanied by AIH in the range of 0.79% to 5.00%[89]. In a series of 52 pediatric patients with AIH, a PIgA deficiency rate of 2.31 % was detected. In this series, the frequency of PIgA deficiency was significantly higher in patients positive for LKM-1 autoantibodies (45%) than in patients positive for ANA and SMA (9%)[90].

APECED syndrome is an IEI characterized by the predominance of autoimmunity, and AIH can occur in up to 43% of cases[91]. In MHC II disorders, autoimmunity may develop against hepatocytes and cholangiocytes in the liver[92]. There is a case report of an association between mucocutaneous candidiasis and AIH in a child with a STAT-1 gain-of-function mutation[93]. In a case series of 274 individuals with a STAT-1 gain-of-function mutation, AIH was reported in six (2%) patients[94]. A high titer positivity for AMA autoantibodies, indicating a predisposition to the development of PBC, has been reported in a case of IPEX syndrome[95]. In a series of 11 patients with hyperimmunoglobulin M syndrome, PSC developed in five (45%) patients. Since *Cryptosporidium parvum* was detected in the stool of four of them, it was thought to play a role in the pathogenesis of PSC[96]. In a series of 90 patients with ALPS, seronegative AIH was detected in three (3.3%) patients (83). A case report of a five-year-old boy with IL-2 receptor alpha (CD25) deficiency provided important information about the pathogenesis of AILD in IEI[97]. He was diagnosed with PBC, a disease that is not normally expected to be observed in this age and sex. It was shown that he had an increase in autoreactive T cells due to a decrease in $CD4^+CD25^+$ Treg cells. After allogeneic bone marrow transplantation, AMA/PDC-E2 positivity disappeared, and PBC findings improved, along with improved T cell composition.

CONCLUSION

The liver has a unique anatomical design to protect the host from potential pathogens passing from the

intestine to the portal circulation, while maintaining a general state of immune hyposensitivity. The liver is the main organ of the innate and adaptive immune systems. As the mechanisms of antigen capture, presentation, and recognition in the liver will be understood, the biological mechanisms of immune tolerance in the liver will become clearer. The balance between immune tolerance and effective immune screening is maintained by interactions between numerous immune cells that are present in and recruited into the liver. This is necessary for normal functioning of the liver. If an inappropriate immune response disturbs this delicate balance, autoimmune liver pathologies can develop. In addition, failure to initiate an effective immune response results in chronic viral infections or failure to clear cancer cells. This function of the liver in maintaining immune responses and tolerance demonstrates the importance of the liver as a vital immune organ.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Clinical characteristics and outcomes of COVID-19 in patients with autoimmune hepatitis: A population-based matched cohort study

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Abstract

BACKGROUND

Patients with autoimmune hepatitis (AIH) require life-long immunosuppressive agents that may increase the risk of poor coronavirus disease 2019 (COVID-19) outcomes. There is a paucity of large data at the population level to assess whether patients with AIH have an increased risk of severe diseases.

AIM

To evaluate the impact of pre-existing AIH on the clinical outcomes of patients with COVID-19.

METHODS

We conducted a population-based, multicenter, propensity score-matched cohort study with consecutive adult patients (≥ 18 years) diagnosed with COVID-19 using the TriNeTx research network platform. The outcomes of patients with AIH (main group) were compared to a propensity score-matched cohort of patients: (1) Without chronic liver disease (CLD); and (2) Patients with CLD except AIH (non-AIH CLD) control groups. Each patient in the main group was matched to a pa-

tient in the control group using 1:1 propensity score matching to reduce confounding effects. The primary outcome was all-cause mortality, and secondary outcomes were hospitalization rate, need for critical care, severe disease, mechanical ventilation, and acute kidney injury (AKI). For each outcome, the risk ratio (RR) and confidence intervals (CI) were calculated to compare the association of AIH with the outcome.

RESULTS

We identified 375 patients with AIH, 1647915 patients with non-CLD, and 15790 patients with non-AIH CLD with COVID-19 infection. Compared to non-CLD patients, the AIH cohort had an increased risk of all-cause mortality (RR = 2.22; 95%CI: 1.07-4.61), hospitalization rate (RR = 1.78; 95%CI: 1.17-2.69), and severe disease (RR = 1.98; 95%CI: 1.19-3.26). The AIH cohort had a lower risk of hospitalization rate (RR = 0.72; 95%CI: 0.56-0.92), critical care (RR = 0.50; 95%CI: 0.32-0.79), and AKI (RR = 0.56; 95%CI: 0.35-0.88) compared to the non-AIH CLD patients.

CONCLUSION

Patients with AIH are associated with increased hospitalization risk, severe disease, and all-cause mortality compared to patients without pre-existing CLD from the diagnosis of COVID-19. However, patients with AIH were not at risk for worse outcomes with COVID-19 than other causes of CLD.

Key Words: Autoimmune hepatitis; SARS-CoV-2; COVID-19; Mortality; Outcomes; Liver disease; Severe

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Core Tip: Autoimmune hepatitis (AIH) is a chronic inflammatory disease of the liver of unknown etiology. Patients with AIH may be at increased risk of severe illness from coronavirus disease 2019 (COVID-19) and have poor outcomes due to underlying chronic liver disease (CLD) and ongoing pre-existing immunosuppression therapies. Patients with AIH are associated with increased hospitalization risk, severe disease, and all-cause mortality compared to patients without pre-existing CLDs from the diagnosis of COVID-19. Patients with AIH had a lower risk of several outcomes, including hospitalization, a necessity for critical care, and acute kidney injury, compared to patients with pre-existing CLDs other than AIH.

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INTRODUCTION

As coronavirus disease 2019 (COVID-19) cases increase in the United States and globally, investigators continue to identify risk factors for adverse outcomes resulting from COVID-19 infection. The known risk factors for severe disease include older age, male gender, and comorbidities such as hypertension, diabetes, chronic obstructive pulmonary disease, and chronic liver diseases (CLDs)[1-3]. In addition, studies have shown that COVID-19 affects the liver, with over one-third of hospitalized COVID-19 patients presenting with abnormal liver function, which was also associated with a longer hospital stay [4,5]. In contrast, a higher risk of mortality and hospitalization rates have been reported in COVID-19 patients with pre-existing liver disease compared to those without liver disease[6].

Autoimmune hepatitis (AIH) is a genetically predisposed CLD. The exact mechanism regarding its immune dysfunction has yet to be elucidated; however, an imbalance between effector and regulatory immunity and molecular mimicry may play a role in its pathogenesis[7]. The prevalence rate of AIH in the United States is estimated to be 31.2/100000, which is similar to the prevalence rates reported in Europe[8]. The association between severe acute respiratory disease coronavirus 2 (SARS-CoV-2) and autoimmune diseases is very complex and only partially understood. In addition, understanding the outcomes of COVID-19 infections in AIH patients is particularly important due to the fact that patients with AIH require lifelong immunosuppressive agents to prevent cirrhosis and end-stage liver disease, which may increase the risk of viral and bacterial infections[9,10]. The clinical impact of the pre-existing use of immunosuppression in patients with COVID-19 remains complex and not clearly defined. Evidence is mixed regarding the impact of immunosuppressive agents on COVID-19 outcomes. Hence,

existing data are controversial on the outcome following COVID-19 infection in patients with autoimmune diseases. Some studies have shown that long-term steroid use in the management of autoimmune conditions prior to COVID-19 diagnosis is associated with adverse outcomes such as hospitalization, intensive care unit (ICU) admission, and mortality[11,12]. At the same time, other results did not support an increased risk of severe COVID-19[13,14]. Thus, study results are inconsistent, with a high degree of heterogeneity. Therefore, a detailed understanding of the clinical course of COVID-19 in patients with AIH is necessary.

On the other hand, increasing vaccine uptake and other public health safety measures have helped reduce the pandemic's burden. However, considerable morbidity and mortality continue to accrue among non-immune and unvaccinated individuals. Furthermore, previous AIH literature has used small samples of patients with AIH. To address these research gaps, we performed a large population-based retrospective cohort study using data from the multicenter research network. Our analysis focused on evaluating AIH as an independent risk factor associated with severe diseases of SARS-CoV-2 and all-cause mortality.

MATERIALS AND METHODS

Study design

This population-based, multicenter, retrospective cohort study was conducted using TriNetX (Cambridge, MA, United States), a federated health research network data set. TriNetX is a multi-institutional health research network that provides de-identified electronic medical records systems (EHRs) from the included healthcare organizations. Clinical variables (referred to as "facts" on the network) are derived directly through EHRs. Robust quality assurance on the network is achieved at the time of extraction before inclusion. The platform only provides aggregate patient counts and statistical summaries to ensure de-identification at all levels of retrieval and dissemination of patient data. TriNetX received a waiver from the Western institutional review board as a federated network since only aggregated counts and statistical summaries of de-identified information are included. No protected health information was obtained, and no study-specific activities were performed in the retrospective analyses. Details of the data source and quality checks are described in the supplementary data.

Study participants

All adult patients (age ≥ 18 years) with AIH and confirmed COVID-19 infection between January 20, 2020, and November 30, 2021, were included. The search criteria for potential patients with COVID-19 were based on specific COVID-19 diagnosis codes or positive laboratory confirmation of COVID-19.

To determine the clinical impact of AIH on clinical outcomes of COVID-19, we compared AIH patients to a control group of patients without any pre-existing CLD, including AIH (non-CLD) and COVID-19. To assess the impact of AIH compared to other liver diseases, we compared AIH patients to a control group of patients with other pre-existing CLD (non-AIH CLD) and COVID-19. Details of the search criteria and diagnosis codes used for patient selection are described in the supplementary data.

Matching process

Each patient in the main group was matched to a patient in the control group using 1:1 propensity score matching (PSM) to reduce confounding effects[15]. Covariates in the propensity score model were adjusted for a priori-identified potential confounders: Age, sex, race/ethnicity (Hispanic, non-Hispanic white, non-Hispanic black, or non-Hispanic other), body mass index (BMI), nicotine dependence, and comorbidities that are listed in Table 1. Logistic regression on these input matrices was used to obtain propensity scores for each patient in both cohorts. Logistic regression was performed in Python 3.6.5 (Python Software Foundation) using standard libraries NumPy and Sklearn. The same analyses were also performed in R 3.4.4 software (R Foundation for Statistical Computing, Vienna, Austria) to ensure outputs match. After calculating propensity scores, matching was performed using a greedy nearest-neighbor matching algorithm with a caliper of 0.1 pooled standard deviations. The order of the rows in the covariate matrix can affect the nearest neighbor matching; therefore, the order of the rows in the matrix was randomized to eliminate this bias.

Study outcomes

The primary study outcome was all-cause mortality from index events within 60 d. The index event was defined as either the time of COVID-19 diagnosis or the first COVID-19 positive test result date, whichever occurred first. Secondary outcomes were hospitalization, severe diseases, acute kidney injury (AKI), and intensive care (requiring extracorporeal membrane oxygenation or mechanical ventilation) in the 30 d from COVID-19 diagnosis. Severe disease was operationalized and defined as a composite outcome requiring intensive care or death within 30 d of COVID-19 diagnosis.

Table 1 Baseline characteristics of patients with autoimmune hepatitis and non-chronic liver diseases patients with a positive test for severe acute respiratory syndrome coronavirus-2

Variables	Before propensity score matching			After propensity score matching		
	AIH (*n* = 375)	Non-CLD (*n* = 1647915)	*P* value	AIH (*n* = 375)	Non-CLD (*n* = 375)	*P* value
Age, yr, mean ± SD	53.1 ± 18.4	46.4 ± 18.8	< 0.01	53.1 ± 8.4	53.2 ± 8.2	0.97
Sex, *n* (%)						
Female	271 (72.2)	893768 (54.2)	< 0.01	271 (72.2)	256 (68.2)	0.23
Ethnicity, *n* (%)						
Hispanic or Latino	37 (9.8)	144535 (8.7)	0.45	37 (9.8)	36 (9.6)	0.90
Race, *n* (%)						
White	238 (63.4)	1028824 (62.4)	0.68	238 (63.4)	228 (60.8)	0.45
Black or African American	64 (17.1)	245820 (14.9)	0.24	64 (17.1)	69 (18.4)	0.63
Other	65 (17.3)	325638 (19.7)	0.24	65 (17.3)	69 (18.4)	0.70
Nicotine dependence, *n* (%)	76 (20.2)	181270 (10.9)	< 0.01	72 (19.2)	74 (19.7)	0.67
BMI (kg/m^2), mean ± SD	28.9 ± 7.05	29.9 ± 7.45	0.07	28.9 ± 7.05	30.8 ± 7.65	0.01
Comorbidities, *n* (%)						
Hypertension	163 (43.4)	384968 (23.3)	< 0.01	163 (43.4)	159 (42.4)	0.77
Ischemic heart diseases	59 (15.7)	119162 (7.2)	< 0.01	59 (15.7)	57 (15.2)	0.84
Heart failure	38 (10.1)	65245 (3.9)	< 0.01	38 (10.1)	37 (9.8)	0.90
Diabetes	74 (19.7)	171727 (10.4)	< 0.01	74 (19.7)	68 (18.1)	0.58
Chronic lower respiratory diseases	82 (21.8)	235406 (14.2)	< 0.01	82 (21.8)	81 (21.6)	0.93
Cerebrovascular diseases	37 (9.8)	73209 (4.4)	< 0.01	37 (9.8)	39 (10.4)	0.81
CKD of any stage	46 (12.2)	78265 (4.7)	< 0.01	46 (12.2)	37 (9.8)	0.29
Neoplasms	125 (33.3)	293362 (17.8)	< 0.01	125 (33.3)	132 (35.2)	0.59

AIH: Autoimmune hepatitis; CLD: Chronic liver diseases; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; SD: Standard deviation; BMI: Body mass index; CKD: Chronic kidney disease.

Statistical analysis

All statistical analyses were performed in real-time using the TriNetX platform. Continuous variables are expressed as means ± SD. Categorical variables were defined as frequency and percentage. For each outcome, the risk ratio (RR) and confidence intervals (CI) were calculated to compare the association of the AIH with the outcome. Numbers were then validated by comparing them with the output from SAS version 9.4. A-priori-defined two-sided alpha of less than ≤ 0.05 was used for statistical significance, and all statistical data analyses were performed utilizing the form of the limitation in real-time.

RESULTS

Baseline characteristics

We identified 15790 non-AIH CLD and 1647915 non-CLD patients during the study period (Figure 1). Major etiologies of non-AIH CLD included alcoholic liver disease (*n* = 4159, 26.3%), NAFLD (*n* = 3085, 19.5%), viral hepatitis (*n* = 1093, 6.9%), and other diseases of the liver (*n* = 3456, 21.8%) (Supplementary Table 1). Baseline characteristics of non-CLD patients are described in Table 1. 893768 (54.2%) were female, and 144535 (8.8%) were Hispanic or Latino. 181270 (11%) reported nicotine dependence. Rates of several common comorbidities were significantly lower in the non-CLD group compared to the AIH cohort, including hypertension (*n* = 384968, 23.3%), neoplasms (*n* = 293362, 17.8%), and chronic lower respiratory diseases (*n* = 235406, 14.2%).

Baseline characteristics of the non-AIH CLD patients are described in Table 2. 6517 (41.2%) patients were female, and 1831 (11.5%) patients had a history of nicotine dependence. Compared to the AIH cohort, non-AIH CLD patients had higher rates of comorbidities, including hypertension (*n* = 11469,

Table 2 Baseline characteristics of patients with autoimmune hepatitis and non-autoimmune hepatitis chronic liver diseases patients with a positive test for severe acute respiratory syndrome coronavirus-2

Variables	Before propensity score matching			After propensity score matching		
	AIH (*n* = 375)	Non-AIH CLD (*n* = 15790)	*P* value	AIH (*n* = 363)	Non-AIH CLD (*n* = 363)	*P* value
Age, yr, mean ± SD	53.1 ± 18.5	60.3 ± 12.1	< 0.01	53.9 ± 18.1	54.3 ± 15.5	0.76
Sex, *n* (%)						
Female	267 (71.2)	6517 (41.2)	< 0.01	262 (72.1)	266 (73.2)	0.74
Ethnicity, *n* (%)						
Hispanic or Latino	82 (21.8)	1579 (10)	< 0.01	82 (22.5)	67 (18.4)	0.19
Race, *n* (%)						
White	236 (62.9)	11132 (70.5)	0.02	235 (64.7)	218 (60.1)	0.19
Black or African American	65 (17.3)	2462 (15.5)	0.41	58 (15.9)	72 (19.8)	0.17
Other	66 (17.6)	1824 (11.5)	0.04	62 (17.1)	61 (16.8)	0.92
Nicotine dependence, *n* (%)	87 (23.2)	1831 (11.5)	< 0.01	89 (24.5)	87 (23.9)	0.94
BMI (kg/m^2), mean ± SD	28.9 ± 7.1	30.4 ± 7.48	0.09	29 ± 7.11	28.7 ± 7.79	0.72
Comorbidities, *n* (%)						
Hypertension	165 (44)	11469 (72.6)	< 0.01	161 (44.3)	200 (55.1)	0.03
Ischemic heart diseases	62 (16.5)	5472 (34.6)	< 0.01	59 (16.2)	56 (15.4)	0.76
Heart failure	39 (10.4)	4196 (26.5)	< 0.01	38 (10.4)	48 (13.2)	0.25
Diabetes	74 (19.7)	7882 (49.9)	0.01	74 (20.4)	66 (18.1)	0.45
Chronic lower respiratory diseases	84 (22.4)	5843 (37.0)	< 0.01	82 (22.5)	79 (21.7)	0.79
Cerebrovascular diseases	37 (9.8)	3079 (19.4)	< 0.01	37 (10.1)	39 (10.7)	0.81
CKD of any stage	49 (13.1)	5102 (32.3)	< 0.01	46 (12.6)	55 (15.1)	0.33
Neoplasms	131 (34.9)	8400 (53.1)	< 0.01	124 (34.1)	131 (36.1)	0.59

AIH: Autoimmune hepatitis; CLD: Chronic liver diseases; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; SD: Standard deviation; BMI: Body mass index; CKD: Chronic kidney disease.

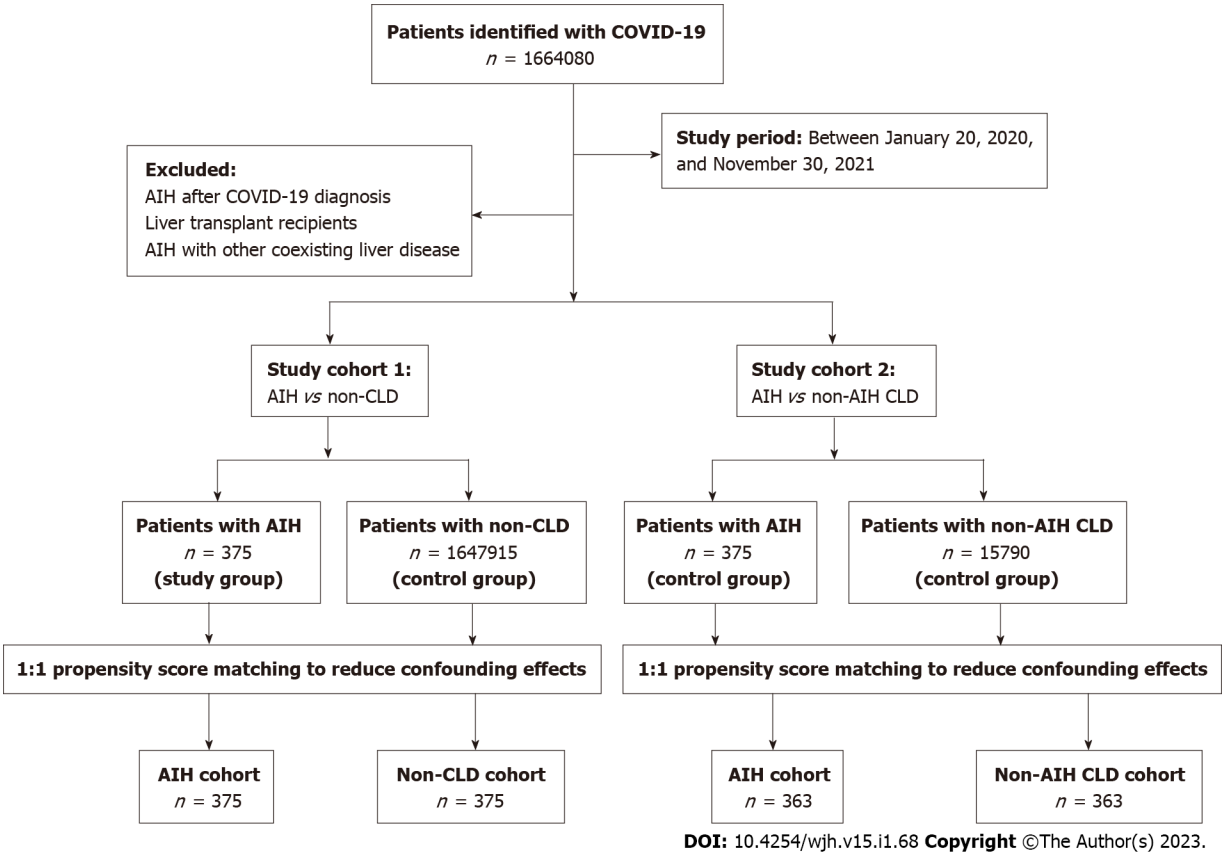
72.6%), neoplasms (*n* = 8400, 53.1%), diabetes (*n* = 7882, 49.9%), and chronic lower respiratory diseases (*n* = 5843, 37.0%).

AIH characteristics

During the same study period, 375 patients with AIH were identified. Baseline characteristics are described in Tables 1 and 2. A majority (*n* = 271, 72.2%) of the patients were female, and 76 (20.2%) patients had a history of nicotine dependence. Common comorbidities were hypertension (*n* = 163, 43.4%), neoplasms (*n* = 125, 33.3%), chronic lower respiratory diseases (*n* = 82, 21.8%), and diabetes (*n* = 74, 19.7%). Coexistence of other immune-mediated disorders in the AIH cohort occurred most often with systemic lupus erythematosus (*n* = 61, 16.2%), rheumatoid arthritis (*n* = 57, 15.2%), Sjögren syndrome (*n* = 45, 12.0%), and ulcerative colitis (*n* = 27, 7.2%) (Supplementary Table 2). The most common immunosuppressive agents used in the AIH cohort were prednisone (*n* = 313, 83.4%), azathioprine (*n* = 170, 45.3%), and budesonide (*n* = 80, 21.3%) (Supplementary Table 2).

Clinical characteristics

Patients with AIH compared to non-CLD: Results of laboratory vitals, symptoms, and laboratory findings between AIH and non-CLD cohorts are presented in Supplementary Table 3. All liver function tests were significantly different between the AIH and non-CLD patients ($P < 0.01$). After a propensity score-matched analysis, all liver function results remained significantly different between the cohorts: The mean alanine aminotransferase (ALT) (65.3 *vs* 23.6 U/L; $P < 0.01$), aspartate aminotransferase (54.9 *vs* 23.7 U/L; $P = 0.01$), total bilirubin (0.86 *vs* 0.53 mg/dL; $P = 0.01$), and alkaline phosphatase (ALP) (105.0 *vs* 88.3 U/L; $P = 0.01$), and serum albumin (3.88 *vs* 4.04 g/dL; $P = 0.04$) (Supplementary Table 3).



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Figure 1 Flow chart showing patient selection for study cohorts. COVID-19: Coronavirus disease-2019; AIH: Autoimmune hepatitis; CLD: Chronic liver disease.

Compared to non-CLD patients, AIH patients had higher ferritin levels (336 *vs* 192 ng/mL; $P = 0.01$) and lower fibrinogen levels (303 *vs* 389 mg/dL; $P = 0.04$). After propensity matching, the higher ferritin level (336 *vs* 118 ng/mL; $P = 0.01$) and lower fibrinogen level (303 *vs* 436 mg/dL; $P = 0.03$) remained. All other inflammatory markers were measured with no significant differences (Supplementary Table 3).

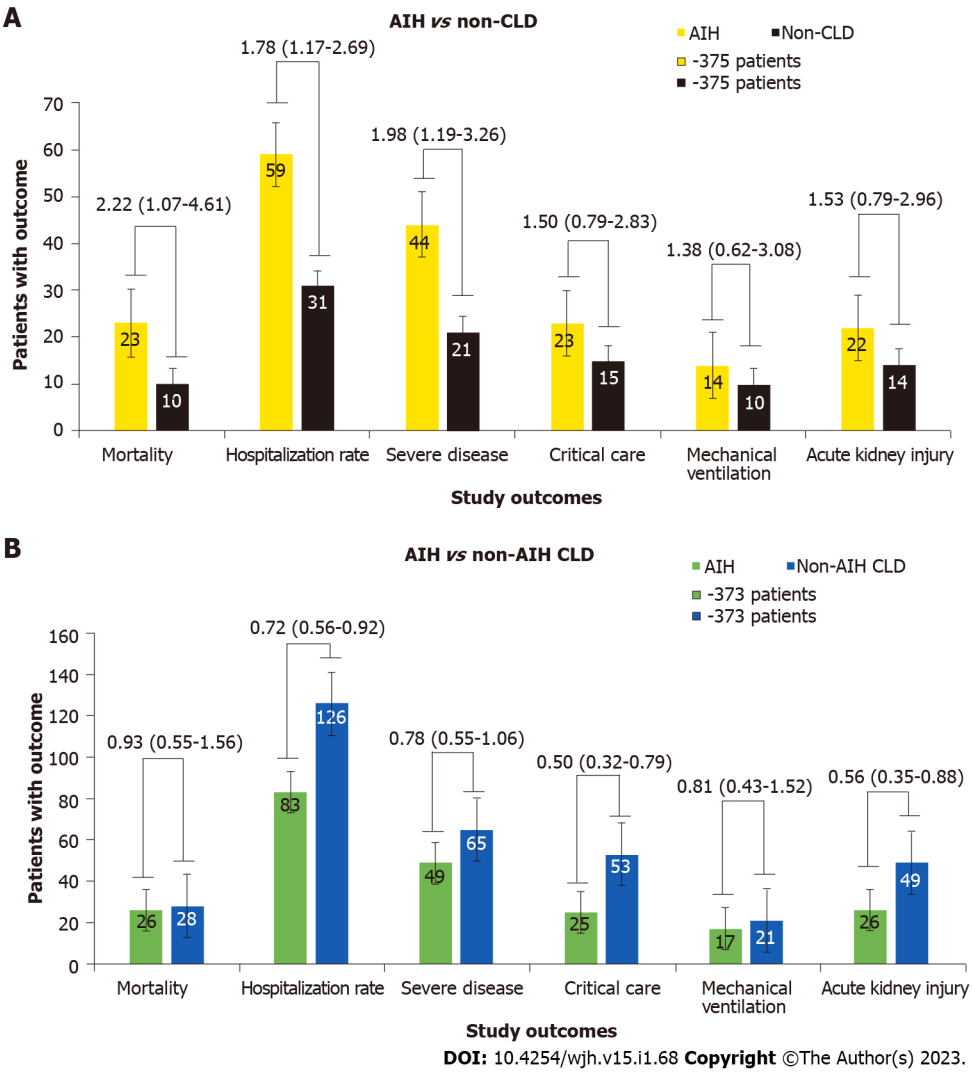
Patients with AIH compared to non-AIH CLD: When compared to non-AIH CLD patients, AIH patients had higher ALT levels (65.6 *vs* 38.3 U/L; $P < 0.01$), lower total bilirubin (0.87 *vs* 1.58 mg/dL; $P = 0.01$), lower ALP (106 *vs* 129 U/L; $P = 0.04$), and higher serum albumin (3.87 *vs* 3.61 g/dL; $P < 0.01$). After PSM, the differences in total bilirubin (0.87 *vs* 2.09 mg/dL; $P < 0.01$), ALP (105 *vs* 133 U/L; $P = 0.04$), and serum albumin (3.87 *vs* 3.71 g/dL; $P = 0.01$) remained significant. Before PSM, AIH patients had higher C-reactive protein levels (15.4 *vs* 28.4 mg/L; $P = 0.01$) and lower erythrocyte sedimentation rate (24.3 *vs* 34.0 mm/h; $P < 0.01$). After PSM, the AIH group had higher fibrinogen levels (307 *vs* 234 mg/dL; $P = 0.01$) (Supplementary Table 4).

Outcomes

Patients with AIH compared to non-CLD: Before PSM, there were significant differences between the AIH and non-CLD cohorts in the rates of all hospitalization-related outcomes. AIH patients had a significantly higher risk of all-cause mortality (RR = 2.33; 95%CI: 1.66-3.28), hospitalization rate (RR = 2.14; 95%CI: 1.75-2.60), critical care (RR = 1.94; 95%CI: 1.35-2.79), severe disease (RR = 2.01; 95%CI: 1.55-2.59), need for mechanical ventilation (RR = 1.99; 95%CI: 1.21-3.27), and AKI (RR = 1.94; 95%CI: 1.38-2.72).

After PSM, the increased risk of all-cause mortality (RR = 2.22; 95%CI: 1.07-4.61), hospitalization rate (RR = 1.78; 95%CI: 1.17-2.69), and severe disease (RR = 1.98; 95%CI: 1.19-3.26), persisted in the AIH group. However, there were no significant differences in the rates of critical care (RR = 1.50; 95%CI: 0.79-2.83), need for mechanical ventilation (RR = 1.38; 95%CI: 0.62-3.08), and AKI (RR = 1.53; 95%CI: 0.79-2.96) (Figure 2A).

Patients with AIH compared to non-AIH CLD: When compared to the non-AIH CLD cohort, the AIH group had a lower risk of all-cause mortality (RR = 0.49; 95%CI: 0.34-0.68), critical care (RR = 0.43; 95%CI: 0.30-0.62), severe disease (RR = 0.57; 95%CI: 0.45-0.74), need for mechanical ventilation (RR = 0.41; 95%CI: 0.24-0.69), and AKI (RR = 0.38; 95%CI: 0.27-0.53), There were no significant differences between the groups in hospitalization rate (RR = 0.93; 95%CI: 0.78-1.10).



DOI: 10.4254/wjh.v15.i1.68 **Copyright** ©The Author(s) 2023.

Figure 2 Risk of all-cause mortality, rates of hospitalization, severe diseases, need for critical care, need for mechanical ventilation, and acute kidney injury, at 30 d from coronavirus disease 2019 diagnosis. A: Between patients with autoimmune hepatitis and patients without any pre-existing liver diseases; B: Between patients with autoimmune hepatitis and patients with pre-existing liver diseases other than autoimmune hepatitis. The numbers inside the bars represent the number of patients who developed outcomes related to the study. AIH: Autoimmune hepatitis; CLD: Chronic liver disease.

After PSM, lower risk persisted for hospitalization rate (RR = 0.72; 95%CI: 0.56-0.92), critical care (RR = 0.50; 95%CI: 0.32-0.79), and AKI (RR = 0.56; 95%CI: 0.35-0.88) among patients with AIH. However, there were no significant differences in all-cause mortality (RR = 0.93; 95%CI: 0.55-1.56), severe disease (RR = 0.78; 95%CI: 0.55-1.06), and need for mechanical ventilation (RR = 0.81; 95%CI: 0.43-1.52) between these groups (Figure 2B).

DISCUSSION

Although the number of COVID-19-related cases, rates of hospitalizations, and deaths are decreasing in the United States, the COVID-19 pandemic is still ongoing worldwide, and significant questions remain. Our data showed that the patients with AIH had a higher risk of hospitalization, severe COVID-19, and all-cause mortality than those without liver disease. Notably, a lower survival probability was also noted for AIH patients. On the other hand, compared to non-AIH CLD, AIH patients had a lower risk of hospitalization, critical care, and AKI without any difference in survival probability between the groups.

The present study is the largest United States-based study investigating severe COVID-19 in AIH patients. Our results showed an increased all-cause mortality risk in AIH patients compared to non-CLD, but no difference in all-cause mortality risk compared to non-AIH CLD. Marjot *et al*[16] demonstrated a similar significant difference in mortality risk between AIH and non-CLD patients; however, their findings showed a similar mortality risk between AIH and non-CLD patients. Efe *et al*[17] compared rates of adverse outcomes of COVID-19 between AIH patients and non-AIH CLD. The AIH cohort included 34 United States patients and 110 total AIH patients[14]. No differences were

found in the risk of severe outcomes[17]. Although outcomes such as mortality, severe COVID-19, need for supplemental oxygen, and hospitalization was addressed in the study[14], the need for intensive care or mechanical ventilation was not. Marjot *et al*[16] also conducted an international retrospective study comparing 70 AIH to non-AIH CLD and non-CLD patients. There was an increased hospitalization risk for AIH patients compared to non-CLD patients[12]; however, there were no differences in the risk of severe outcomes between the AIH group and patients with other causes of CLD[13,14]. Outcomes assessed in this study included hospitalization, ICU requirement, ICU admission, the new requirement for renal replacement therapy, the need for invasive ventilation, and mortality. In AIH patients, age and advanced liver disease, but not immunosuppression, were found to be factors associated with mortality[13]. While both studies investigated hospitalization and mortality, neither study included AKI as an adverse outcome. Additionally, a small case series demonstrated a clinical course of COVID-19 in 10 AIH patients that were similar to the general population[15]. Our study addresses these gaps in knowledge while building on previous work with a focus on a larger cohort of United States-based AIH patients.

Our results may differ from previous studies due to the covariables included in our propensity score-matched analysis. Strengths of the present study include an expansion of clinical outcomes addressed in previous studies and an adjustment for confounders such as BMI, race, ethnicity, chronic kidney disease, neoplasms, and obstructive sleep apnea. The chronic, low-grade inflammation that is characteristic of obesity causes immune dysregulation, and obesity has been established as an independent risk factor for severe COVID-19 disease[14]. The inclusion of race and ethnicity as covariables are also important, as minority groups, including African American, Hispanic, and Asian American individuals, have higher rates of comorbidities that are associated with an increased risk of severe COVID-19 disease [18,19].

Autoimmune disease diagnosis has been associated with more severe COVID-19 disease[20,21]. The immunosuppressive agents used to treat AIH may increase the risk of viral and bacterial infections and delay viral clearance[22]. Our contrasting finding that AIH patients had a lower risk of several adverse outcomes compared to non-AIH CLD patients may be explained by the immunosuppressive agents that are used in its treatment. Other investigators have found a decreased risk of severe COVID-19 outcomes such as mechanical ventilation, death, and severe acute respiratory distress syndrome[23]. In contrast, a different study found a higher risk of severe COVID-19 in AIH patients who were on thiopurine or glucocorticoid therapy prior to COVID-19 infection[24]. Further investigation is needed to clarify the relationship between immunosuppression and COVID-19 outcomes.

Of note, we found that AIH patients had higher fibrinogen levels than the non-CLD group but lower than the non-AIH CLD group. This is consistent with the literature, as higher fibrinogen levels have been associated with disease severity and ICU admission in COVID-19 patients[25], which may be explained by the role of the cytokine storm that follows COVID-19 infection in disseminated intravascular coagulation[26].

Strengths and limitations

Our study has several strengths. Firstly, our study is the first to examine severe COVID-19 outcomes in a large cohort of United States-based AIH patients. While other studies have also compared AIH COVID-19 outcomes to non-CLD and non-AIH CLD groups, our study includes a larger sample size of 375 patients with AIH. Additionally, we included AKI as an adverse outcome. AKI is a common complication of COVID-19, reported in approximately 29% of hospitalized patients and 78% of patients that require intubation[27]. The investigation of AKI in COVID-19 is important, as there may be differences in pathophysiology between AKI related to COVID-19 and non-COVID sepsis-associated AKI[25]. Secondly, we included a robust control and adjustment for baseline and potential confounders. Thirdly, the large sample in the propensity-matched analyses resulted in narrow confidence intervals. It allowed us to capture a significant number of outcomes, which lends strength to the conclusions that we have derived. Lastly, our cohort was derived from a multicenter database, increasing the generalizability of our findings within the United States.

The study had some notable limitations. First, the data derived from an EHRs-based database is susceptible to errors in coding or data entry when patient information is translated into the diagnosis and procedure codes. However, care was taken to use standardized measures to identify cases to minimize documentation errors. Second, even though we adjusted our analyses, it is still possible that there is some residual confounding we did not account for. Third, patients who were asymptomatic throughout the course of infection and who did not undergo COVID-19 testing were not captured in the study. Fourth, our data were not able to include COVID-19 vaccines or SARS-CoV-2 variants to assess the impact on accuracy in patients with AIH. Another limitation includes the absence of cirrhosis prevalence and Child-Pugh scores in our cohort. Our study did not examine the changes made in immunosuppressive therapy after COVID-19 diagnosis in AIH patients. Finally, we could not obtain long-term outcomes due to a comparatively short observation period.

CONCLUSION

In conclusion, in this cohort, we found that AIH patients have an increased hospitalization risk, severe COVID-19, and all-cause mortality compared to non-CLD patients. Compared to the large group of patients with non-AIH CLD, AIH patients had a lower risk of several outcomes, including hospitalization, a necessity for critical care, and AKI. These results confirm that many patients with existing AIH are at high risk and should continue to follow recommended preventive measures against SARS-CoV-2 exposure.

ARTICLE HIGHLIGHTS

Research background

Severe illness and clinical outcomes can directly correlate with the underlying comorbidities of patients infected with coronavirus disease 2019 (COVID-19), including patients with autoimmune diseases. However, the clinical course of COVID-19 in patients with autoimmune hepatitis (AIH) is still not well studied.

Research motivation

AIH is a chronic inflammatory liver disease of unknown etiology in which autoimmune-mediated factors against hepatocytes are thought to play a key role. Patients with AIH may be at increased risk of severe illness from COVID-19 and have poor outcomes due to underlying chronic liver disease (CLD) and ongoing pre-existing immunosuppression therapies. Notably, there is a wide research gap in the perceived impact of COVID-19 on patients with AIH due to a high degree of heterogeneity in the existing literature.

Research objectives

This study aimed to evaluate the impact of pre-existing AIH on the clinical outcomes of patients with COVID-19.

Research methods

A population-based, multicenter, propensity score-matched cohort study included 375 patients with AIH, 1647915 patients with non-CLD, and 15790 patients with non-AIH CLD with COVID-19 infection. To reduce confounding effects, we performed a 1:1 propensity score matching with each patient in the main group to a patient in the control group. The primary outcome was all-cause mortality at 60 d, and secondary outcomes were hospitalization rate, need for critical care, severe disease, mechanical ventilation, and acute kidney injury (AKI) at 30 d.

Research results

Patients with AIH had an increased risk of all-cause mortality [risk ratio (RR) = 2.22; 95% confidence interval (CI): 1.07-4.61], hospitalization rate (RR = 1.78), and severe disease (RR = 1.98) compared to the non-CLD controls. However, compared to the non-AIH CLD group, patients in the AIH cohort had a lower risk of hospitalization rate (RR = 0.72), critical care (RR = 0.50), and AKI (RR = 0.56).

Research conclusions

This multicenter, propensity score-matched cohort study reveals that patients with AIH are at risk of worse COVID-19 outcomes than those without pre-existing CLD. However, patients with AIH were not at increased risk of COVID-19 adverse outcomes compared to matched patients with other causes of CLD.

Research perspectives

Further studies with long-term follow-up of these patients are needed to understand the long-term impact of COVID-19 on the liver and elucidate the pathogenic mechanisms among patients with AIH.

FOOTNOTES

Author contributions: Krishnan A conceptualized and designed the research; Alqahtani SA and Woreta TA supervised the project; Krishnan A performed the formal analysis and interpretation of the data; Krishnan A and Patel RA wrote the original draft; Krishnan A, Patel RA, Hadi YB, Mukherjee D, Woreta TA, and Alqahtani SA performed the review and editing of the draft; Krishnan A and Hadi YB performed a critical revision of the manuscript; and all authors revised the manuscript for important intellectual content; and all authors approved the article's final version, including the authorship list.

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ORIGINAL ARTICLE

Retrospective Study

Influence of non-alcoholic fatty liver disease on non-variceal upper gastrointestinal bleeding: A nationwide analysis

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disease globally with an estimated prevalence of 25%, with the clinical and economic burden expected to continue to increase. In the United States, non-variceal upper gastrointestinal bleeding (NVUGIB) has an estimated incidence of 61-78 cases per 100000 people with a mortality rate of 2%-15% based on co-morbidity burden.

AIM

To identify the outcomes of NVUGIB in NAFLD hospitalizations in the United States.

METHODS

We utilized the National Inpatient Sample from 2016-2019 to identify all NVUGIB hospitalizations in the United States. This population was divided based on the presence and absence of NAFLD. Hospitalization characteristics, outcomes and complications were compared.

RESULTS

The total number of hospitalizations for NVUGIB was 799785, of which 6% were found to have NAFLD. NAFLD and GIB was, on average, more common in younger patients, females, and Hispanics than GIB without NAFLD. Interestingly, GIB was less common amongst blacks with NAFLD. Multivariate logistic regression analysis was conducted, controlling for the multiple covariates. The primary

outcome of interest, mortality, was found to be significantly higher in patients with NAFLD and GIB [adjusted odds ratio (aOR) = 1.018 (1.013-1.022)]. Secondary outcomes of interest, shock [aOR = 1.015 (1.008-1.022)], acute respiratory failure [aOR = 1.01 (1.005-1.015)] and acute liver failure [aOR = 1.016 (1.013-1.019)] were all more likely to occur in this cohort. Patients with NAFLD were also more likely to incur higher total hospital charges (THC) [$2148 ($1677-$2618)]; however, were less likely to have a longer length of stay [0.27 d (0.17-0.38)]. Interestingly, in our study, the patients with NAFLD were less likely to suffer from acute myocardial infarction [aOR = 0.992 (0.989-0.995)]. Patients with NAFLD were not more likely to suffer acute kidney injury, sepsis, blood transfusion, intubation, or dialysis.

CONCLUSION

NVUGIB in NAFLD hospitalizations had higher inpatient mortality, THC, and complications such as shock, acute respiratory failure, and acute liver failure compared to those without NAFLD.

Key Words: Non-alcoholic fatty liver disease; Non-variceal gastrointestinal bleeding; Outcomes; Mortality; Complications

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Core Tip: Non-alcoholic fatty liver disease (NAFLD) is a growing problem. The national inpatient database was used to identify patients with non-variceal upper gastrointestinal bleeding who were categorized based on NAFLD status. Statistically significant differences were observed between the two cohorts with respect to mortality, utilization of healthcare resources and complications. We believe this will be beneficial for physicians in terms of predicting morbidity and prognosis in these patients.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease worldwide[1]. It has a disease spectrum ranging from hepatic steatosis to non-alcoholic steatohepatitis, which may ultimately lead to liver cirrhosis[2]. Major risk factors for NAFLD include obesity, metabolic syndrome, diabetes mellitus, hypertriglyceridemia, hypertension, and increasing age. The primary pathophysiological mechanism implicated in the development of NAFLD involves *de-novo* synthesis and uptake of triglyceride by hepatocytes leading to the development of 'fatty liver'[3]. Per current literature, NAFLD is associated with significant morbidity and all-cause mortality, with mortality rates ranging from 5% to 40%[4-6]. Furthermore, with increasing rates of NAFLD in the global population, associated complications such as gastrointestinal bleeding (GIB) are also on the rise.

Upper GIB can be divided into 2 main categories, namely variceal and non-variceal upper GIB (NVUGIB). Variceal GIB is usually seen in patients with portal hypertension in a setting of underlying liver cirrhosis[7,8]. However, the most common cause of NVUGIB is peptic ulcer disease. Other causes include but are not limited to gastritis, duodenitis, angiodysplasia, non-variceal esophageal hemorrhage secondary to mucosal tears, *etc*. All the causes included in the study are mentioned in the Supplementary material, malignancy as a cause of NVUGIB was not included in the study. In the United States, it is estimated that NVUGIB has an incidence rate of 61-78 cases per 100000 persons with a mortality rate ranging from 2%-15% depending on the co-morbidity burden. Although there is a significant paucity of data on the rates of NVUGIB in NAFLD populations, current literature has described a positive association between *Helicobacter pylori* (*H. pylori*) infection and NAFLD, which could in turn lead to higher rates of GIB. Hence, in this study, we investigate and compare hospitalization characteristics, clinical outcomes, and complications of NVUGIB in NAFLD and non-NAFLD hospitalizations in the United States.

MATERIALS AND METHODS

Study design and data source

The study population was derived from the National Inpatient Sample (NIS) which is a part of the Healthcare Cost and Utilization Project (HCUP) databases. It is one of the largest publicly available, multi-ethnic databases derived from a collection of billing data submitted by United States hospitals to state-wide data organizations. As the NIS collects data from almost all hospitals across the United States, it covers greater than 95% of the United States population. It approximates a 20% stratified sample of discharges from United States community hospitals and the dataset is further weighted to obtain national estimates. For our study period between 2016 and 2019, the NIS database was coded using the International Classification of Diseases, Tenth Clinical Modification/Procedure Coding System (ICD/PCS-10).

Study population

We identified all adult (≥ 18 years) hospitalizations with NVUGIB in the United States from 2016-2019. The study population was further divided into two distinct subgroups based on the presence or absence of NAFLD. Individuals ≤ 18 years of age, and those with a diagnosis of liver disease other than NAFLD were excluded from the analysis. Details on inclusion and exclusion criteria are included in the Supplementary material.

Outcome measures

The primary outcome of interest was mortality. Secondary outcomes of interest included length of stay (LOS), hospital charges, and complications such as acute kidney injury, shock, sepsis, acute respiratory failure, acute myocardial infarction, acute liver failure, blood transfusion, need for early endoscopy, need for intubation, and need for dialysis.

Ethical considerations

The NIS does not contain patient or hospital-specific identifiers. Hence, an Institutional Review Board (IRB) approval was not required for this study as per the guidelines put forth by our IRB on the analysis of HCUP databases.

Statistical analysis

The statistical analysis was conducted using R software (version 4.2.1) to account for weights in the stratified survey design for the NIS database. The weights were considered during the statistical estimation process by incorporating variables for strata, clusters, and weights for discharges in the NIS database. Descriptive statistics were provided, including the mean (standard error) for continuous variables and count (percentage) for categorical variables. Mann-Whitney tests with Bonferroni corrections were used for testing differences in continuous variables, while chi-squared tests with Bonferroni corrections were used for testing the homogeneity of categorical variables. Furthermore, a multivariate regression analysis was performed to compare outcomes such as in-patient mortality, healthcare burden (mean LOS and mean total hospital charges), and complications. All analyses with *P*-values ≤ 0.05 were considered statistically significant.

RESULTS

Hospitalization characteristics

We identified a total of 799785 patients admitted with a primary diagnosis of NVUGIB between the years 2016 and 2019 that met our inclusion criteria. Of these 752980 (94.15%) belonged to the cohort without NAFLD and 46805 (5.85%) belonged to the cohort with NAFLD.

Compared to the group without NAFLD, the patients with NAFLD were significantly younger (69.3 *vs* 64.6, $P < 0.001$). In both groups, GIB was more common in females. Furthermore, there were statistically significant racial differences noted, with GIB and NAFLD being less common in blacks (8.5% *vs* 14.4%, $P < 0.001$) and more common in Hispanics (15% *vs* 8.2%, $P < 0.001$). The Elixhauser comorbidities index was almost similar in both groups, with most patients having 2 or more comorbidities. Compared to the group without NAFLD, we noted that the NAFLD group had a higher proportion of patients with diabetes (44.1% *vs* 30%, $P < 0.001$) and obesity (18% *vs* 11%, $P < 0.001$). The patient and hospital characteristics are summarized in Table 1 and Figure 1.

Clinical outcomes

After adjusting for the variables shown in Table 1, the group with NAFLD had higher odds of inpatient mortality [4.2% *vs* 2.7%, adjusted odds ratio (aOR) = 1.018 (1.013-1.022), $P < 0.01$] compared to those without NAFLD.

Table 1 Comparison of demographics, comorbidities, and hospital stay information

Variable	GI bleeding without NAFLD (*n* = 752980)	GI bleeding with NAFLD (*n* = 46805)	*P* value
Age (yr)			< 0.001
mean ± SD	69.3 ± 0.1	64.6 ± 0.2	
Sex			< 0.001
Male	374615 (49.8%)	21805 (46.6%)	
Female	378210 (50.2%)	24985 (53.4%)	
Race			< 0.001
White	515935 (68.5%)	31705 (67.7%)	
Black	108520 (14.4%)	3965 (8.5%)	
Hispanic	61990 (8.2%)	7030 (15%)	
Other	46220 (6.1%)	3000 (6.4%)	
Insurance			< 0.001
Medicare	521895 (69.3%)	28550 (61%)	
Medicaid	67665 (9%)	5460 (11.7%)	
Private	122560 (16.3%)	9670 (20.7%)	
Self-pay	23575 (3.1%)	1805 (3.9%)	
Other	16250 (2.2%)	1240 (2.6%)	
Hospital location			< 0.001
Rural	82535 (11%)	3745 (8%)	
Urban nonteaching	189130 (25.1%)	11245 (24%)	
Urban teaching	481315 (63.9%)	31815 (68%)	
Hospital bedsize			< 0.001
Small	162810 (21.6%)	8810 (18.8%)	
Medium	236145 (31.4%)	14245 (30.4%)	
Large	354025 (47%)	23750 (50.7%)	
Hospital region			
Northeast	152290 (20.2%)	7440 (15.9%)	
Midwest	163005 (21.6%)	9370 (20%)	
South	301330 (40%)	19790 (42.3%)	
West	136355 (18.1%)	10205 (21.8%)	
Chronic pulmonary disease			< 0.001
0	608815 (80.9%)	39505 (84.4%)	
1	144165 (19.1%)	7300 (15.6%)	
Hypertension			< 0.001
0	241235 (32%)	18195 (38.9%)	
1	511745 (68%)	28610 (61.1%)	
Diabetes			< 0.001
0	527140 (70%)	26170 (55.9%)	
1	225840 (30%)	20635 (44.1%)	
Obesity			< 0.001
0	670225 (89%)	38385 (82%)	
1	82755 (11%)	8420 (18%)	

Peripheral vascular disease			< 0.001
0	700625 (93%)	44630 (95.4%)	
1	52355 (7%)	2175 (4.6%)	
Smoker			0.598
0	658350 (87.4%)	40765 (87.1%)	
1	94630 (12.6%)	6040 (12.9%)	
Valvular disease			< 0.001
0	736900 (97.9%)	46255 (98.8%)	
1	16080 (2.1%)	550 (1.2%)	
Colorectal cancer			0.287
0	747345 (99.3%)	46380 (99.1%)	
1	5635 (0.7%)	425 (0.9%)	
Number of Elixhauser comorbidities			< 0.001
0	89900 (11.9%)	5195 (11.1%)	
1	191920 (25.5%)	12280 (26.2%)	
2	233635 (31%)	14935 (31.9%)	
3 +	237525 (31.5%)	14395 (30.8%)	
Disposition			< 0.001
Routine	13685 (1.8%)	825 (1.8%)	
Short-term hospital	448085 (59.5%)	29620 (63.3%)	
Skilled nursing facility	21130 (2.8%)	1775 (3.8%)	
Home health care	148465 (19.7%)	6550 (14%)	
Died in-hospital	100955 (13.4%)	6090 (13%)	
Other	20205 (2.7%)	1920 (4.1%)	

GI: Gastrointestinal; NAFLD: Non-alcoholic fatty liver disease.

Healthcare utilization

The difference between the total charge of hospitalizations was also statistically significant, being higher in the NAFLD group ($35092 *vs* $32275, $P < 0.01$). Patients with GIB and NAFLD were less likely to have a longer LOS (4.47 ± 4.92 *vs* 4.27 ± 4.53, $P < 0.01$). Routine discharges were the same in both groups; however, patients with NAFLD were more likely to go to a short-term rehab facility (63.3% *vs* 59.5%, $P < 0.001$).

Complications

Patients with NVUGIB and NAFLD were more likely to have worse outcomes in terms of complications including shock [13% *vs* 12%, aOR = 1.015 (1.008-1.023), $P < 0.01$], acute respiratory failure [5.2% *vs* 4.1%, aOR = 1.01 (1.005-1.015), $P < 0.01$), and acute liver failure [2% *vs* 0.3%, aOR = 1.016 (1.013-1.019), $P < 0.01$]. Peculiarly, patients with NAFLD were less likely to suffer from an acute myocardial infarction (MI). However, they were 1.04 times more likely to undergo an endoscopy. The clinical outcomes, healthcare utilization, and complications are summarized in Table 2.

DISCUSSION

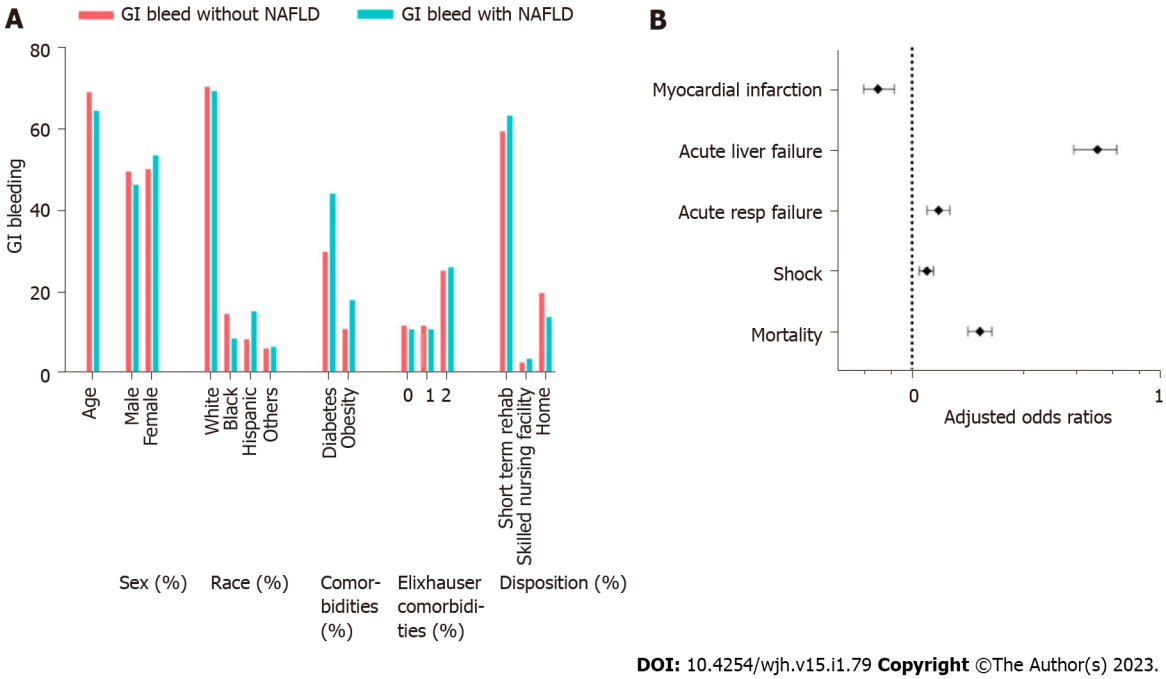
Many studies have been conducted to evaluate variceal bleeding in liver disease and cirrhosis. There is a paucity of published data evaluating NVUGIB in patients with NAFLD without cirrhosis[9]. Given the increasing incidence of NAFLD, understanding the patient demographics, clinical outcomes and associations is of practical importance to gastroenterologists and hepatologists[10-13].

In our analysis, it was noted that patients with both GIB and NAFLD were younger, with a higher incidence in the Hispanic population, and were seen more in population groups with diabetes and

Table 2 Regression analysis showing effect of non-alcoholic fatty liver disease on outcomes in patients with gastrointestinal bleeding

Outcomes	GI bleeding with NAFLD (*n* = 45215)	GI bleeding without NAFLD (*n* = 726490)	Univariate *P* value	OR or regression coefficient (95%CI)	Multivariate *P* value
Mortality	1920 (4.2%)	20205 (2.7%)	< 0.01	1.018 (1.013-1.022)	< 0.01
Length of stay	4.47 ± 5.03	4.26 ± 4.51	< 0.01	0.27 (0.17-0.38)	< 0.01
Total charges	35092 ± 21749	32275 ± 21011	< 0.01	2148 (1677-2618)	< 0.01
Acute kidney injury	10150 (22.4%)	159955 (21.2%)	1	1.012 (1.003-1.021)	1
Shock	6015 (13.3%)	87425 (11.6%)	< 0.01	1.015 (1.008-1.023)	< 0.01
Sepsis	1000 (2.2%)	12640 (1.7%)	0.14	1.005 (1.002-1.008)	1
Acute respiratory failure	2330 (5.2%)	30540 (4.1%)	< 0.01	1.01 (1.005-1.015)	< 0.01
Acute MI	955 (2.1%)	22635 (3%)	< 0.01	0.992 (0.989-0.995)	< 0.01
Acute liver failure	915 (2%)	2560 (0.3%)	< 0.01	1.016 (1.013-1.019)	< 0.01
Blood transfusion	12505 (27.7%)	210580 (28%)	0.14	1.003 (0.993-1.012)	1
Endoscopy	12500 (27.6%)	169385 (22.5%)	< 0.01	1.038 (1.028-1.048)	< 0.01
Intubation	140 (0.3%)	1255 (0.2%)	0.28	1.001 (1-1.003)	1
Dialysis	750 (1.7%)	11525 (1.5%)	1	1.001 (0.998-1.003)	1

All *P* values were corrected for multiple comparisons using Bonferroni correction. Length of stay and total charges were calculated with regression coefficients, while all other outcomes were calculated with odds ratios. GI: Gastrointestinal; NAFLD: Non-alcoholic fatty liver disease; OR: Odds ratio; CI: Confidence interval; MI: Myocardial infarction.



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Figure 1 Gastrointestinal bleeds and patient characteristics. A: Gastrointestinal bleeds and patient characteristics; B: Outcomes. GI: Gastrointestinal; NAFLD: Non-alcoholic fatty liver disease.

obesity. Al-though the length of hospitalization was almost similar in both groups, patients with NAFLD and NVUGIB had higher inpatient costs with increased discharges to short-term rehab facilities. Patients were also noted to have higher mortality and were likely to have acute liver failure, respiratory failure, and shock but less chance of having an acute MI during the hospital course.

Our study found increased odds of patients with NAFLD presenting with GIB at a younger age. This is in contrast to available literature[14]. This is probably related to patients having an increased risk of developing NAFLD at a younger age with the increasing risk factors especially the increasing

prevalence of metabolic syndrome in young adults, which is one of the major risk factors for NAFLD [15]. Patients with NAFLD are more prone to atherosclerotic cardiovascular disease (ASCVD) including coronary artery disease (CAD)[16-18]. With the increased CAD prevalence and percutaneous interventions for CAD, an increasing number of patients are on antiplatelet medications such as aspirin and clopidogrel which likely predispose them to GIB. Despite ASCVD still being the highest cause of mortality in NAFLD patients, in our study, we found that the odds of NAFLD patients with GIB developing an acute MI were actually less[19]. There are studies with conflicting data regarding acute cardiac events in patients admitted for other NAFLD-related complications[14,20].

Studies have also demonstrated a positive association between *H. pylori* infection and predisposition to NAFLD incidence[21,22]. This underlying relationship can also explain the increased risk of developing gastric ulcers and subsequent bleeding[23]. Studies have shown that aspirin can decrease the progression of fibrosis in NAFLD. Although it is not known if this has led to increased use of aspirin in this population but could also be a contributing factor.

Previous studies have shown that NAFLD has an increased prevalence in the Hispanic population[24-26]. This also resonates with our results, as NAFLD with GIB was higher in Hispanics. Non-variceal GIB from ulcer disease is seen more in the African-American population[27,28]. However, in our study we found that patients with NAFLD were less likely to have NVUGIB, indicating a possible protective effect. The mechanism for the same is unclear. This association needs to be further studied.

Patients with NAFLD and GIB were found to have a longer LOS and showed increased odds of having higher hospital charges and discharges to short-term rehab facilities, thus leading to increased utilization of healthcare resources and an increased economic burden. This trend has been seen in multiple studies and was associated with the established risk factors of NAFLD and metabolic syndrome, especially diabetes[29,30]. Another reason for the economic burden could be the higher incidence of complications among these patients[31,32].

Murine models have shown that hepatic steatosis and NAFLD lead to aberrant corticosterone release which could put patients at increased risk of developing and delayed recovery from shock[33]. It was shown that reduced lung function is an independent risk factor for the development of NAFLD which can theoretically increase the risk of developing acute respiratory failure[34]. It was also shown that NAFLD and metabolic syndrome can be associated with impaired lung function predominantly due to abdominal obesity[35]. Along with the increased risk of shock and respiratory failure, the NAFLD population is inherently at risk for the development of acute on chronic liver failure from chronic hepatocyte inflammation and increased mortality in the presence of multiple comorbidities[36].

Strengths and limitations

Using the NIS database gives nationwide generalizability, a large patient population, and multiple clinical parameters. It provides an excellent representative sample with results in a reliable and valid range[37]. Our study should be prudently interpreted as the NIS database has its own limitations. It does not include how NAFLD was diagnosed and the specific diagnostic modality that was used. This contributes to variations in the prevalence of NAFLD amongst various geographical regions and income groups.

Another drawback was that given it is a nationwide sample and with the use of ICD-10 CM coding, there may have been imprecision and erroneous coding causing an over or underestimation of the cases. ICD nomenclature does not include the spectrum of liver disease to further stratify based on severity in the NAFLD population. Although Elixhauser comorbidity indices were used to account for the various systemic comorbidities, the calculation of liver-specific indices such as model for end-stage liver disease score was not possible given the non-availability of laboratory data.

Areas for future research

With the increasing worldwide incidence of liver disease from NAFLD and with the rising frequency of hospitalizations[37,38], emphasis should be placed on aggressive risk factor modification and secondary prevention of the disease and its numerous complications. Further longitudinal studies are needed to study NVUGIB in the NAFLD population and develop tools to help guide clinicians in the early detection of patients at risk for NVUGIB. This will help reduce multiple hospitalizations, increasing financial burden with prolonged hospital stays and mortality.

CONCLUSION

Our analysis showed that patients with NVUGIB have higher mortality, increased complications, longer LOS and higher hospital charges demonstrating the increased morbidity and economic burden of NAFLD.

ARTICLE HIGHLIGHTS

Research background

With the increasing prevalence, morbidity and mortality of non-alcoholic fatty liver disease (NAFLD), and worse outcomes with concomitant conditions, we wanted to determine the effect of NAFLD on a commonly seen in-patient presentation, non-variceal upper gastrointestinal bleeding (NVUGIB).

Research motivation

There are studies showing the effect of alcoholic liver disease on both variceal and NVUGIB, along with studies showing an increased risk of variceal bleeding and screening in patients with NAFLD. However, there have been no studies showing the influence of NAFLD on NVUGIB. Our aim was to try to bridge this gap.

Research objectives

Our objective was to examine whether the presence of NAFLD led to worse outcomes in patients with NVUGIB.

Research methods

We used the National Inpatient Sample database to ensure generalizability of findings. We compared the two cohorts of NAFLD with and without NVUGIB on the basis of mortality which was the primary outcome and secondary outcomes such as the length of stay, hospital charges, and complications.

Research results

It was shown that patients with NVUGIB and NAFLD had higher odds of mortality, higher hospital charges and more complications such as shock, acute respiratory failure and acute liver failure.

Research conclusions

Co-existence of NAFLD and NVUGIB was associated with higher mortality, morbidity and economic burden.

Research perspectives

Because of increased morbidity and mortality due to NAFLD, aggressive risk management should be a focus. Also, further studies should be performed to stratify patients with NAFLD that are at higher risk of NVUGIB so that they can be identified by clinicians and the mortality, morbidity and economic burden can be reduced.

FOOTNOTES

Author contributions: Soni A and Yekula A contributed to the conceptual design of the study; Soni A, Yekula A, and Soni A independently screened the medical records and extracted the data; Sood N performed the statistical analysis; Soni A, Yekula A, and Dahiya DS contributed to the write-up and submission of the study; Abraham G reviewed the final manuscript; and all authors reviewed and agreed the final content of the article.

Institutional review board statement: We utilized data from the National Inpatient Sample database, which meets all relevant ethical and regulatory standards. National Inpatient Sample is a publicly available database provided by the Agency of Healthcare Research and Quality. It includes a national representative sample of discharge-level files and does not include patient or hospital-identifiable information. Due to the nature of its complex sampling method, and being a deidentified database available for public use, institutional board review policy was met and as per the IRB policy at Saint Vincent Hospital no review or approval was required.

Informed consent statement: We utilized data from the National Inpatient Sample database, which meets all relevant ethical and regulatory standards. National Inpatient Sample is a publicly available database provided by the Agency of Healthcare Research and Quality. It includes a national representative sample of discharge-level files and does not include patient or hospital-identifiable information. Due to the nature of its complex sampling method, and being a deidentified database available for public use, informed consent was met and as per the IRB policy at Saint Vincent Hospital no review or approval was required.

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

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Observational Study

Rising incidence, progression and changing patterns of liver disease in Wales 1999-2019

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Abstract

BACKGROUND

Liver disease incidence and hence demand on hepatology services is increasing.

AIM

To describe trends in incidence and natural history of liver diseases in Wales to inform effective provision of hepatology services.

METHODS

The registry is populated by International Classification of Diseases-10 (ICD-10) code diagnoses for residents derived from mortality data and inpatient/day case activity between 1999-2019. Pseudo-anonymised linkage of: (1) Causative diagnoses; (2) Cirrhosis; (3) Portal hypertension; (4) Decompensation; and (5) Liver cancer diagnoses enabled tracking liver disease progression.

RESULTS

The population of Wales in 2019 was 3.1 million. Between 1999 and 2019 73054 individuals were diagnosed with a hepatic disorder, including 18633 diagnosed with cirrhosis, 10965 with liver decompensation and 2316 with hepatocellular carcinoma (HCC). Over 21 years the incidence of liver diseases increased 3.6 fold,

predominantly driven by a 10 fold increase in non-alcoholic fatty liver disease (NAFLD); the leading cause of liver disease from 2014. The incidence of cirrhosis, decompensation, HCC, and all-cause mortality tripled. Liver-related mortality doubled. Alcohol-related liver disease (ArLD), autoimmune liver disease and congestive hepatopathy were associated with the highest rates of decompensation and all-cause mortality.

CONCLUSION
A 10 fold increase in NAFLD incidence is driving a 3.6 fold increase in liver disease in Wales over 21 years. Liver-related morbidity and mortality rose more slowly reflecting the lower progression rate in NAFLD. Incidence of ArLD remained stable but was associated with the highest rates of liver-related and all-cause mortality.

Key Words: Epidemiology; Cirrhosis; Liver failure; Non-alcoholic fatty liver disease hepatitis; Hepatocellular carcinoma

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Core Tip: In this paper we describe the following: (1) Novel methodology for developing a national liver registry; (2) The incidence of liver disease has increased 3.6-fold in Wales between 1999-2019 driven by a 10-fold increase in non-alcoholic fatty liver disease (NAFLD); (3) 3-fold increase in cirrhosis, portal hypertension, decompensation and hepatocellular carcinoma, 2-fold increase in liver disease related mortality between 1999-2019; and (4) Actuarial tables of 10-year liver disease progression: Alcohol-related liver disease, autoimmune liver disease and congestive hepatopathy are associated with increased rates of decompensation and death compared to viral hepatitis and NAFLD. Description of the proportion of patients dying from liver disease as directly, as a contributory cause or where liver disease has not been recording on the death certificate.

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INTRODUCTION

Liver disease is the third most common cause of premature death in the United Kingdom[1]. Since 1970 mortality from liver disease has increased fourfold in the United Kingdom whilst all other major causes of death have declined[2]. The major causes of liver disease are alcohol-related liver disease (ArLD), non-alcoholic fatty liver disease (NAFLD) and viral hepatitis.

Liver disease typically progresses through several stages with increasing mortality regardless of the underlying aetiology[3]. Persistent inflammation may drive the accumulation of fibrosis resulting in cirrhosis and the development of portal hypertension over months to years[4]. Decompensation of chronic liver disease is defined by a deterioration in hepatic function and is represented by the sequalae of portal hypertension on a background of cirrhosis: Jaundice, ascites, encephalopathy, hepatorenal syndrome and variceal bleeding[5]. Up to a third of individuals with cirrhosis will develop liver cancer over their life time[6], which can precipitate liver decompensation. Decompensation may be the initial symptoms and first presentation to medical services and is associated with a high mortality[2,7]. Treatment of the underlying liver disease is key to improving prognosis, and can result in improvement in liver function, re-compensation of liver failure and even regression of cirrhosis[8,9]. Access to specialist care, surveillance for liver cancers and variceal bleeding and early discharge specialist follow up are all associated with reduced all-cause mortality for patients with cirrhosis[10,11].

Over the last decade there have been significant clinical advances in the management of liver disease, a striking example is the impact of direct acting antiviral drugs to eradicate hepatitis C virus (HCV) infection[12]. It is reasonable to assume that public health initiatives addressing lifestyle risk factors for liver disease may impact on the liver disease incidence and morbidity. Minimum unit pricing for alcohol in Scotland has resulted in an initial reduction in alcohol consumption, and taxation on sugary drinks has been introduced in an attempt to counter the obesity epidemic[13,14]. The long term impact of such interventions on liver disease incidence and mortality has yet to be established. Accurate epidemiological data may inform the effective provision of public health, primary and secondary care

initiatives for liver disease and assess the future effectiveness of these interventions. Previous epidemiological studies have been limited by use of either mortality data alone[15,16], focusing of cohorts with advanced disease severity[3,10,17,18], or by specific disease aetiology such as autoimmune hepatitis, HCV or ArLD[4,19,20].

We set out to define the incidence of inpatient presentation, progression through significant stages of disease over time, and mortality of all liver diseases in Wales. We have developed a national liver disease registry populated by routinely coded diagnoses related to hospital admissions and death certificates. These diagnoses are recorded using the International Classification of Diseases (ICD)-10 classification[21]. Incorporating routinely coded data into a registry remains problematic for the following reasons: (1) ICD-10 codes for liver disease may be specific to individual aetiologies; descriptive of a disease process; or reflect the stage of disease *i.e.*, 'chronic viral hepatitis C' (B18.2), 'inflammatory liver disease, unspecified' (K76.9) and portal hypertension (K76.6) respectively; (2) Diagnoses are recorded in the order of presentation rather than reflecting the natural history of disease described above. For example, variceal bleeding (I85.01) may be the initial presentation of autoimmune hepatitis (K75.4), however, a period of investigation including liver biopsy is commonly required before the aetiological diagnosis is later made. A code of 'inflammatory liver disease, unspecified' (K76.9) may be recorded during the diagnostic work up for this patient which becomes redundant once autoimmune hepatitis is confirmed; and (3) The progression of liver disease requires different levels of surveillance and monitoring and defining the stage of liver disease can be challenging. As a consequence, disease registries do not typically reflect multiple stages of disease.

In this paper we have applied a novel methodology to define: (1) The point of entry into a national liver registry; (2) Aetiological diagnoses from routinely coded data whilst removing redundant diagnoses; and (3) A decision tree to define the ordering of diagnostic codes for stages of liver disease. The aim was to develop novel insights into the changing aetiology and progression of liver disease, hence providing an analytic pipeline that will allow assessment of public health interventions, define clinical service requirements and aid redesign.

MATERIALS AND METHODS

Data sources

Between 1999 and 2019 the population of Wales increased from 2.9 to 3.1 million people; of whom 2.5 million are adults. Health care is devolved to the Welsh Government from the United Kingdom Government in Westminster. Primary and secondary health care services are delivered by 7 health boards each covering a population of approximately 400000 people. All medical diagnoses documented in medical notes from hospital admissions and day case procedures are recorded by dedicated teams of disease coders within health boards using the ICD-10 classification[21]. Mortality data including diagnoses recorded on death certificates are derived from the Office for National Statistics (ONS). The ONS record the ICD-10 code of the underlying cause of death, defined as "the disease or injury which initiated the train of morbid events leading directly to death" detailed in Part Ia-c of the United Kingdom death certificate[22]. Contributory diseases not part of the direct sequence resulting in death are recorded and are typically reported in part II of the death certificate. For all Welsh residents these data from hospitals in Wales or admissions to English hospitals are uploaded into the NHS Wales Informatics Services (NWIS) data warehouse. It is not possible to access primary care records to link risk factors for liver disease for all patients in Wales at present. Individuals with liver disease diagnosis recorded between 1991 and 1998 were excluded to reduce the risk of secular trend analysis bias through over-estimation of incidence in the early period of the study. All coded diagnoses between 1st January 1999 and 31st December 2019 were captured. Individuals were anonymised and given a unique identifier to link all demographic characteristics, 4-digit ICD-10 codes, and mortality data. The European Age Standardised Rate (EASR) of liver diseases was calculated using ONS census data for Wales from 2001-2019; the years for which census data has been used to estimate the European Age Standardised population for Wales[22].

Definitions of liver disease aetiology and stages of liver disease

ICD-10 classifications include codes for aetiology of liver disease, cirrhosis, portal hypertension, liver cancers and decompensation. We have sought to categorise ICD-10 codes into these stages of liver diseases as laid out below.

Aetiology: Building upon the recoding mapping in the Hepahealth project[15] and 2021 expert panel consensus[23], we grouped ICD-10 codes to represent the aetiology of liver diseases. In order to manage overlapping and compound liver diseases we designated a hierarchy of 3 tiers groups based upon perceived clinical importance and relevance to public health, primary and secondary care intervention (Table 1). Tier 1 diagnoses were defined as the 6 major categories of liver disease hepatitis B virus (HBV), HCV, autoimmune liver disease, ArLD, NAFLD, and metabolic liver diseases (haemochromatosis, alpha 1 antitrypsin deficiency and Wilson's disease). Individuals with more than one of these

Table 1 International Classification of Diseases-10 code case definition for liver disease aetiology, stratified into hierarchical tiers based of clinical importance

Tier 1			Tier 2			Tier 3		
Descriptor		ICD-10 code	Descriptor		ICD-10 code	Descriptor		ICD-10 code
Autoimmune liver disease	Autoimmune hepatitis	K75.4	Hepatitis not specified	Inflammatory liver disease unspecified	K76.9	Miscellaneous	Peliosis hepatis	K76.4
	Primary biliary cholangitis	K74.3		Other specified inflammatory liver disease	K75.8		Other specified diseases of the liver	K76.8
	Granulomatous hepatitis not elsewhere specified	K75.3		Chronic hepatitis not elsewhere classified	K73.0-K73.9		Liver disorders in diseases classified elsewhere	K77
Metabolic liver disease	Haemochromatosis	E83.11	Congestive hepatopathy	Chronic passive congestion of the liver	K76.1			
	Alpha 1 anti-trypsin deficiency	E88.01		Central haemor-rhagic necrosis of the liver	K76.2			
	Wilson's disease	E83.01		Hepatic veno-occlusive disease	K76.5			
HBV	Hepatitis B without D	B18.1	Toxic liver disease	Toxic liver disease	K71			
	Hepatitis B with D	B18.0						
HCV	Hepatitis C	B18.2						
Alcohol-related liver disease	Alcoholic liver disease	K70-K70.9						
Non-alcoholic fatty liver disease	Non-alcoholic fatty liver disease	K76.0						

ICD-10: International Classification of Diseases-10; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Tier 1 diagnoses are recorded as an overlap aetiology. If alcohol was one of the overlapping Tier 1 aetiologies individuals were defined as 'alcohol-overlap', if alcohol was not one of multiple Tier 1 diagnoses they were defined as 'not alcohol-overlap'. All codes recorded by clinical coders were included in this registry methodology and the relative position of codes complied by the clinical coders did not impact on the aetiology definition.

Tier 2 diagnoses were toxic liver injury (K71), congestive hepatopathy, indicating passive venous congestion of the liver including right heart failure (K76.1, K76.2, K76.5 and I82.0), and hepatitis not specified (K76.9, K75.8 and K73.0-K73.9 capturing undefined liver inflammation). We propose that whilst these diagnoses may result in significant chronic liver disease, either they are not specific or they do not constitute the majority of hepatology workload within either primary or secondary care. Additionally, in the presence of Tier 1 diagnoses they are of secondary importance to understanding the epidemiology of liver disease. Tier 3 was designated as miscellaneous diagnoses (K76.4 peliosis hepatis, K76.8 'other specified diseases of the liver' and K77 'liver disorders in diseases classified elsewhere'). Tier 3 diagnoses were only recorded in the absence of Tier 1 or 2 diagnoses. Finally, individuals without one of these aetiological codes but with a diagnosis of cirrhosis were grouped as 'cirrhosis without defined aetiology'. Individuals were pseudo-anonymised with linkage of diagnoses allowing repeat codes to be removed.

Stages of liver disease

We propose that liver disease is typically considered in 5 discrete stages (Supplementary Figure 1). The first stage is the underlying aetiological disease process in the absence of cirrhosis. The management of this stage focuses upon reversing causes of inflammation and, in specific subsets of patients with HBV, hepatocellular carcinoma (HCC) surveillance. The ICD-10 case definitions for stages 2-5 are detailed in Table 2 and described below. Stage 2 is cirrhosis without portal hypertension or synthetic failure. At this point HCC should be considered and signs of potentially significant portal hypertension sought to screen for varices by gastroscopy. Stage 3 is defined as portal hypertension without decompensation and represents an important group which should be screened for varices and medium/large varices treated to prevent bleeding. The presence of clinically significant portal hypertension is associated with

Table 2 International Classification of Diseases-10 codes to define the advanced stages of liver disease

Aetiological diagnoses (stage 1)	Cirrhosis (stage 2)	Portal hypertension (stage 3)	Hepatic decompensation (stage 4)	Hepatocellular carcinoma (stage 5)
As defined in Table 1	Alcoholic cirrhosis, K70.3	Portal hypertension, K76.6	Chronic hepatic failure, K72.1	Primary liver cancer C22.0
	Hepatic fibrosis or sclerosis, K74.0-K74.2	Portal vein thrombosis, I81	Hepatorenal syndrome, K76.7	Hepatocellular carcinoma C22.1
	Secondary biliary cirrhosis, K74.4	Oesophageal varices without bleeding I85.9	Oesophageal varices with bleeding, I85.0	
	Biliary cirrhosis unspecified, K74.5	Oesophageal varices without bleeding in diseases specified elsewhere, I98.2	Hepatic failure unspecified K72	
	Cirrhosis, other, K74.6			

increased mortality[5]. Portal vein thrombosis is included within this group as a significant complication of portal hypertension. Stage 4 denotes liver decompensation associated with variceal bleeding, hepatic synthetic failure, ascites and hepatorenal syndrome. Stage 5 represents development of HCC. Whilst this may develop spontaneously or in HBV in the absence of cirrhosis, HCC most commonly arises in cirrhotic livers. It is well recognised that liver injury may regress and decompensation may improve. There are no recorded codes for this process however, liver transplantation may be considered as a separate code applied to a small proportion of individuals with liver disease. As described above, patients may progress through these stages in turn but can present either in a stepwise fashion or at a later stage depending on symptoms, screening or incidental diagnosis. Each of these stages requires varying levels of surveillance and specialist input to ameliorate the risk of liver-related mortality.

The acute liver diseases are considered separately to these acquired chronic liver diseases (Supplementary Table 1) as they carry a different challenge to public health, primary, and secondary care. Whilst these codes are captured, they are not included within the aetiology of chronic liver diseases described above. Alcohol-related codes that did not indicate liver disease (for example F10; alcohol use disorder) were not recorded to maintain a focus on individuals with evidence of liver disease requiring hepatology services rather than broader substance misuse services.

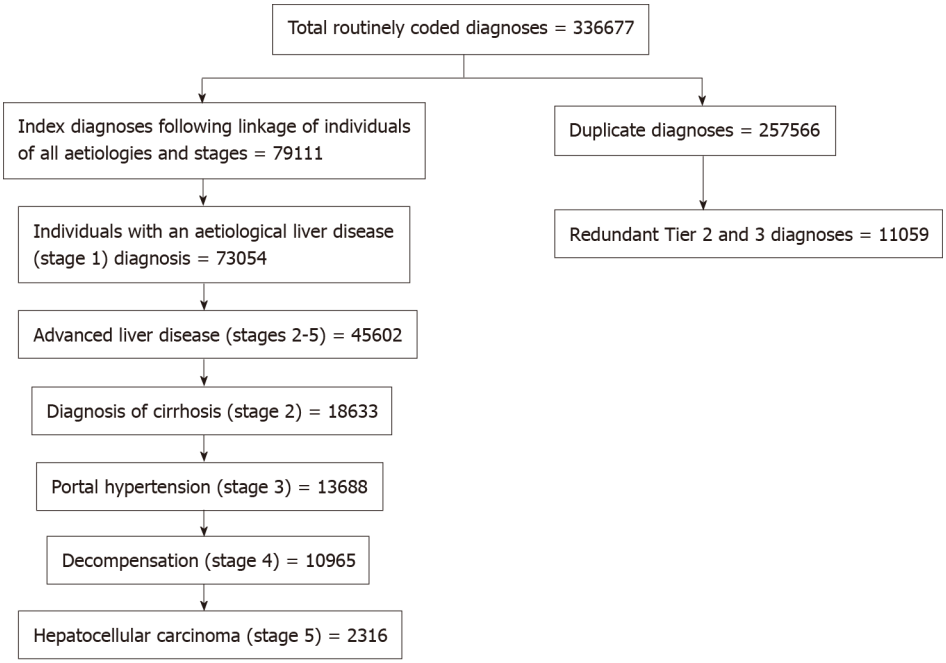
Time of registry entry and progression analysis

We proposed that the time of the first liver-related diagnosis listed in either Table 1 or Table 2 is used as the point of entry into the registry and was defined as the index diagnosis. In order to assess the proportion of individuals presenting late the diagnoses were ordered in keeping with the natural history of disease. Thus, the highest tier aetiological diagnosis is always the first entry into the liver registry, followed by cirrhosis, portal hypertension, decompensation and liver cancer. The application of the Wales Liver Registry methodology to a hypothetical patient is described in Supplementary Figure 2. The proportion of patients at each stage of liver disease was recorded. Mortality was recorded and deaths were divided into liver disease underlying cause, liver disease contributing, and non-liver-related.

We also set out to assess the quality of routinely coded inpatient, day case and mortality diagnoses in Wales in comparison to outpatient liver disease data to identify if significant liver diagnoses are under-recorded. Consultant hepatologists within the Gwent region (Aneurin Bevan Health Board), have routinely entered ICD-10 liver disease codes at the end of each outpatient appointment since 2012. These diagnoses were made with the full benefit of clinical investigations and could be updated at subsequent appointments to reflect evolving clinical manifestations of liver disease and progression of liver disease. We defined this as the gold standard of aetiological diagnosis to compare the diagnoses recorded within hospitals and on death certificates. To assess the impact of our proposed methodology we interrogated the NWIS data warehouse and report the impact of this approach. Scripts were written in SQL to perform the data linkage and reordering. Data was then collated and reported as fold change in incidence and time to event by Kaplan Meier analysis in Power BI (Microsoft, United Kingdom) and Prism (GraphPad, United States). This study was conducted in keeping with the Helsinki declaration. The design of this study was discussed with the South Wales research ethics committee and was not classed as research requiring regulatory approvals by Health Research Authority.

RESULTS

Between January 1, 1999 and December 31, 2019 there were 336677 routinely coded diagnoses of liver disease in Wales. There were 79111 index ICD-10 diagnosis of liver disease of any aetiology or stage in 73054 individuals (Figure 1). This indicates that there were approximately 3 times as many repeat



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Figure 1 Modified CONSORT flow chart of liver disease diagnoses Wales 1999-2019. There were 336677 liver disease diagnoses recorded by routine inpatient, day case and death certificate coding. 257566 were duplicate diagnoses. 11059 aetiological diagnoses were considered redundant by superior tier diagnosis (*e.g.*, hepatitis C virus is higher tier than hepatitis not specified in the same individual). The total number of index liver disease stage diagnoses are recorded. Each subsequent box shows the number of individuals within the total who have advanced liver disease.

diagnoses as indexed diagnoses during subsequent admissions. Fifty two percent of individuals (n = 37877) with a liver disease diagnosis between 1999 and 2019 died from all causes. A third of the individuals (n = 13266) who died, died from an underlying liver disease cause; equating to 18% of all individuals in the registry. In 2019 there were 35177 individuals alive with a secondary care diagnosis of chronic liver disease equating to a prevalence of 1.1% of the 3.1 million Welsh population.

The proposed hierarchy of aetiological diagnoses was applied to the liver registry cohort; 11059 Tier 2 and 3 diagnoses (including miscellaneous and hepatitis not specified) recorded in the presence of a Tier 1 diagnosis and therefore considered redundant. Following application of the staging criteria methodology there were 16992 individuals with cirrhosis, including 12858 diagnosed with portal hypertension and 10399 with an index diagnoses of decompensation, and 2316 with HCC (Figure 1). The aetiologies of liver disease in Wales are listed in Table 3; across the study period, the most frequent causes of liver disease in Wales were ArLD (26.1%), NAFLD (21.6%) metabolic disease (11.5%) and HCV (4.7%). The mean age of the entire cohort at diagnosis was 59.7 years and there was a slight male preponderance (54.5%, n = 39875). The mean age of diagnosis varied by aetiologies, ranging from 43 years in HCV to 64.7 years in congestive hepatology. Similarly, the proportion of males ranged from 20.5% to 67.8% in autoimmune liver diseases and ArLD respectively (Table 3).

Impact of outpatient diagnosis

Inpatient and day case coding may fail to capture the incidence of disease diagnosed in the outpatient clinic. We wished to assess the variation in incidence of aetiological diagnoses and staging diagnoses in the outpatient setting. When including outpatient coded diagnoses there was a substantial increase in the number of HBV (additional 112%), HCV (77%), autoimmune liver diseases (66%), NAFLD (40%), hepatitis not specified (34%), metabolic (23%), and ArLD (23%) cases captured (Supplementary Figure 3A). There was no increase in number of diagnoses of congestive hepatopathy, miscellaneous or toxic liver disease diagnoses. Of note there was no increase in the number of diagnoses of cirrhosis, decompensation, portal hypertension or HCC with the additional of outpatient coding to inpatient/day case coding (Supplementary Figure 3B).

The rising incidence of liver disease in Wales

Between 1999 and 2019 the total number of new liver disease diagnosis rose 3.6-fold from 1916 to 6932 individuals per annum (Figure 2A). The total EASR of liver diseases increased from 75.9 to 199 per 100000 (Figure 2B) between 2001 and 2019. There was a marked increase in all liver disease diagnoses over this period apart from ArLD and toxic liver disease. NAFLD demonstrated a 10-fold increase over the 20-year period and in 2014 became the most common aetiological diagnosis of liver disease in Wales. Importantly, between 1999 and 2019 there was at least a 3-fold increase in the first recorded diagnosis of

Table 3 The number, age and sex of individuals with aetiological liver disease diagnoses in Wales 1999-2019 following application of aetiology hierarchy

Aetiology	*n*	% of total cohort	Mean/median age (yr)	% male
Tier 1				
ArLD	16143	26.1%	54.9/55	67.8%
NAFLD	13390	21.6%	57/58	47%
Metabolic	7131	11.5%	63/67	52.5%
HCV	2889	4.7%	43/41	66.8%
ArLD overlap	2979	4.8%	51.2/51	65.3%
Autoimmune liver diseases	2312	3.7%	61.8/64	20.5%
HBV	904	1.5%	43.5/39	53.3%
Non ArLD overlap	955	1.5%	51.2/51	55.8%
Tier 2				
Hepatitis not specified	6069	9.8%	63/66	50.9%
Congestive hepatopathy	894	1.4%	64.7/70	49.6%
Toxic liver disease	820	1.3%	46.3/44	38.4%
Tier 3				
Miscellaneous	7430	12%	66.7/70	42%

ArLD: Alcohol-related liver disease; NAFLD: Non-alcoholic fatty liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

the clinically significant sequelae of chronic liver disease; cirrhosis, (435 to 1533) portal hypertension (282 to 1214), decompensation (173 to 785), and liver cancers (146 to 449, Figure 2B). The absolute number of deaths per year in a longitudinal cohort may be influence by lead time bias; the increasing age of the cohort, progressive and accumulation of comorbidities. However, between 2001 and 2019 the number of deaths in Wales with an underlying liver disease cause or in which liver disease contributed doubled (451 to 890 and 199 to 381 respectively, Figure 2C). In 2019 the number of all-cause deaths in the Wales Liver Registry was 3398.

Aetiology drives liver disease morbidity

Different aetiologies of liver disease will have different rates of progression to advanced liver disease and risk of all-cause mortality thus requiring varying levels of specialist care and surveillance. For each aetiological group we analysed the proportion of patients by their most advanced stage of liver disease (aetiology only, cirrhosis, portal hypertension, decompensation, and liver cancer) and all-cause mortality from entry into the liver registry up until death or censure at the end of the study period. The proportion who had further diagnoses indicating progression to each stage of liver disease was recorded at baseline, and by 6 mo, 1, 2, 3, 5 and 10 years (Table 4, Supplementary Figures 4A-F). The aetiology of liver disease resulted in significantly different proportions of each stage of liver disease at each time point (Figures 3A-F; $P < 0.001$ one way ANOVA). In this respect, advanced liver disease and decompensation were more frequent in ArLD, autoimmune liver disease, HCV, and congestive hepatopathy than NAFLD, HBV, and metabolic liver disease. This ranking of more progressive liver diseases did not change significantly at different time points (Figure 3). A notable exception was the proportion of patients living with HCC diagnosis was greatest in those with HBV up to year 3, then it was greatest in those with HCV (Figure 3E). The highest rates of cirrhosis were in ArLD (35%-20%) and autoimmune liver diseases (23.5%-18.5%) over the 10 years of follow up. The rate of cirrhosis in NAFLD (2.5%-1.98%) was over 10-fold lower than for ArLD (Table 4). In the final year of the study the number of individuals with index ArLD aetiology, cirrhosis and decompensation diagnoses were 638, 537 and 238 respectively. In the same year index NAFLD aetiology, cirrhosis and decompensation diagnoses were 2242, 164 and 63 respectively (data not shown). The proportion of ArLD and autoimmune liver patients with cirrhosis decreased over 10 years whilst the proportion with portal hypertension and decompensation increased to a peak at 1 year and 3 years respectively before falling below baseline (Figures 3C and 3D). This suggests progression of liver disease drives the increasing morbidity for these aetiologies. Congestive hepatopathy had the greatest mortality increasing for 21% at entry into the registry, 42% at 1 year and 64% at 10 years but relatively low rates of cirrhosis. Alcohol and metabolic liver disease had similar high levels of all-cause mortality whilst HBV and HCV had the lowest levels of

Table 4 Progression of liver disease over 10 years by aetiology

	0	6 mo	1 yr	2 yr	3 yr	5 yr	10 yr
ArLD							
Stage 1	48.75	40.27	36.14	31.54	28.36	24.21	19.18
Stage 2	16.52	14.12	12.93	11.75	10.82	9.65	7.87
Stage 3	13.47	13.93	13.92	12.8	11.86	10.18	8.1
Stage 4	4.49	4.8	5.09	5.3	5.33	4.97	4.25
Stage 5	0.63	0.5	0.49	0.44	0.38	0.34	0.34
Stage 6	16.14	26.38	31.43	38.17	43.25	50.64	60.25
NAFLD							
Stage 1	86.3	82.52	80.42	77.98	76.25	73.93	70.7
Stage 2	2.54	2.28	2.31	2.21	2.22	2.18	1.98
Stage 3	2.87	2.99	2.87	2.7	2.52	2.29	2.07
Stage 4	0.82	0.93	0.98	1.01	1.01	0.95	0.91
Stage 5	0.4	0.38	0.36	0.28	0.3	0.22	0.24
Stage 6	7.07	10.89	13.07	15.82	17.71	20.43	24.1
Autoimmune liver disease							
Stage 1	72.94	66.33	63.31	59.57	56.44	51.89	44.83
Stage 2	10.79	10.53	10.12	9.67	9.07	8.51	7.73
Stage 3	8.7	9.29	9.41	9.07	8.77	7.69	6.83
Stage 4	2.43	2.8	2.84	2.95	3.02	3.21	2.99
Stage 5	0.86	0.9	0.82	0.67	0.63	0.56	0.6
Stage 6	4.29	10.15	13.51	18.07	22.06	28.14	37.03
HBV							
Stage 1	88.96	86.25	84.58	82.6	81.46	79.69	78.02
Stage 2	2.5	1.77	1.98	1.98	1.88	1.88	1.88
Stage 3	2.71	2.71	2.5	2.5	2.4	1.98	2.08
Stage 4	0.94	1.04	1.04	0.94	0.94	1.04	1.25
Stage 5	2.19	1.46	1.25	1.25	1.25	0.94	0.83
Stage 6	2.71	6.77	8.65	10.73	12.08	14.48	15.94
HCV							
Stage 1	88.02	85.5	83.54	80.68	78.51	75.38	71.59
Stage 2	4.15	3.88	3.94	4.06	4.12	4.06	3.61
Stage 3	2.8	2.59	2.53	2.53	2.59	2.35	2.29
Stage 4	0.87	1.05	1.14	1.17	1.2	1.17	1.2
Stage 5	1.38	1.2	1.17	1.11	1.08	1.17	1.17
Stage 6	2.77	5.78	7.67	10.44	12.49	15.86	20.13
Metabolic							
Stage 1	82.03	67.35	63.3	59.25	56.3	52.51	47.7
Stage 2	1.73	1.37	1.29	1.21	1.16	1.11	1.05
Stage 3	1.36	1.24	1.16	1.2	1.07	0.99	0.96
Stage 4	0.63	0.52	0.53	0.57	0.56	0.56	0.49
Stage 5	0.56	0.4	0.24	0.23	0.23	0.28	0.23

Stage 6	13.69	29.12	33.48	37.54	40.69	44.55	49.57
Congestive hepatopathy							
Stage 1	70.36	55.3	50.05	43.44	38.58	34.79	31.1
Stage 2	2.24	1.65	1.65	1.07	0.78	0.58	0.58
Stage 3	3.21	3.01	2.62	2.33	2.04	1.94	1.46
Stage 4	2.04	1.75	1.75	1.75	1.94	1.85	2.04
Stage 5	0.68	0.29	0.29	0.29	0.29	0.19	0.19
Stage 6	21.48	38	43.63	51.12	56.37	60.64	64.63
Toxic							
Stage 1	80.87	76.09	73.96	71.31	69.48	67.45	62.87
Stage 2	1.22	0.92	1.02	0.81	0.71	0.61	0.61
Stage 3	1.63	1.93	1.73	1.42	1.73	1.53	1.42
Stage 4	3.46	3.15	3.36	3.26	3.36	3.15	2.95
Stage 5	0.31	0.2	0.2	0.2	0.2	0.2	0.1
Stage 6	12.51	17.7	19.74	22.99	24.52	27.06	32.04
Miscellaneous							
Stage 1	91.24	80.46	75.76	70.45	67.05	63.04	57.4
Stage 2	0.62	0.57	0.49	0.42	0.35	0.31	0.29
Stage 3	0.94	0.97	0.99	0.94	0.94	0.9	0.75
Stage 4	0.37	0.34	0.37	0.39	0.39	0.35	0.37
Stage 5	0.62	0.64	0.57	0.45	0.38	0.35	0.28
Stage 6	6.21	17.02	21.82	27.35	30.89	35.04	40.91

The percentage of the individuals with a liver disease diagnosis who have progressed from aetiology only (stage 1), aetiology and cirrhosis (stage 2), aetiology, cirrhosis and portal hypertension (stage 3), aetiology, cirrhosis and portal hypertension and decompensation (stage 4), aetiology and liver cancer stage 5, and aetiology and mortality by any cause (stage 6). At baseline entry of registry between 1999 and 2019 and up to 10 yr of follow up. ArLD: Alcohol-related liver disease; NAFLD: Non-alcoholic fatty liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

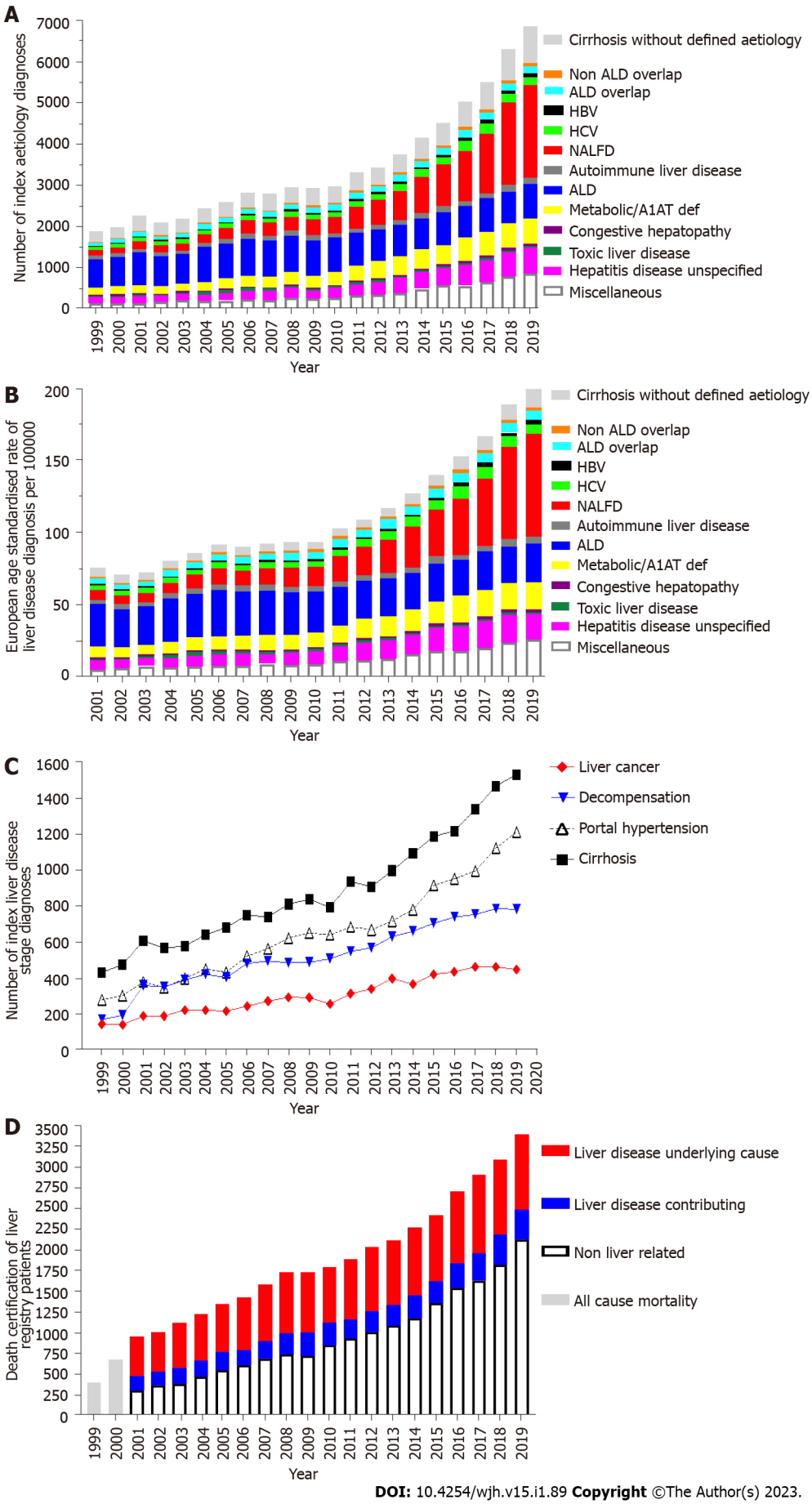
mortality over 10 years (Figure 3F). Progression to advanced stages of liver disease appeared to most frequently occurred in the first 3 years following entry into the registry (Figures 3A-F).

Liver disease recorded on death certification

The variation in progression to advanced stages of liver disease suggests that liver-related mortality will vary considerable between aetiologies of liver disease. The ONS records diagnoses that are the underlying cause of death and contributary causes. We compared the number of individuals who had a liver disease diagnosis listed as the underlying cause of death, other recording of liver disease or no mention of liver disease on the death certificate. Absolute number of deaths caused directly by liver disease was highest in the cohort ArLD (n = 6238) and this was approximately 10-fold higher than deaths due to liver disease in hepatitis not specified (n = 704), NAFLD (n = 559) or autoimmune liver diseases (n = 334, Figure 4A). ArLD, overlap and autoimmune liver diseases were associated with the greatest proportion of liver-related deaths. A third of individuals with NAFLD who died had a liver disease diagnosis recorded on their death certificate. The lowest rates of liver-related death were associated with metabolic liver disease, congestive hepatopathy and miscellaneous liver disease diagnoses (Figure 4B). These data are in keeping with diagnoses associated with highest rates of progression to cirrhosis and decompensation (Figure 3).

DISCUSSION

Accurate epidemiological data covering the broad arc of liver disease diagnoses and stages has the potential to improve the planning of hepatology services. In this paper we have proposed a novel epidemiological approach to analysing routinely coded data with 3 key features: (1) Defined groups of ICD-10 codes with the application of a hierarchy order of the most clinically relevant codes; (2) Linkage



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Figure 2 Incidence of liver disease in Wales 1999-2019. A: The incidence of liver disease by aetiology recorded by routine coding following inpatient and

day case admission and on death certification in Wales between 1999 and 2019; B: The European Age Standardised Rate of the incidence of liver disease in Wales between 2001 and 2019; C: The incidence of index diagnosis of liver disease stage (cirrhosis, portal hypertension, decompensation and liver cancer) caused by liver diseases of all aetiologies in Wales; D: All cause mortality in individuals with a preceding liver disease diagnosis in Wales 1999-2019. ArLD: Alcohol related liver disease; NALFD: Non-alcoholic fatty liver disease; HCV: Hepatitis C virus; HBV: Hepatitis B virus; A1AT: Alpha 1 antitrypsin deficiency.

of diagnoses on an individual level to assess the clinical stage of liver disease; and (3) Monitor progression and mortality. To knowledge this approach to capture all diagnoses and stages of liver disease has not been applied previously in the United Kingdom or internationally. We have applied this methodology to the Welsh national data warehouse to establish the first dedicated national liver disease registry providing novel insight into the changing incidence and progression associated with different liver diseases.

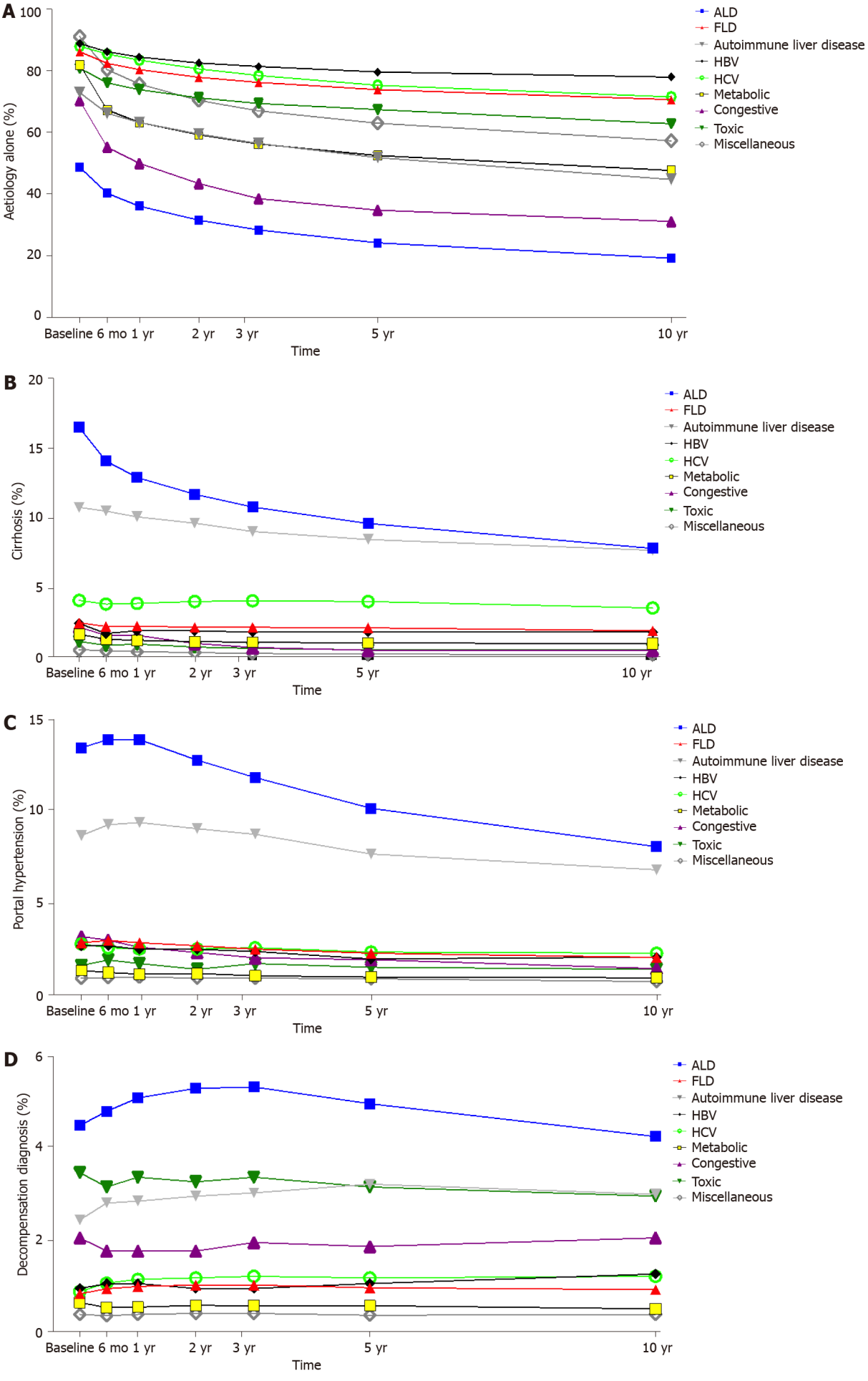
The Wales Liver Registry methodology builds on the Hepahealth project grouping of ICD-10 code which was predominantly applied to mortality data[15]. To increase the relevance to longitudinally collected routine hospital coding data these groupings have been modified with the addition of a hierarchical ordering of aetiological diagnoses removing less specific overlapping codes. We have prioritised diagnoses that predominantly drive the long-term liver disease outcomes to better inform appropriate service level interventions. Analysis of outpatient data suggests that routine inpatient coding will underestimate the incidence of most aetiologies, in particular viral hepatitis (Supplementary Figure 3). Importantly, however, the significant sequalae of chronic liver disease are well captured within routine inpatient coding compared to consultant led outpatient diagnoses. In addition to being key diagnoses related to patient symptoms, quality of life and mortality[24], this data informs the provision of secondary care resources screening for varices and HCC. This approach has also allowed analysis of subsequent diagnoses indicating progression, and liver disease related deaths. This has highlighted aetiologies that frequently present in an advanced disease state and are associated with poor outcomes such as ArLD and congestive hepatology (Table 4). Conversely in 2019 NALFD had a 4-fold higher incidence than ArLD but was associated with fewer index cases of cirrhosis, decompensation and liver-related deaths underlying the importance of defining morbidity in addition to incidence and mortality. This approach has the potential to be applied to a variety of chronic conditions to inform practical registries which are reflective of disease progression with increased diagnostic specificity.

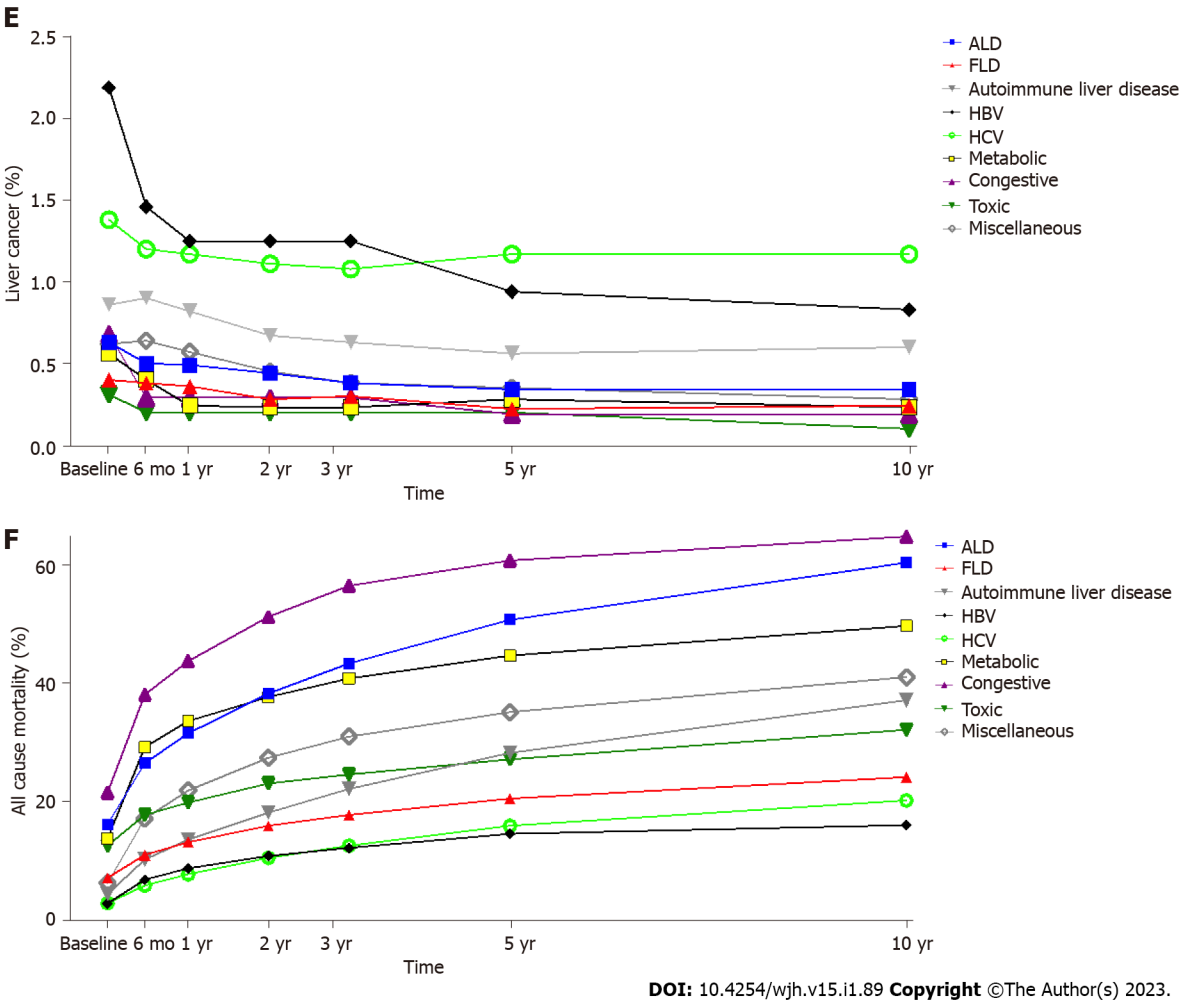
Incidence and outcomes of liver disease

The incidence of liver disease has increased 3.6-fold over the last 21 years in Wales, predominantly driven by a 10-fold increase in NAFLD diagnoses (Figure 2). This is in keeping with trends in NAFLD around the world[25]. Between 2004/5 and 2018/19 the proportion of overweight and obese individuals in Wales increased from 55% to 60% and obesity from 18% to 25%[26,27]. The rapid increase in incidence of NAFLD in the registry may reflect the evolution of establishing NAFLD in individuals over several years. Other co-morbidities that contribute to the incidence of NAFLD include diabetes and insulin resistance. The incidence of diabetes in Wales has doubled in the last 21 years and Wales has the highest prevalence of diabetes in the United Kingdom (7.4%)[28]. The precise proportion of the overweight and obesity population that will develop NAFLD remains to be defined, nonetheless, at present, the trajectory of NAFLD diagnoses continues to climb. The proportion of individuals with NALFD who had cirrhosis increased by 70% over the 21 year period (Figure 4A). This increase is likely to reflect more severe and earlier onset of obesity in Wales in combination with earlier detection using novel diagnostic approaches including fibroscan. The rising incidence of NAFLD may also be related to increased recognition and diagnosis of NAFLD through national guidelines and primary care abnormal liver function test pathways and an increasing number of hepatologists in Wales altering clinical practice.

ArLD has a high rate of cirrhosis and advanced liver disease at presentation (35%) which will have a significant demand on healthcare resources. Interestingly, the incidence of ArLD peaked between 2006 and 2008 (approximately 925 diagnoses per year), before reducing towards baseline levels seen in 1999 (630 diagnoses). The fall in ArLD incidence coincides with a trend to a reduction in the consumption of alcohol within the United Kingdom[29], and the introduction of the alcohol duty escalator between 2008 and 2013. This increased duty on alcohol by 2% above the rate of inflation reduced alcohol affordability [14,30]. Such measures demonstrate how targeted policy intervention in the community may further reduce the proportion of individuals with ArLD presenting with decompensation. Minimum unit pricing, introduced in Wales in March 2020, may further impact trends in ArLD incidence and disease behavior.

The annual all-cause mortality of individuals with a liver disease diagnosis has increased 30% over the last 21 years. This increase is a tenth of the increment of liver disease incidence. All cause and liver related mortality was greatest in individuals with an ArLD diagnosis and considerably lower for individuals with NAFLD. Advances in the management of chronic liver diseases are likely to have impacted upon mortality rates. In particular hepatitis C viral eradication with the advent of diatom diazotroph associations. Other factors that are likely to have had a significant impact are variceal


A
Aetiology alone (%)
100
80
60
40
20
0
Baseline 6 mo 1 yr 2 yr 3 yr 5 yr 10 yr
Time
ALD
FLD
Autoimmune liver disease
HBV
HCV
Metabolic
Congestive
Toxic
Miscellaneous
B
Cirrhosis (%)
20
15
10
5
0
Baseline 6 mo 1 yr 2 yr 3 yr 5 yr 10 yr
Time
ALD
FLD
Autoimmune liver disease
HBV
HCV
Metabolic
Congestive
Toxic
Miscellaneous
C
Portal hypertension (%)
15
10
5
0
Baseline 6 mo 1 yr 2 yr 3 yr 5 yr 10 yr
Time
ALD
FLD
Autoimmune liver disease
HBV
HCV
Metabolic
Congestive
Toxic
Miscellaneous
D
Decompensation diagnosis (%)
6
4
2
0
Baseline 6 mo 1 yr 2 yr 3 yr 5 yr 10 yr
Time
ALD
FLD
Autoimmune liver disease
HBV
HCV
Metabolic
Congestive
Toxic
Miscellaneous



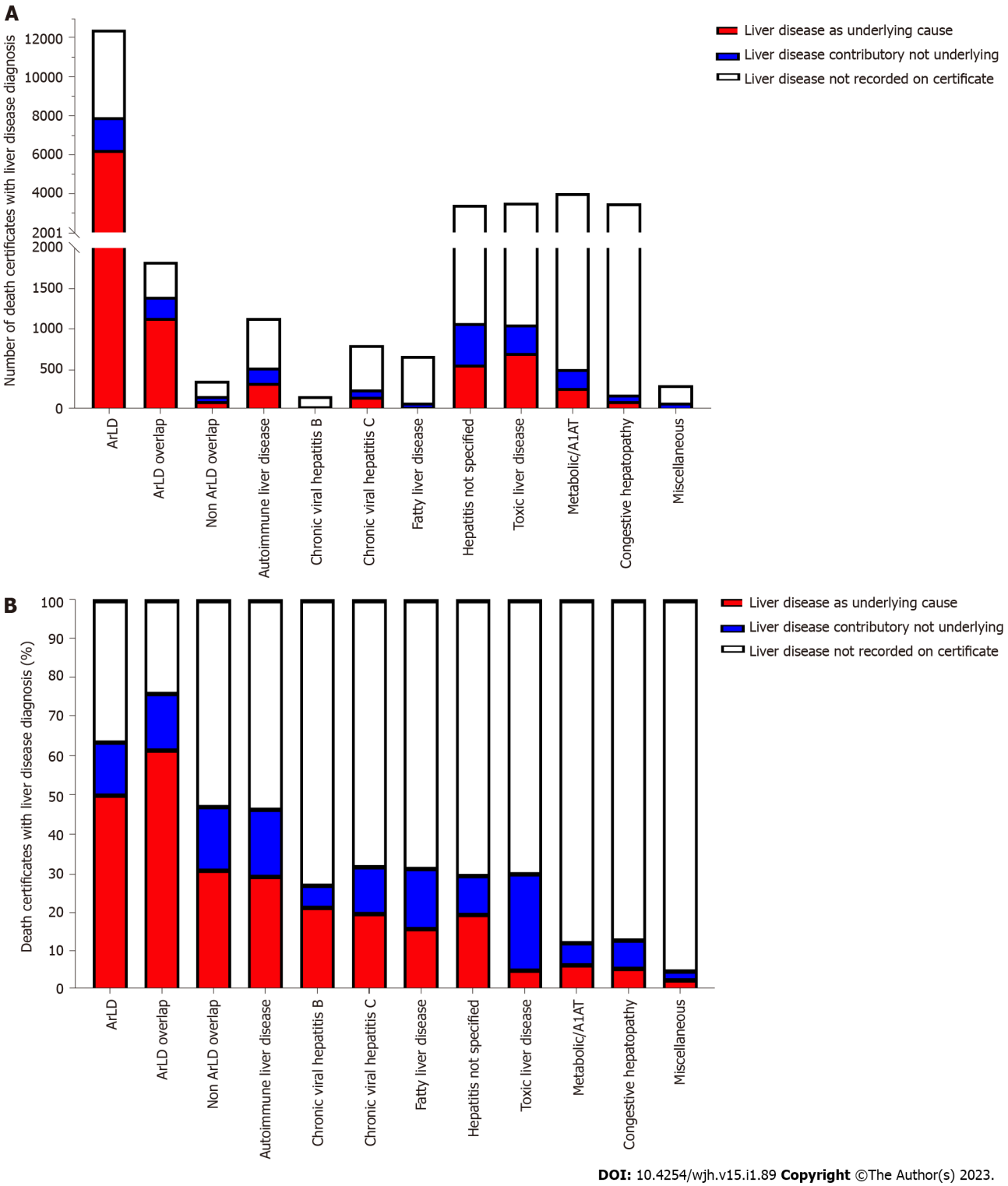
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Figure 3 Progression of liver diseases in Wales. The proportion of individuals at their most advanced stage of liver disease at baseline, 6 mo, 1, 2, 3, 5 and 10 years in Wales by aetiology. A: Aetiology (stage 1) diagnosis alone (not progressed); B: Cirrhosis (stage 2); C: Portal hypertension (stage 3); D: Decompensation (stage 4); E: Liver cancer (stage 5); F: All cause mortality (stage 6).

screening programmes and alcohol and lifestyle interventions. Our data suggests that the early period following initial diagnosis is associated with the greatest morbidity and mortality (Figure 3). This supports previous data suggesting that early specialist follow up and presumably optimal management is associated improved outcomes following decompensation[11].

Taken together these data confirm that the major causes of liver disease in Wales are driven by population health behaviors and lifestyle, which are, crucially, modifiable. Given the scale of the obesity and diabetes epidemics in Wales, hepatology services will need to adapt and expand to manage the 5%-6% of individuals who develop cirrhosis and advanced liver disease. To improve population health and the impact on clinical services, further targeted interventions on alcohol excess and body weight are required to reverse the rising trends observed in this study and prevent the adverse health consequences also identified. Identification of patients who will most benefit from long term follow up requires further investigation.

We recognise the limitations of this study in regard to the lack of primary care and complete outpatient data. Firstly, there is a reduction in the capture of aetiology diagnoses compared to the outpatient department particularly for viral hepatitis (Supplementary Figure 3B). This may in part reflect the efficacy of nucleos(t)ide inhibitors in suppressing HBV[31,32] and direct acting antivirals to eradicate HCV[33], with a reduction in these patients developing HCC or decompensation requiring inpatient hepatology services[34,35]. Secondly whilst up to 50% of index liver disease diagnoses in the United Kingdom occur within the inpatient setting this is associated with a worse outcome[36], this is likely to reflect more advanced underlying liver disease. The described natural history of liver disease is likely to be less severe if primary care data is included. However, acute hospital admission marks a milestone in the prognosis of liver disease defining outcomes from this time point is useful when counselling the need for major lifestyle interventions such as alcohol cessation. In future, primary care diagnoses of liver disease could be included in the Liver Registry by mapping SNOMED or read codes onto ICD-10 codes and applying the same analytic approach. This would provide further insight into the time from first presentation to medical services to disease progression. The major practical challenge



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Figure 4 Liver disease diagnoses on death certificates reported for individuals in the Wales Liver Disease Registry 1999-2019. A: The number of liver disease diagnoses that were reported as the underlying cause, reported on the death certification and certificates which were did not have any mention of liver disease for all individuals with an index diagnosis of liver disease in Wales between 1999-2019; B: The proportion of deaths by aetiology that were recorded with an underlying liver disease cause, liver disease reported on the death certificate or liver disease was not mentioned. ArLD: Alcohol related Liver Disease; A1AT: Alpha 1 antitrypsin deficiency.

to accessing this data in Wales, as elsewhere, is the lack of primary care coding data in the national secondary care data warehouse. Thirdly routinely coded diagnoses do not define non-cirrhotic disease is mild or more advanced. There are inherent assumptions associated with applying a framework to a complex disease natural history and the current approach lacks a certain level of granularity, for example, HBV serological markers of disease activity or levels of fibrosis and steatohepatitis in NAFLD. This level of detail would require significant specialised data input and would probably be best served

by separate or combined disease specific registries with additional clinical details and prospective data entry. Similarly cirrhosis could be stratified by inclusion of clinical scoring systems such as Childs Pugh or model for end-stage liver disease, however, it is not possible to access these results to incorporate into the Liver Registry.

CONCLUSION

We propose that the Wales Liver Registry methodology to group aetiological diagnoses and monitor time to progression is robust and has provided novel insights into liver disease in Wales prior to the coronavirus disease 2019 (COVID-19) pandemic. Development of an analytic pipeline will allow rapid assessment of COVID-19 which presents significant challenges in terms of a worsening picture in terms of liver disease lifestyle risk factors as well as impacting healthcare capacity. This data can be used to identify potential targets for the provision of hepatology services, including public health interventions, and assess their impact.

ARTICLE HIGHLIGHTS

Research background

The incidence, morbidity, and mortality related to liver disease is not well understood.

Research motivation

To develop a national liver disease registry to inform the provision of hepatology services.

Research objectives

Develop and apply a novel methodology for a national liver registry incorporating aetiology, stage and mortality related to liver disease.

Research methods

Novel methodology for developing a national liver registry using routinely coded secondary care and death certificate datasets.

Research results

The incidence of liver disease has increased 3.6-fold in Wales between 1999-2019 driven by a 10-fold increase in non-alcoholic fatty liver disease (NAFLD) 3-fold increase in cirrhosis, portal hypertension, decompensation and hepatocellular carcinoma, 2-fold increase in liver disease related mortality between 1999-2019. Actuarial tables of 10-year liver disease progression: Alcohol-related liver disease, autoimmune liver disease and congestive hepatopathy are associated with increased rates of decompensation and death compared to viral hepatitis and NAFLD.

Research conclusions

The Wales Liver Registry methodology provides a novel approach to understand the progression of liver disease in the setting of a rapidly altering incidence of liver disease in Wales.

Research perspectives

We have developed an analytic pipeline and will using this methodology to assess the impact of improvements in service provision.

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FOOTNOTES

Author contributions: Pembroke TPI, John G, Salmon J, and Yeoman A developed the concept and design of the study; Yousuf F, Czajkowski M and Yeoman A collected data; Pembroke TPI, John G, Puyk B, Howkins K, Clarke R and Yeoman A analysed the data; Pembroke TPI, Godkin A, Salmon J, and Yeoman A prepared the manuscript.

Institutional review board statement: This study was initiated as a dedicated workstream of the Liver Disease Implementation Group and Public Health Wales to establish a Liver Registry for Wales as a core workstream for this project. This manuscript represents the report of this commissioned work. As such it not been reviewed by the university review board.

Informed consent statement: This study was conducted in keeping with the Helsinki declaration. The design of this study was discussed with the South Wales research ethics committee and was not classed as research requiring regulatory approvals by Health Research Authority, United Kingdom.

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

Data sharing statement: No additional data are available.

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

Observational Study

Detection of colorectal adenomas using artificial intelligence models in patients with chronic hepatitis C

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Abstract

BACKGROUND

Hepatitis C virus is known for its oncogenic potential, especially in hepatocellular carcinoma and non-Hodgkin lymphoma. Several studies have shown that chronic hepatitis C (CHC) has an increased risk of the development of colorectal cancer (CRC).

AIM

To analyze this positive relationship and develop an artificial intelligence (AI)-based tool using machine learning (ML) algorithms to stratify these patient populations into risk groups for CRC/adenoma detection.

METHODS

To develop the AI automated calculator, we applied ML to train models to predict the probability and the number of adenomas detected on colonoscopy. Data sets were split into 70:30 ratios for training and internal validation. The Scikit-learn standard scaler was used to scale values of continuous variables. Colonoscopy findings were used as the gold standard and deep learning architecture was used to

train six ML models for prediction. A Flask (customizable Python framework) application programming interface (API) was used to deploy the trained ML model with the highest accuracy as a web application. Finally, Heroku was used for the deployment of the web-based API to https://adenomadetection.herokuapp.com.

RESULTS

Of 415 patients, 206 had colonoscopy results. On internal validation, the Bernoulli naive Bayes model predicted the probability of adenoma detection with the highest accuracy of 56%, precision of 55%, recall of 55%, and F1 measure of 54%. Support vector regressor predicted the number of adenomas with the least mean absolute error of 0.905.

CONCLUSION

Our AI-based tool can help providers stratify patients with CHC for early referral for screening colonoscopy. Along with providing a numerical percentage, the calculator can also comment on the number of adenomatous polyps a gastroenterologist can expect, prompting a higher adenoma detection rate.

Key Words: Machine learning; Calculator; Artificial intelligence; Hepatitis C; Screening

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Core Tip: Hepatitis C is associated with a wide array of extra-hepatic manifestations. In this study, we evaluated the incidence of colorectal adenomas and adenoma detection rates in hepatitis C patients. We developed an artificial intelligence-based tool to guide physicians in the detection and diagnosis of pre-malignant and malignant colorectal pathologies in these patient populations.

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INTRODUCTION

Hepatitis C is the most common blood-borne infection worldwide despite being gravely under-diagnosed[1,2]. Data from the National Health and Nutrition Examination Survey 2013-2016 and four other high-risk populations; homeless, incarcerated, active-duty military, and nursing homes estimates that approximately 4.1 million persons in the United States (approximately 1.7% of the population) were hepatitis C virus (HCV) antibody-positive indicating past exposure and 2.4 million persons (approximately 1% of the population) were HCV RNA positive indicating an active infection[3].

Although HCV is thought of as a primary disease of the liver, it has a wide array of extrahepatic manifestations, including skin, blood, lymphatic and intestinal pathologies[4,5]. The United States Chronic Hepatitis Cohort Study showed an increased incidence of rectal cancer with increased mortality in chronic HCV, but not colon cancers[4-6]. Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second most common cause of cancer-related death worldwide[3,7].

The current gold standard for diagnosing CRC is colonoscopy. The United States Preventive Services Task Force, American College of Gastroenterology, and American Cancer Society recommend screening for CRC starting at age 45 years in average-risk individuals[8-10]. There are no specific screening guidelines for CRC in patients with hepatitis C, despite there being an increased association.

Importance of artificial intelligence

Artificial intelligence (AI) is the new silk road. It is a technique that allows machines to store large amounts of data from various sources, process them accurately, reason, and even simulate human intelligence to provide a plan for clinical treatment. AI has augmented medical research on an enormous scale in recent years and continues to do so. It has significantly helped reduce the workload of clinicians and healthcare staff[11].

There are multiple facets of AI used in gastroenterology such as convolutional neural network (CNN), deep learning (DL), machine learning (ML), and computer-aided design (CAD)[12]. ML is a subset of AI, DL is a subset of ML and neural networks make up the backbone of DL algorithms. Multiple AI-assisted systems such as EndoBRAIN CAD by Kudo *et al*[13] and a CNN-based auxiliary

model by Ding *et al*[14] have been used to detect and diagnose bowel pathologies including colonic adenomas and neoplasms with higher sensitivity and specificity as compared to trained endoscopic experts[13,14].

ML is one of the subsets of AI where algorithms are trained for specific tasks. This is being increasingly used in modern medicine for analyzing large volumes and complex data. ML can be applied in any clinical setting to include mathematical and statistical assumptions that are unfamiliar to most clinicians. It can be performed in a two-step process. Firstly, the clinical question. Secondly, which factors in the clinical question/process can be optimized. Data management is another aspect of ML that is crucial. The availability of high-quality data can train the ML algorithms and avoid data bias and errors that can erroneously skew the results.

The use of AI has shown an increase in both polyp detection rate and adenoma detection rate (ADR) which are the primary colonoscopy quality indicators. Each 1% increase in ADR leads to an approximately 3% decrease in the future risk of cancer[15]. As timely intervention in the form of screening colonoscopy can help detect pre-neoplastic or early stages of CRC[15], it is imperative to develop a tool that can help clinicians distinguish which patients with chronic hepatitis C (CHC) infection require early referral for colonoscopy. Rustagi *et al*[16] in a case-control study showed a significantly higher number of adenomas detected on screening colonoscopy in the CHC group. In addition, building an ML model which can help predict the expected number of adenomas can reduce missed adenomas, decreasing subsequent cancer and the overall burden of CRC on healthcare. This is the aim of our research in developing an AI/ML-based tool.

MATERIALS AND METHODS

Study design and setting

This observational study with cross-sectional data collection and analysis was conducted at a community hospital in Massachusetts, USA. The institutional review board approved the study. The results were tabulated and statistically analyzed using computer software (SPSS version 25 for Windows, SPSS Inc., Chicago, IL, USA). Descriptive statistics for continuous variables were calculated with the Mann-Whitney U test, and categorical variables were calculated with the Chi-square test. The level of significance was set at $P < 0.05$.

Model training: Classification

Several ML models were trained and tested, and the model that could predict percentage probability with the highest accuracy was saved for the deployment stage.

Training and testing dataset characteristics

The dataset used to train and test the ML algorithms was collected manually and stored in a comma-separated file format. The dataset contained several attribute vectors from 415 patients [*i.e.*, sex, age, body mass index (BMI), obesity class, oral contraceptive use, significant alcohol use, hypothyroidism, intravenous drug use, diabetes mellitus, human immunodeficiency virus, concomitant statin use, controlled attenuation parameter (CAP) grade, HCV status, genotype, aspartate aminotransferase, alanine aminotransferase, platelet count, hemoglobin A1c, and triglycerides]. Some patients had missing data, which were replaced by zeros for training and testing. The task was to predict the percentage probability of adenoma occurrence in colonoscopy, followed by calculating the number of polyps. For this purpose, two ML models, *i.e.*, classification and regression models, were trained, and the best models were saved from both ML categories.

Preprocessing

The dataset was loaded onto a pandas DataFrame. The output label was colorectal adenomatous polyps. Numerical feature vectors were replaced with categorical variables. Categorical attributes such as HCV genotype and gene polymorphisms underwent label encoding. The variables with numerical values were scaled using the scikit-learn StandardScaler function to scale down to the desired range of 0-1. The complete dataset was split into a 70:30 ratio, in which 70% of the total dataset was used for training. The remaining 30% of the entire dataset was used to test internal validity and select the ML model with the highest predictive value.

ML algorithm internal testing

Several ML algorithms were trained to predict hepatic steatosis and CAP grades using the above-listed vectors. These models included: Support vector classifier, random forest, Bernoulli naïve Bayes (BNB), Gradient boosting classifier (GBC), logistic regression, and stochastic gradient descent classifier. All models were trained using 70% of the dataset. After training, each model was tested using the remaining 30% of the dataset The model with the highest testing accuracy, the GBC model, was chosen for external validity testing.

GBC model

The GBC model is a set of ML algorithms that additively combines multiple weaker ML models to produce a final predictive model. Our model assigned a binary classification to datasets and used multiple regressions along several decision trees to refine its attempts to predict the steatosis classification correctly. The model graded each attempt on a loss function which evaluates the extent to which the previous tree was inaccurate.

RESULTS

Patients were divided into cases (HCV) and controls (non-HCV). Data was tabulated as described in Table 1. Figure 1 is a strobe diagram with an overview of the methodology and results. The performance of different ML algorithms for training and testing to detect colorectal adenomatous polyps is shown in Table 2.

Using the colonoscopy results determined by the pathology of the biopsied polyp as the output, we applied a DL architecture using the above variables to train and test several ML models using a 415-patient dataset. As shown in Table 2, our results demonstrated that the BNB model had the highest testing accuracy (56%), precision (55%), recall (55%), and F1 score (54%). The distribution of actual and predicted labels during internal testing can be seen in Figure 2. As depicted in Table 3, our results demonstrated that the support vector regressor model had the lowest mean absolute error - 0.905, indicating it was the most suitable ML model to calculate an approximate number of polyps.

Application development phase

A flask-based web app was developed using the model with the highest accuracy for the application phase. A flash application programming interface (API) was used to deploy the trained ML model as a web application. The web-based API was then deployed into a web server using Heroku, a cloud application platform (https://adenomadetection.herokuapp.com/).

DISCUSSION

The initial analyses of the variables have demonstrated that median age of 62 years, with higher BMI, smoking, alcohol use, and concomitant aspirin use are related to adenoma detection with statistical significance among the data collected from patients with CHC who underwent colonoscopies. Using these initial datasets, DL architecture was used to train six ML models, which were prepared and validated using a 70:30 ratio of the dataset. Of the multiple models, the BNB model predicted the probability of adenoma detection with the highest accuracy, precision, and recall. Flask API was used to deploy the ML model, and Heroku web API was used to develop the AI tool. The model training and performance are shown in Tables 2-4.

The AI tool may be useful in the clinical setting to triage patients with hepatitis C, who have not received a formal CRC screening to stratify them into high-risk and low-risk groups. This can guide the gastroenterologist during the colonoscopy to help increase the ADRs. This knowledge of the increased risk of CRC incidence in hepatitis C and isolating patients that are high risk can prompt physicians to start CRC screening early and more frequently in these specific subsets.

Although multiple review articles have been published, there are very few clinical studies. To our knowledge, this is one of two retrospective analyses performed comparing CRC in hepatitis C and non-hepatitis C individuals. One of the main limiting factors was that the study was performed in the patient population that visited the primary care clinic at a community hospital in a single city in the northeast United States; it is limited by the geographical and socioeconomic aspects of the patient population. Analysis and validation were carried out on this limited sample size. Another drawback was that the study was primarily a retrospective analysis. Timing of the colonoscopy and the patient having active hepatitis infection might not always be documented, and timing was not specific in many patients, which limited the sample size that was analyzed.

AI application to healthcare has been increasingly pursued in the past decade, changing how we deliver advanced patient care[17,18]. AI and ML ensure the automation of many time-consuming activities and provide better insight into patient data that are not evident to providers[17]. Although the hype behind AI is promising, various barriers prevent the real-world application of these new AI systems. AI has helped in increased interoperability of extensive data, reasonable data-driven decisions involving evidence-based medicine, and hence a higher quality of care.

CRC is a commonly diagnosed malignancy in men and women with increasing mortality[19]. Many epidemiological studies have shown a positive association of CHC infection with extra-hepatic malignancies, especially gastrointestinal malignancies[16,19]. Our study focused on this positive association between CHC and CRC and on using AI and ML models to further ease the diagnosis in these patient populations.

Table 1 Patient demographics

Variables	Case (*n* = 109)	Control (*n* = 97)	OR	95%CI	*P* value
Age (mean ± SD in yr)	62.73 ± 9.146	60.20 ± 7.062			0.02[a]
Age median (yr), *n* (%)	49 (50.5)	58 (53.2)	0.54	0.31-0.95	0.03[a]
Gender, *n* (%) - female	48 (44)	46 (47.4)	0.87	0.50-1.51	0.62
BMI, median (IQR)	28 (7)	32 (7)			0.001[a]
Family history of hepatitis, *n* (%)	10 (9.2)	6 (6.2)	1.53	0.55-4.38	0.42
Aspirin use, *n* (%)	22 (20.2)	35 (36.1)	0.44	0.24-0.83	0.01[a]
Smoking, *n* (%)			2.31	1.31-4.07	0.004[a]
No	51 (46.8)	65 (67)			
Yes	38 (34.9)	15 (15.5)			
Former	20 (18.3)	17 (17.5)			
Total pack years, median (IQR)	25 (21)	25 (11)			0.75
Alcohol use, *n* (%)	12 (11)	24 (24.7)	0.37	0.17-0.80	0.01[a]
DM, *n* (%)	27 (24.8)	31 (32)	0.87	0.50-1.51	0.25[a]
HIV, *n* (%)	3 (2.8)	1 (1)	2.71	0.27-26.56	0.62[a]
Adenomatous polyps present	58 (53.2)	33 (34)	2.20	1.25-3.87	0.006[a]
Bowel preparation (%)					0.14
Good	90 (82.6)	88 (90.7)			
Fair	11(10.1)	7 (7.2)			
Poor	8 (7.3)	2 (2.1)			

[a]$P < 0.05$

OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; IQR: Interquartile range; DM: Diabetes mellitus; HIV: Human immunodeficiency virus.

Table 2 Machine learning model training

ML model	Test accuracy (%)	Precision (%)	Recall (%)	F1 score (%)
Support vector classifier	52	51	51	51
Random forest	53	53	53	53
Bernoulli naïve Bayes	56	55	55	54
Gradient boosting	50	49	49	48
Logistic regression	50	48	48	47
Deep neural networks	53	53	53	51

ML: Machine learning.

AI is integrated into our everyday life to such an extent that we barely remember what life was before it. This includes face recognition to unlock our phones, self-driven cars, chatbots for almost every business, and even ML-based financial fraud detection. AI-driven models are increasingly utilized to screen, diagnose and monitor multiple clinical conditions. Many AI algorithms have been used in CRC detection. Hu *et al*[20] performed ML simulations using S-Kohonen, Backpropagation and SVM neural networks, showing the S-Kohonen method's effectiveness for colon cancer classification[20].

Zhang *et al*[21] derived a cost-effective and sensitive method for detecting BRAF mutations in CRC using a counter propagation artificial neural network to distinguish mutant BRAF *vs* wild type[20,21]. Many such ML models are increasingly being utilized in precision oncology to precisely guide diagnosis and management decisions in CRC patients[19-21].

Table 3 Machine learning models and their mean absolute error

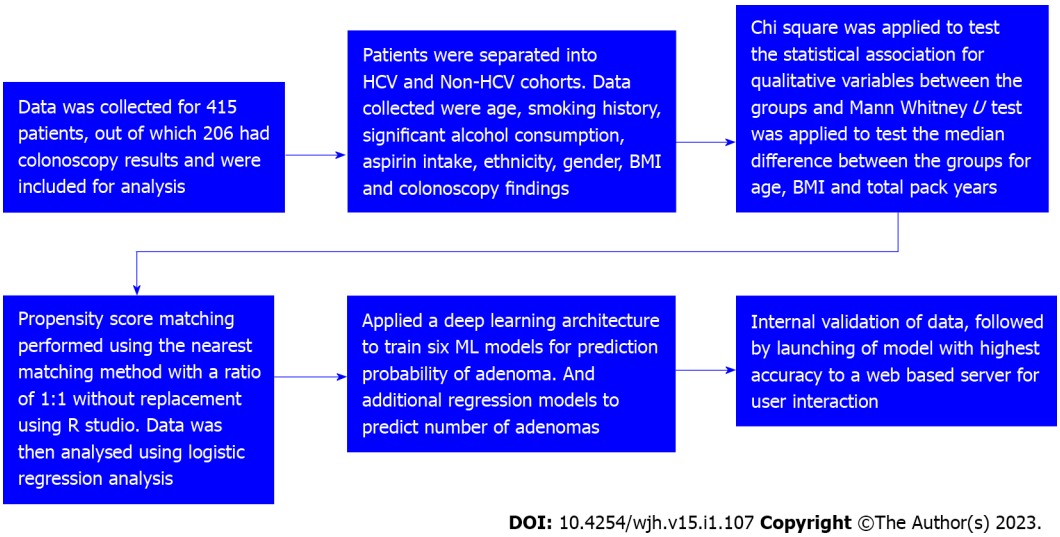
ML model	Mean absolute error
Linear regression	1.072
LGBM regressor	1.106
XGBoost regressor	1.273
ElasticNet	0.941
Gradient boosting regressor	1.139
Support vector regressor	0.905

ML: Machine learning.

Table 4 Machine learning models and their performance

ML model	Performance
Support vector classifier	Good
Random forest	Good
Bernoulli naïve Bayes	Optimal
Gradient boosting	Inadequate
Logistic regression	Inadequate
Deep neural networks	Good

ML: Machine learning.



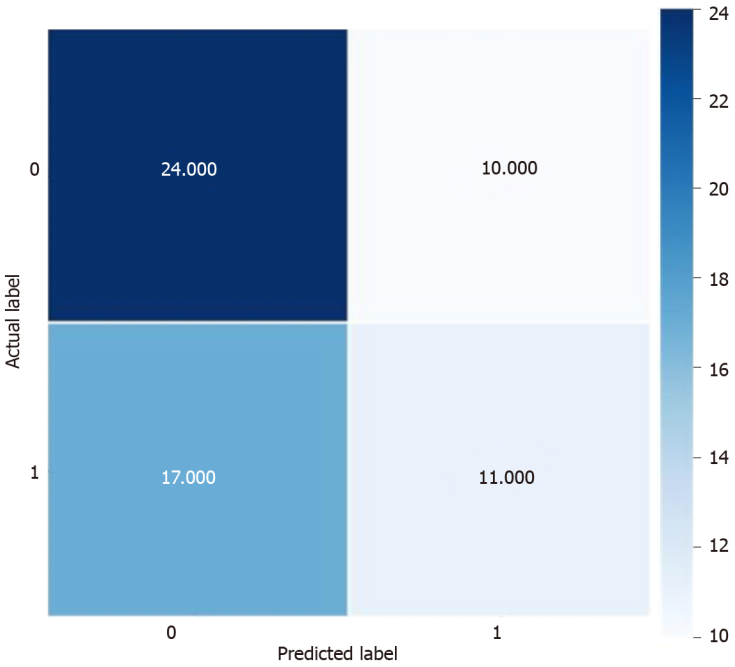
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Figure 1 Strobe diagram. HCV: Hepatitis C virus; ML: Machine learning.

AI tools will potentially transform our practice by leveraging massive amounts of data to personalize care to the right patient, in the right amount, at the right time. These novel tools assist physicians in the detection and early diagnosis of pre-malignant and malignant lesions in general and high-risk populations.

CONCLUSION

Our AI tool can be further modified based on the treatment of hepatitis C with the new direct-acting



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Figure 2 Distribution of actual label *vs* predicted label during internal testing.

antivirals, and how treated and cured hepatitis C alters the incidence of CRC in these groups. Long-term prospective studies, including a subgroup analysis between patients cured of hepatitis C, who had a relapse of the disease, and who refused or were untreated and how it affected CRC detection, would help guide diagnostics. Further validation with randomized controlled trials and multicenter participation will ensure replicability and repeatability of the results for the smooth incorporation of such AI-based tools into clinical practice[19-22].

ARTICLE HIGHLIGHTS

Research background

Several studies have shown that chronic hepatitis C virus (HCV) increases the risk of developing colorectal cancer (CRC). We conducted a study to analyze this positive relationship. We developed an artificial intelligence (AI) based tool using machine learning (ML) algorithms to stratify these patient populations into risk groups for CRC/adenoma detection.

Research motivation

We acknowledge the increased applications of AI with ML in medicine. Gastroenterology and hepatology have immense potential for AI integration. Hence, to develop an AI automated calculator, we applied ML to train models to predict the probability and the number of adenomas detected on colonoscopy.

Research objectives

Our objective was the create a readily available AI tool in the form of a calculator that healthcare providers throughout the globe can access to predict the prevalence of adenoma/CRC.

Research methods

We used colonoscopy findings as the gold standard and applied a deep learning architecture to train ML models for prediction. The institutional review board approved the study.

Research results

Data on 415 patients were collected. We discovered a higher incidence of adenoma/CRC in patients with chronic HCV in the untreated patient population. On internal validation, the Bernoulli naive Bayes ML model showed the highest predictive accuracy and recall for adenoma detection rates.

Research conclusions

Our AI-based tool shows an association between HCV and colorectal adenomas. This tool can help providers stratify their patients at increased risk of CRC and prompt early referral for colonoscopy.

Research perspectives

In the future, we would like to see this calculator being used in clinical practice as a preventative measure to increase early diagnosis of high-risk adenomas/CRC.

FOOTNOTES

Author contributions: Singh Y and Gogtay M contributed to the conceptual design of the study; Singh Y, Gogtay M, Yekula A, and Soni A independently screened the medical records and extracted the data; Tripathi K conducted the statistical analysis; Singh Y, Gogtay M, Yekula A and Soni A contributed to the write-up and submission of the study; Tripathi K, Mishra AK, and Abraham G reviewed the final manuscript; and all authors reviewed and agreed the final content of the article.

Institutional review board statement: We obtained approval from the joint institutional review board at MetroWest Medical Center (IRB#2021-035) before initiation and complied with the study protocol. The IRB waived the requirement of consent from study participants.

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LETTER TO THE EDITOR

Prognostic role of ring finger and WD repeat domain 3 and immune cell infiltration in hepatocellular carcinoma

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Abstract

We have found that the expression of ring finger and WD repeat domain 3 (RFWD3) is significantly higher in unpaired and paired hepatocellular carcinoma (HCC) tissues than in normal tissues. Moreover, this expression has a significant correlation with the infiltration level of 14 immune cell types and when the detected RFWD3 expression levels were grouped as high and low, a prominent difference was revealed for overall survival, disease-specific survival, and progression-free interval. Through statistical analysis (univariate Cox), we were also able to identify RFWD3 as an independent prognostic element for HCC, with RFWD3 having an ability to accurately predict HCC prognosis (area under the curve of 0.863). Finally, we have generated prognostic nomograms for probabilities of 1-, 3- and 5-year overall survival in HCC *via* integrating the factors of age, pathologic stage, alpha-fetoprotein level, and RFWD3 expression.

Key Words: Hepatocellular carcinoma; Ring finger and WD repeat domain 3; Immune cell infiltration; Bioinformatics

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Core Tip: We have discovered that ring finger and WD repeat domain 3 (RFWD3) expression is remarkably higher in tumor tissues compared to corresponding non-tumor tissues, regardless of hepatocellular carcinoma (HCC) tissue type (unpaired or paired). The RFWD3 expression also showed a significant correlation with the infiltration level of 14 immune cell types and was identified as an independent prognostic element in HCC by univariate Cox regression analysis. Our collective findings suggest that RFWD3 has the ability to accurately predict prognosis of HCC.

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TO THE EDITOR

We perused the recently published paper by Liang *et al*[1] with much interest. The authors reported on their assessments of ring finger and WD repeat domain 3 (RFWD3) expression levels in hepatocellular carcinoma (HCC) patients. Their findings included RFWD3 effects on HCC prognosis, the processes of proliferation, invasion and metastasis, and the underlying mechanisms, specifically regulation *via* the Wnt/β-catenin signaling pathway. We have a particular appreciation for these authors' novel investigation into the prognostic implication of RFWD3 in HCC as we have also discovered that the expression of RFWD3 is prominently higher in both unpaired and paired HCC tissues from HCC patients than in their corresponding normal tissues (Figure 1A and B).

According to the current literature, cancer cells, endothelial cells, stromal cells, immune cells, and cancer-associated fibroblasts cells all exist in the tumor microenvironment (TME)[3,4]. While the TME is known to play crucial roles in development, invasion and metastasis of HCC, the immune escape of HCC cells has yet to be fully understood and continues to complicate cancer treatment[5]. Due to the ongoing and well-known limitations of chemotherapy in general, immunotherapies are a hot topic of bench and clinical research. This newly emerging cancer therapy exploits immune cells both inside and outside the TME to target and attack cancer cells; its demonstrated advantages are high specificity and low side-effects[6]. The power of this therapeutic method's potential lies in the fact that different types of immune-related cells serve diverse roles; for HCC, the research into defining and developing those immune cells that inhibit/promote tumor processes has a long way to go[7].

Upon reading the report that Liang *et al*[1] found RFWD3 is able to affect the prognosis of HCC, we tested a hypothesis that the expression of RFWD3 may be associated with immune cell infiltration in HCC. Detailed information is shown in Table 1. Following our initial positive data, we systematically explored the correlation between RFWD3 expression and infiltration level of 24 immune cell types, using a single-sample gene set analysis (also known as ssGSEA) algorithm and Spearman coefficient correlation analysis[8]. We found that RFWD3 expression has a remarkable correlation with the infiltration level of 14 immune cell types (Figure 2A). Among them, RFWD3 expression was positively associated with the infiltration level of T helper (Th) cells in general, Th2 cells in particular, T follicular helper (TFH) cells, T central memory (Tcm) cells, activated dendritic cells (DCs), natural killer (NK) $CD56^{bright}$ cells, and eosinophils (all $P < 0.05$; Figure 2B-H). There were negative associations with cytotoxic cells, DCs, plasmacytoid DCs (pDCs), neutrophils, T gamma delta (Tgd) cells, T regulatory cells (Tregs), and Th17 cells (all $P < 0.05$; Figure 2I-O). We hope our findings will encourage further investigations into RFWD3 as an HCC immunotherapy. Detailed information on this aspect is presented in Table 2.

Importantly, we agree with the finding of Liang *et al*[1] that indicates higher RFWD3 expression is related to worse overall survival (OS) in HCC. We have found that OS, disease-free survival, and progression-free interval were prominently shorter in HCC patient tissues with high RFWD3 expression than in those with low RFWD3 expression (all $P < 0.05$; Figure 3A-C). Our further statistical analysis *via* univariate Cox regression identified RFWD3 as an independent prognostic element for HCC (Table 3). Generation of the receiver operating characteristic curve showed that RFWD3 has the ability to accurately predict prognosis in HCC (area under the curve of 0.863). Finally, we generated prognostic nomograms for probabilities of 1-, 3- and 5-year OS in HCC *via* integrating the factors of age, pathologic stage, alpha-fetoprotein level, and RFWD3 expression; each element was assigned a score according to its contribution to survival (Figure 3E).

Ultimately, our new findings highlight that the research of Liang *et al*[1] is worthy of attention and that subsequent efforts to build upon it, such as our related discoveries, may promote the next generation of effective and safe therapeutics, such as immunotherapies.

Table 1 Detailed statistical results of differential expression of ring finger and WD repeat domain 3 in hepatocellular carcinoma and normal tissues

Gene	Group	*n*	Minimum	Maximum	Median	IQR	Lower quartile	Upper quartile	Mean	SD	SE
RFWD3	Normal	50	0.504	1.504	0.98	0.251	0.883	1.133	1.013	0.208	0.029
	Tumor	374	0.62	3.5	1.544	0.755	1.245	2	1.647	0.559	0.029
	Normal	50	0.504	1.504	0.98	0.251	0.883	1.133	1.013	0.208	0.029
	Tumor	50	0.707	2.939	1.577	0.716	1.204	1.92	1.578	0.521	0.074

RFWD3: Ring finger and WD repeat domain 3; IQR: Interquartile range; SD: Standard deviation; SE: Standard error.

Table 2 Detailed information on the statistical correlation between ring finger and WD repeat domain 3 expression and immune cell infiltration

Gene	Immune cell type	Pearson's correlation coefficient	Pearson's *P* value	Spearman's correlation coefficient	Spearman's *P* value
RFWD3	Th2 cells	0.499	< 0.001	0.501	< 0.001
	Th cells	0.434	< 0.001	0.436	< 0.001
	Cytotoxic cells	-0.304	< 0.001	-0.314	< 0.001
	DCs	-0.281	< 0.001	-0.304	< 0.001
	pDCs	-0.261	< 0.001	-0.261	< 0.001
	Neutrophils	-0.210	< 0.001	-0.214	< 0.001
	TFH cells	0.226	< 0.001	0.213	< 0.001
	Tcm cells	0.187	< 0.001	0.164	0.002
	Tgd cells	-0.105	0.043	-0.142	0.006
	Tregs	-0.155	0.003	-0.121	0.019
	aDCs	0.141	0.006	0.114	0.027
	NK $CD56^{bright}$ cells	0.128	0.013	0.112	0.031
	Th17 cells	-0.170	< 0.001	-0.110	0.033
	Eosinophils	0.077	0.135	0.106	0.041
	Macrophages	0.096	0.063	0.071	0.171
	Th1 cells	0.090	0.081	0.064	0.214
	iDCs	-0.034	0.507	-0.061	0.241
	Mast cells	-0.053	0.309	-0.060	0.247
	CD8 T cells	-0.047	0.368	-0.058	0.260
	T cells	-0.013	0.796	-0.033	0.522
	Tem cells	0.085	0.100	0.020	0.704
	B cells	0.033	0.525	0.017	0.744
	NK cells	0.035	0.494	-0.012	0.810
	NK $CD56^{dim}$ cells	0.020	0.699	-0.001	0.979

aDCs: Activated dendritic cells; DCs: Dendritic cells; iDCs: Immature dendritic cells; NK: Natural killer; pDCs: Plasmacytoid dendritic cells; RFWD3: Ring finger and WD repeat domain 3; Tcm: T central memory; Tem: T effector memory; TFH: T follicular helper; Tgd: T gamma delta.

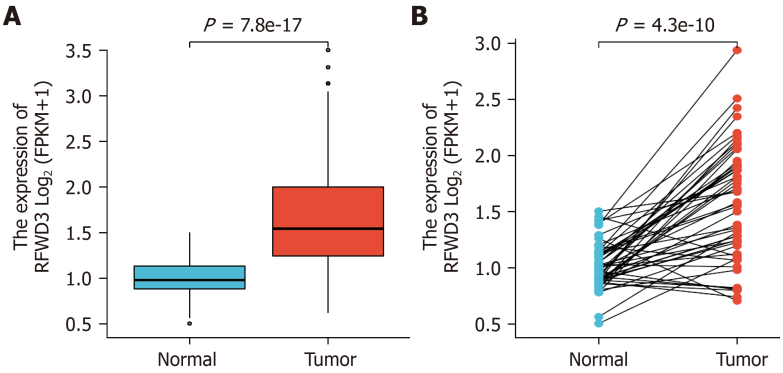
Statistical analysis

R statistical software (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/) was used for all statistical analyses. Wilcoxon rank-sum test was used to perform the differential expression analysis of RFWD3 between HCC samples and corresponding

Table 3 Univariate Cox regression analysis in hepatocellular carcinoma

Characteristics	Total, *n*	Univariate analysis	
		Hazard ratio (95%CI)	*P* value
Pathologic stage	349		
I	173		
II	86	1.417 (0.868-2.312)	0.164
III	85	2.734 (1.792-4.172)	< 0.001
IV	5	5.597 (1.726-18.148)	0.004
Child-Pugh grade	240		
A	218		
B	21	1.595 (0.757-3.361)	0.219
C	1	2.138 (0.294-15.544)	0.453
Fibrosis Ishak score	214		
0	75		
1/2	31	0.935 (0.437-2.002)	0.864
3/4	28	0.698 (0.288-1.695)	0.428
5/6	80	0.737 (0.410-1.325)	0.308
Histologic grade	368		
G1	55		
G2	178	1.162 (0.686-1.969)	0.576
G3	123	1.185 (0.683-2.057)	0.545
G4	12	1.681 (0.621-4.549)	0.307
RFWD3	373	1.557 (1.148-2.110)	0.004

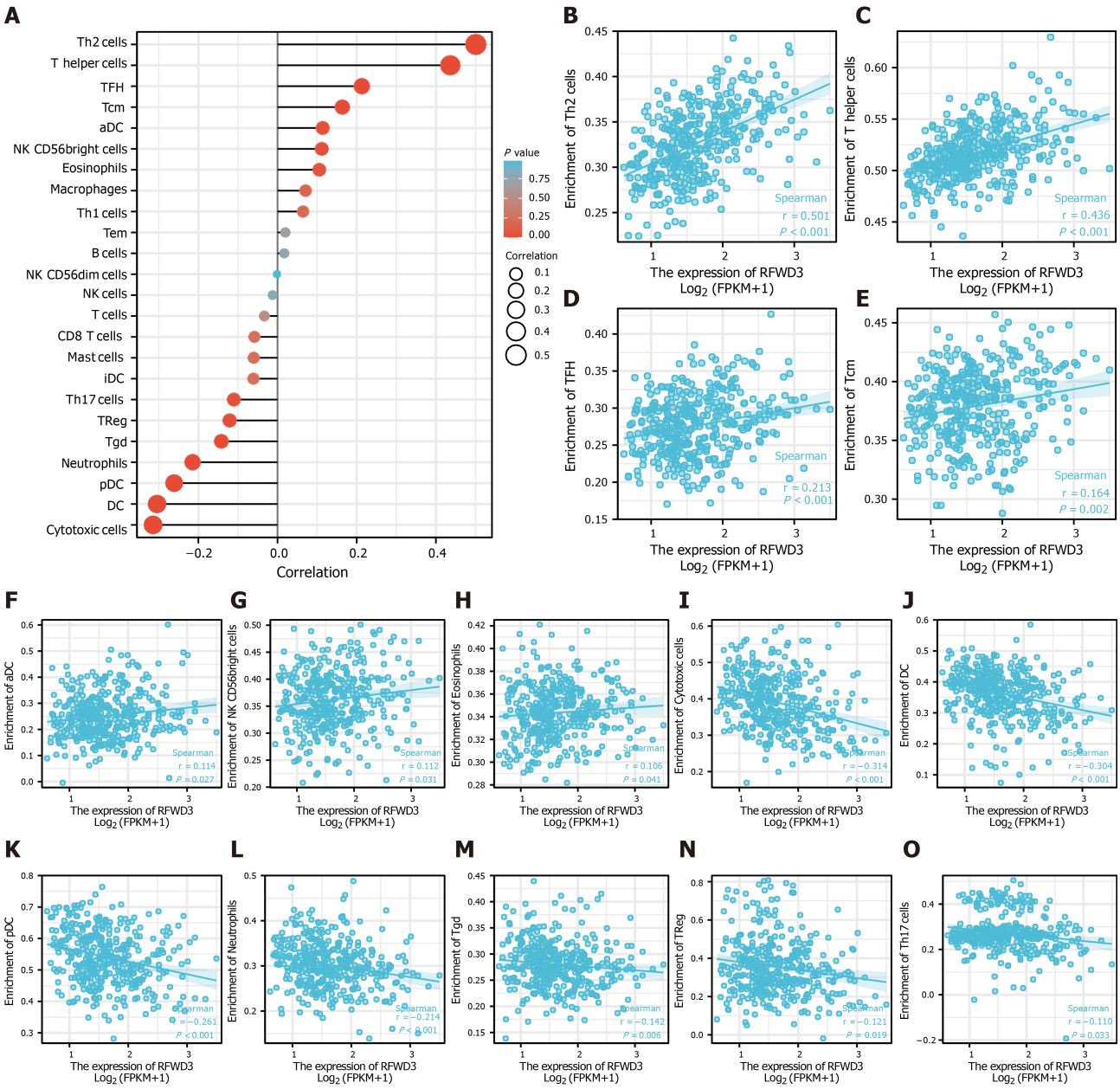
CI: Confidence interval; RFWD3: Ring finger and WD repeat domain 3.



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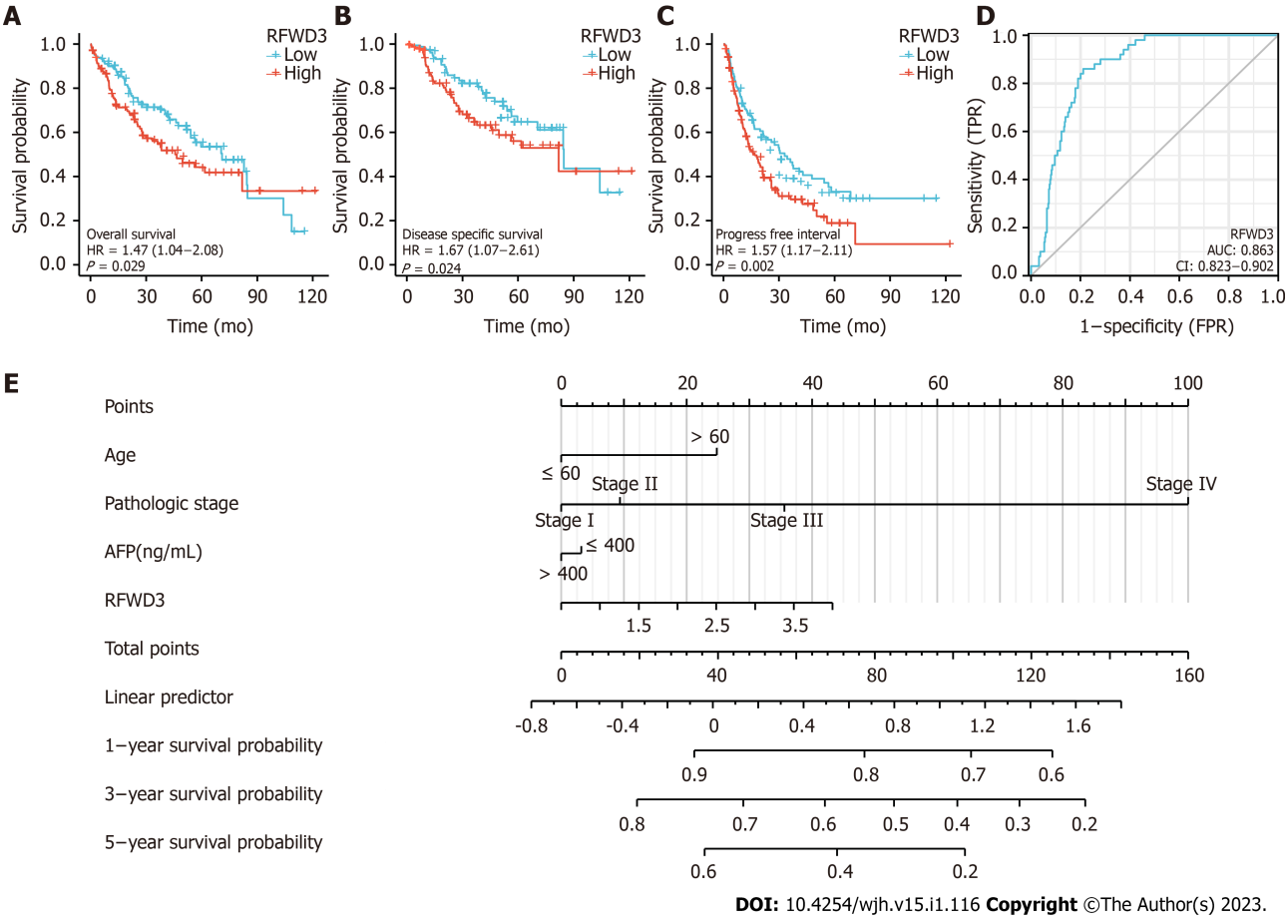
Figure 1 Differential expression levels of ring finger and WD repeat domain 3 in hepatocellular carcinoma and normal tissues. A: Non-paired hepatocellular carcinoma (HCC) and normal samples; B: Paired HCC and normal samples. Data source: mRNA-Seq data from the Genotype-Tissue Expression project (GTEx) of The Cancer Genome Atlas processed through the Toil process in the UCSC Xena database.(https://xenabrowser.net/datapages/)[2].

normal samples, with results demonstrated by the "ggplot2" R package[10]. Survival analysis was carried out by log-rank test and univariate Cox regression. The association between RFWD3 expression and immune cell infiltration were performed by Spearman and Pearson analysis. Positive values of correlation coefficient indicate positive correlation, negative values indicate negative correlation.



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Figure 2 Correlation analysis of ring finger and WD repeat domain 3 expression and immune cell infiltration in hepatocellular carcinoma.
A: Lollipop plot manifesting the correlation between ring finger and WD repeat domain 3 (RFWD3) expression and the infiltration level of 24 immune cell types; B-H: The infiltration levels of 7 immune cell types have significant positive correlation with RFWD3 expression; B: T helper (Th)2 cells; C: Th cells; D: T follicular helper (TFH) cells; E: T central memory (Tcm) cells; F: Activated dendritic cells (aDCs); G: Natural killer (NK) CD56^{bright} cells; H: Eosinophils; I-O: The infiltration levels of 7 immune cell types have significant negative correlation with RFWD3 expression; I: Cytotoxic cells; J: Dendritic cells (DCs); K: Plasmacytoid dendritic cells (pDCs); L: Neutrophils; M: T gamma delta (Tgd) cells; N: T regulatory cells (Tregs); O: Th17 cells.



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Figure 3 Survival analysis of ring finger and WD repeat domain 3 in hepatocellular carcinoma and the nomogram for prognosis. A-C: Ring finger and WD repeat domain 3 (RFWD3) expression is related to overall survival, disease-specific survival (B) and progression-free interval (C)[9] in the The Cancer Genome Atlas-liver hepatocellular carcinoma (HCC) data; D: Receiver operating characteristic curves for the RFWD3 gene's prognosis predictive ability. The nomogram can predict 1-, 3- and 5-year overall survival of HCC based on clinicopathological features and the expression of RFWD3.

FOOTNOTES

Author contributions: Miao YD and Zhang F designed the research; Miao YD wrote the paper; Quan WX and Wang JT performed the data analysis, Gan J and Dong X prepared the tables and figures; Zhang F and Miao YD reviewed the manuscript; Miao YD and Quan WX contributed equally to the article; All authors approved the final manuscript.

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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

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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.

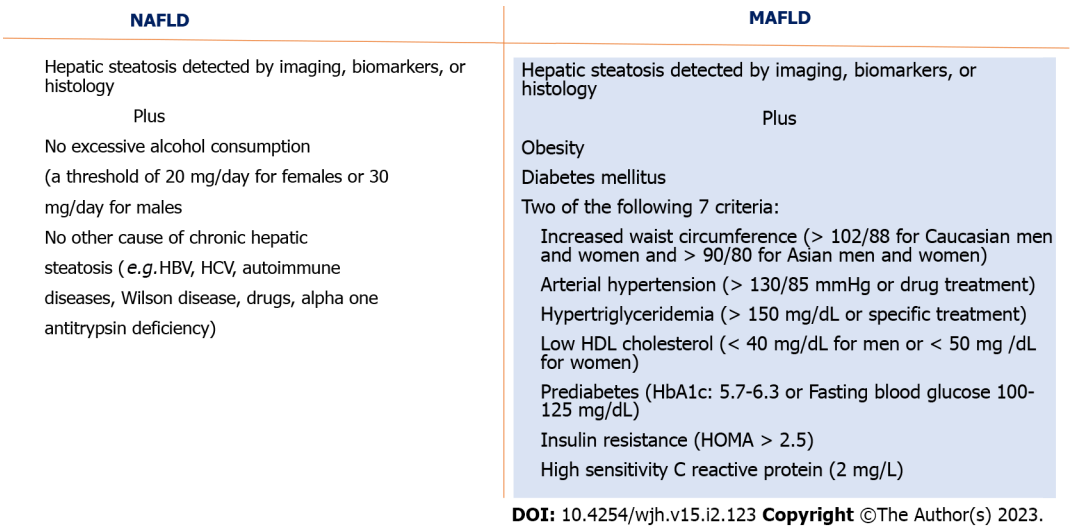
Among the many important findings, MAFLD criteria could better identify patients at risk of liver fibrosis than the NAFLD criteria in the American population[6]. High diagnostic ability of fatty liver index in the detection of steatosis was seen in patients with MAFLD[7]. Fibrosis-4 index and NAFLD fibrosis 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

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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

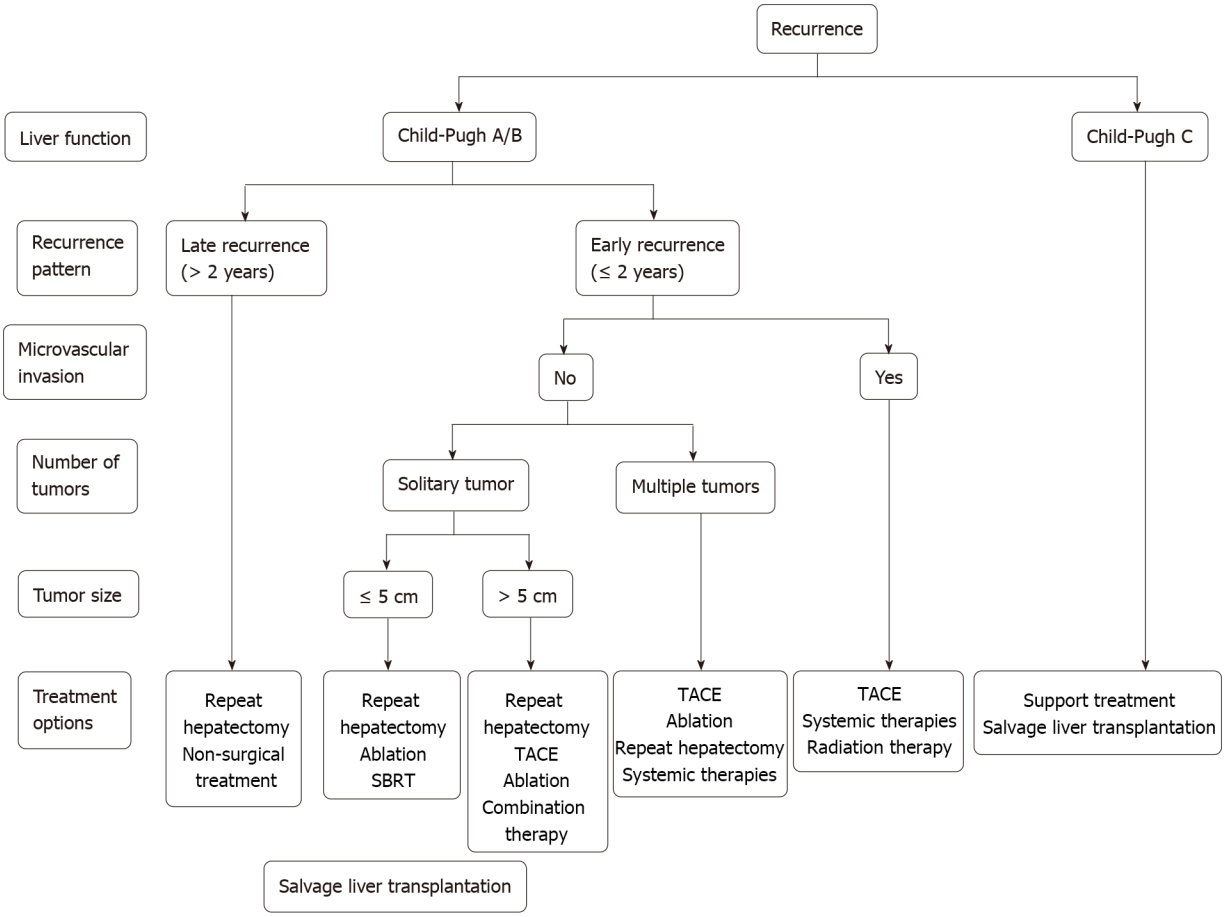
Ref.	Type	Years	*n*	1-, 3-, and 5-yr OS	1-, 3-, and 5-yr RFS
Sun *et al*[172]	Retrospective	2002-2014	57	98%/77%/53%	61%/27%/17%
Ho *et al*[103]	Retrospective	2001-2007	54	-/-/83%	N/A
Liang *et al*[182]	Retrospective	1999-2007	66	77%/49%/40%	N/A
Song *et al*[173]	Retrospective	1994-2012	178	99%/83% 71%	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

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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

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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

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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].

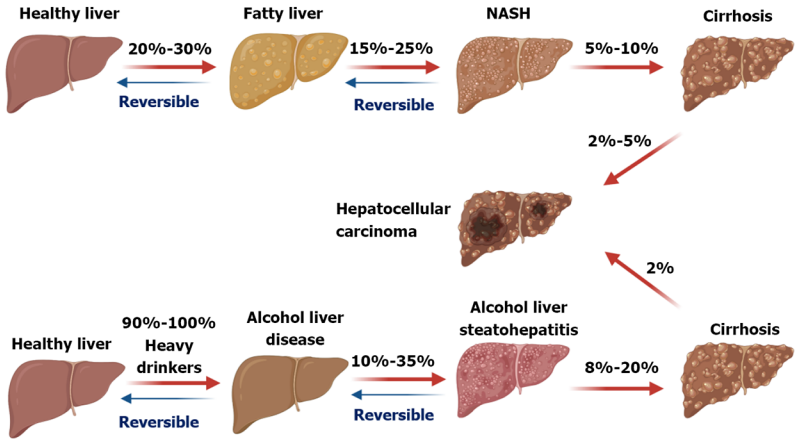
NAFLD is the most common CLD with a broad spectrum, ranging from non-alcohol fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) with the progression of liver inflammation and different degrees of fibrosis[12]. NASH also can progression to HCC (Figure 1)[13]. The global prevalence of NAFLD was 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.

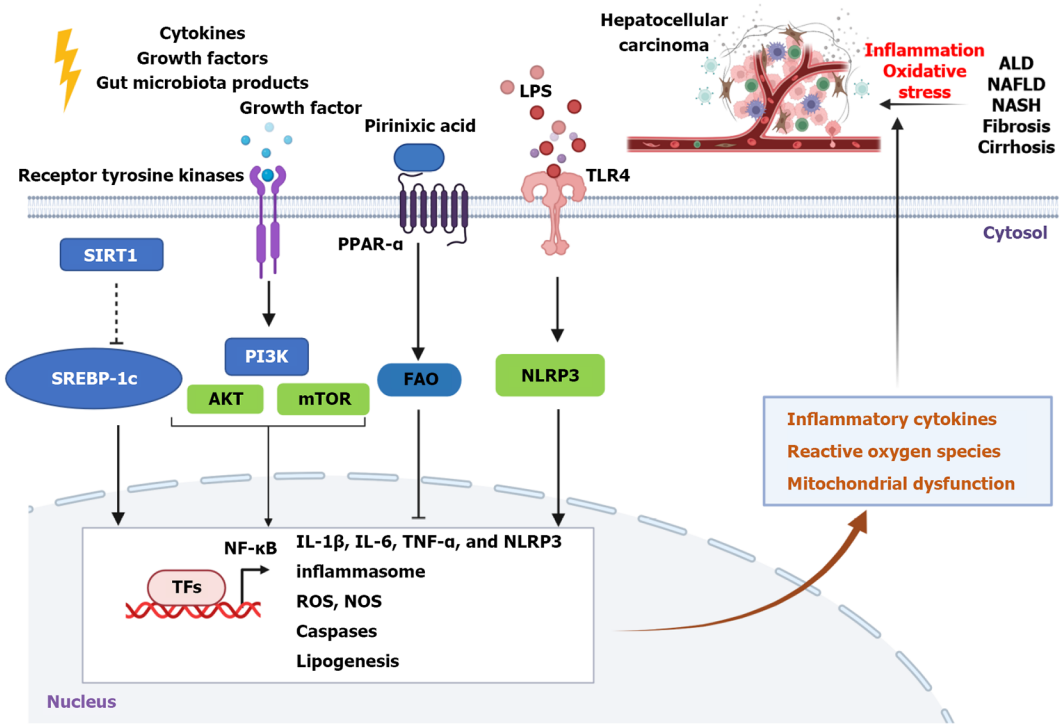
AMP-activated protein kinase

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

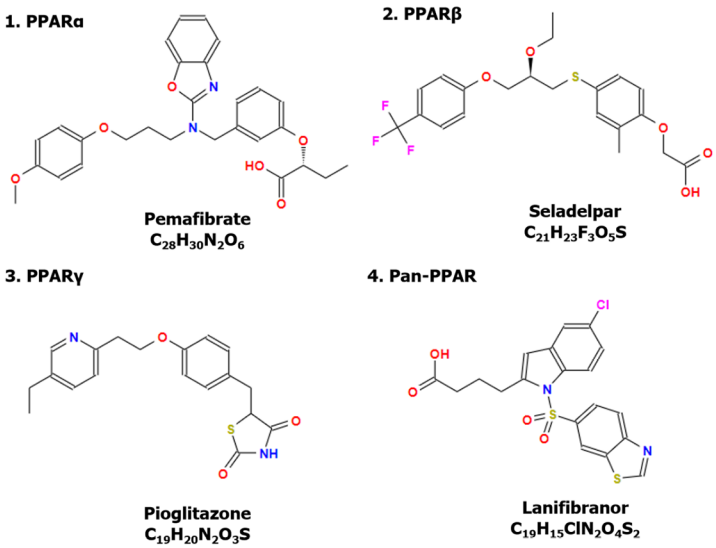
LF is an iron-binding protein in mammalian milk and displays multiple functions, including antioxidant, anti-cancer, and anti-inflammatory activities. During NASH progression, LF treatment can inhibit NF-κB activation to downregulate a high-fat diet and chemical dimethylnitrosamine-induced liver injury, 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

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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

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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].

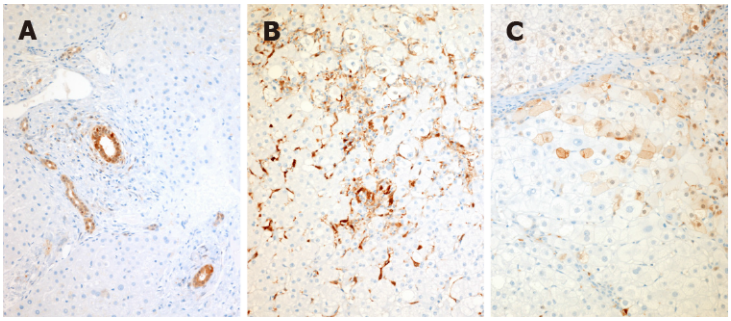


Figure 1 Immunohistochemical staining of normal, non-alcoholic steatohepatitis, and non-alcoholic steatohepatitis cirrhosis liver tissue with a galectin-3 antibody. A: Normal liver: In the normal liver, immunoreactive galectin-3 is present in epithelial cells of all large and small bile ducts and in 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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FOOTNOTES

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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

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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

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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

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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

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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

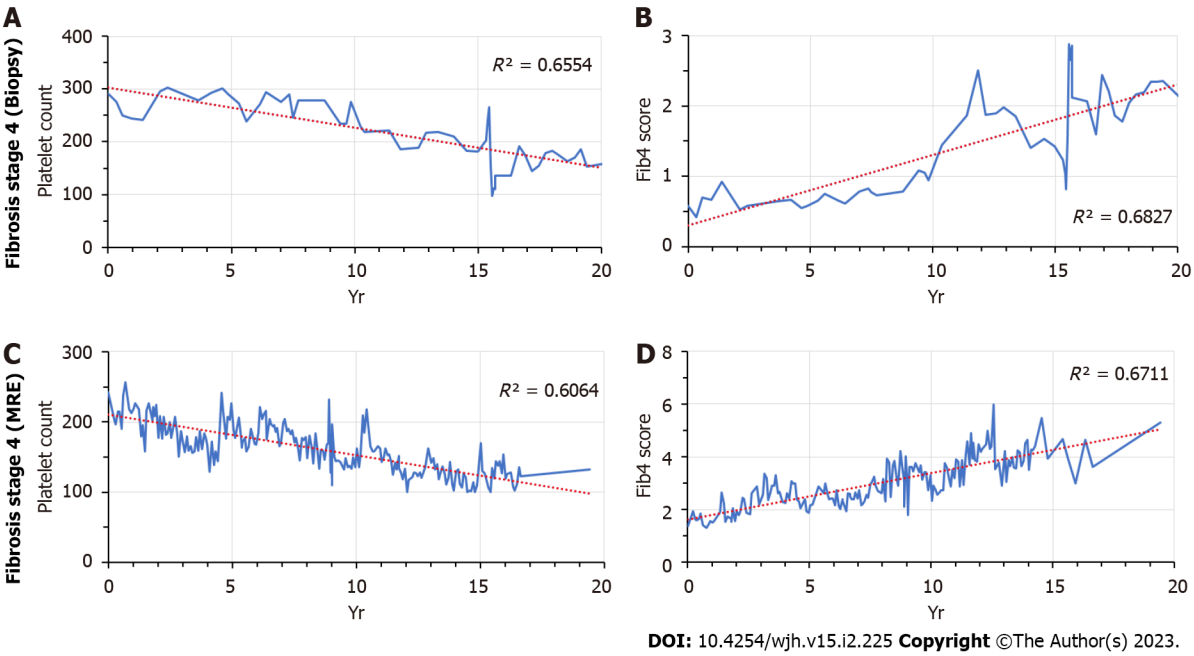
Predicator variable	Estimate	Std error	*t* value	Prob >|*t*|
Outcome: Fibrosis score on biopsy				
Fib-4 (last score)	0.214	0.048	4.43	**< 0.0001**
Correlation-platelets	-0.461	0.227	-2.03	**0.044**
Diabetes mellitus	-0.436	0.111	-3.94	**0.0001**
Hypertension	-0.181	0.112	-1.63	0.1062
BMI	0.02	0.016	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.

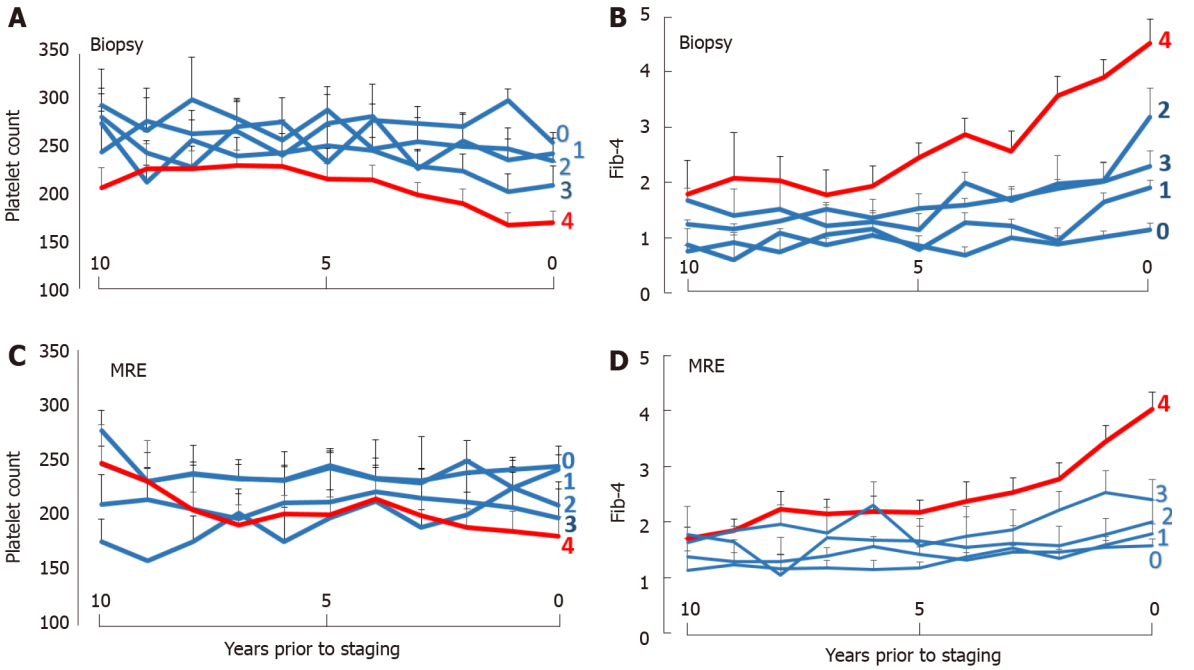
The development of thrombocytopenia in patients with chronic liver disease – regardless of its underlying disease etiology – is well known to hepatologists. Several mechanisms have been implicated for this phenomenon, including portal hypertension, and decreases in hepatic thrombopoietin production[14]. 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.

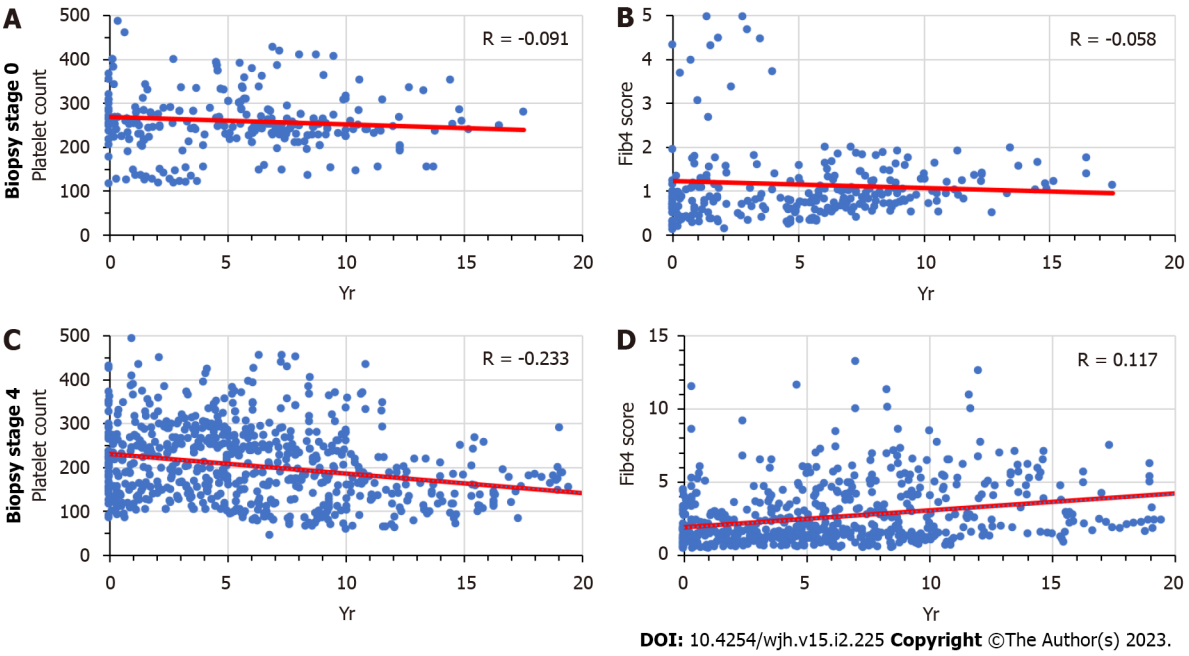


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Figure 2 Time-dependent changes in platelet counts and Fib-4 scores: Cohort averages. A: Biopsy cohort, platelet counts; B: Biopsy cohort, Fib4 scores; C: Magnetic resonance elastography (MRE) cohort, platelet counts; D: MRE cohort, Fib4 scores. Data 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.

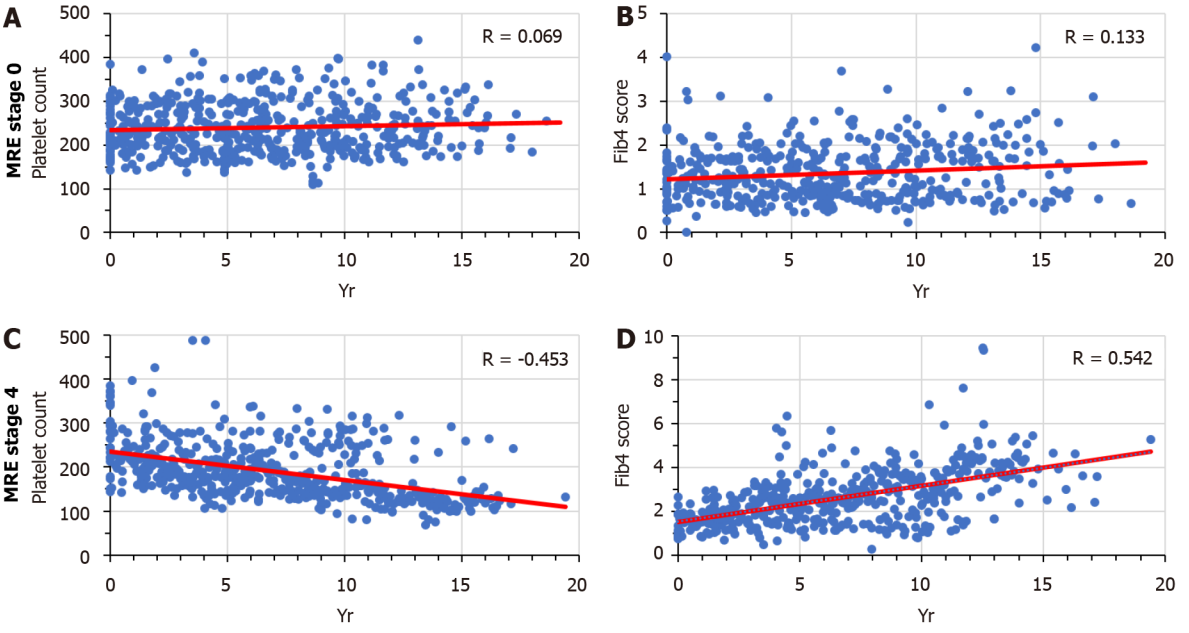
"advanced fibrosis", and we only included patients in whom the fibrosis stage had been determined by liver biopsy or MR elastography, the two modalities with the best performance characteristics.

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

You-Wen Tan, Jia-Min Wang, Xing-Bei Zhou

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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

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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

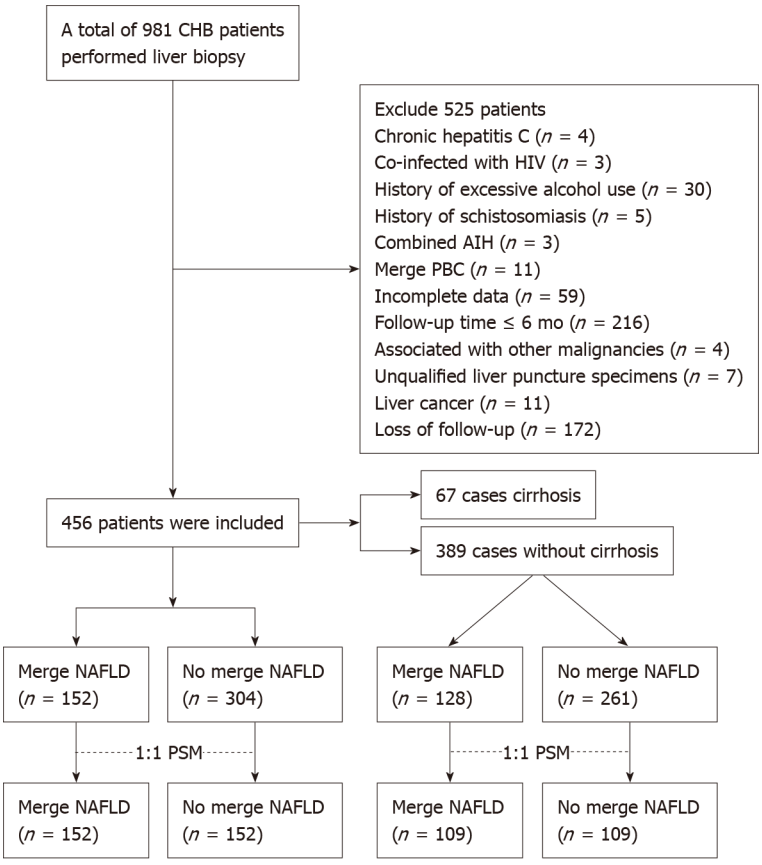
NASH: Nonalcoholic steatohepatitis; NAS: Activity score of nonalcoholic fatty liver disease; NAFLD: Nonalcoholic fatty liver disease.

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.

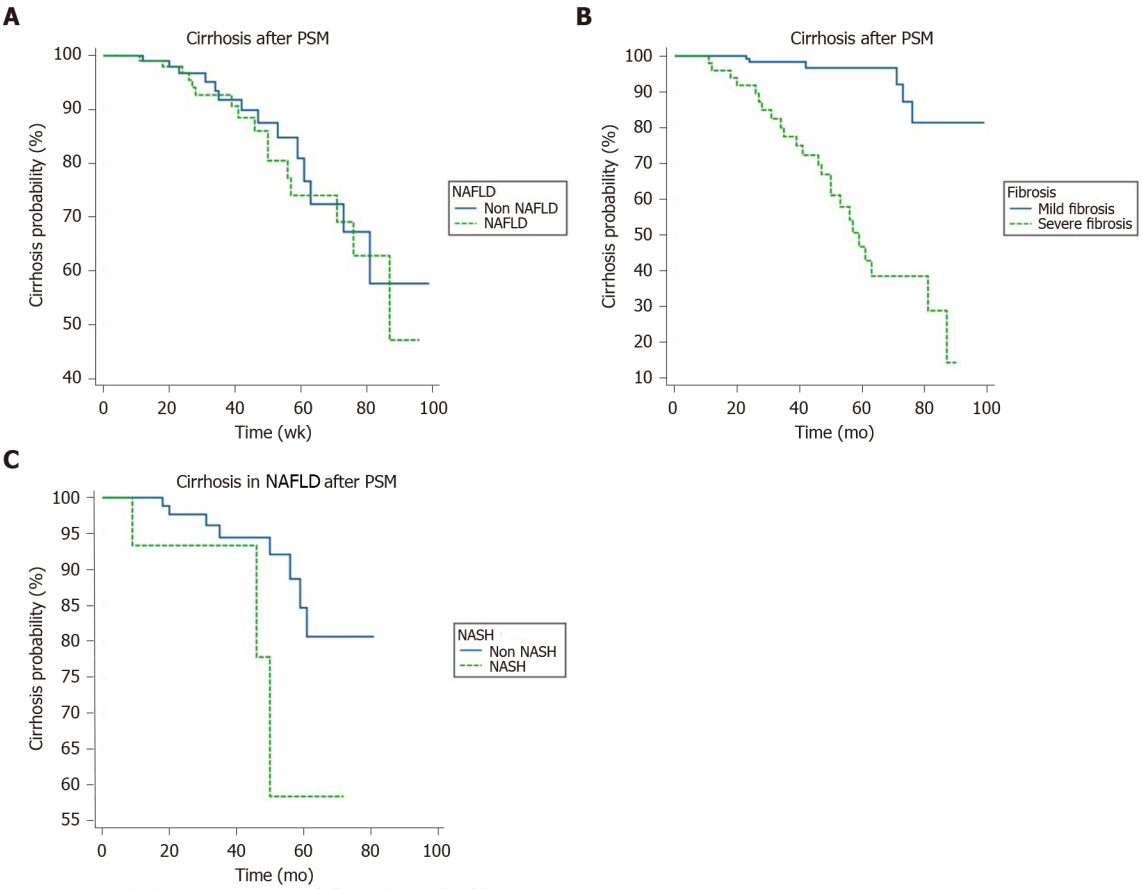
$P > 0.05$) (figures ignored).

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).

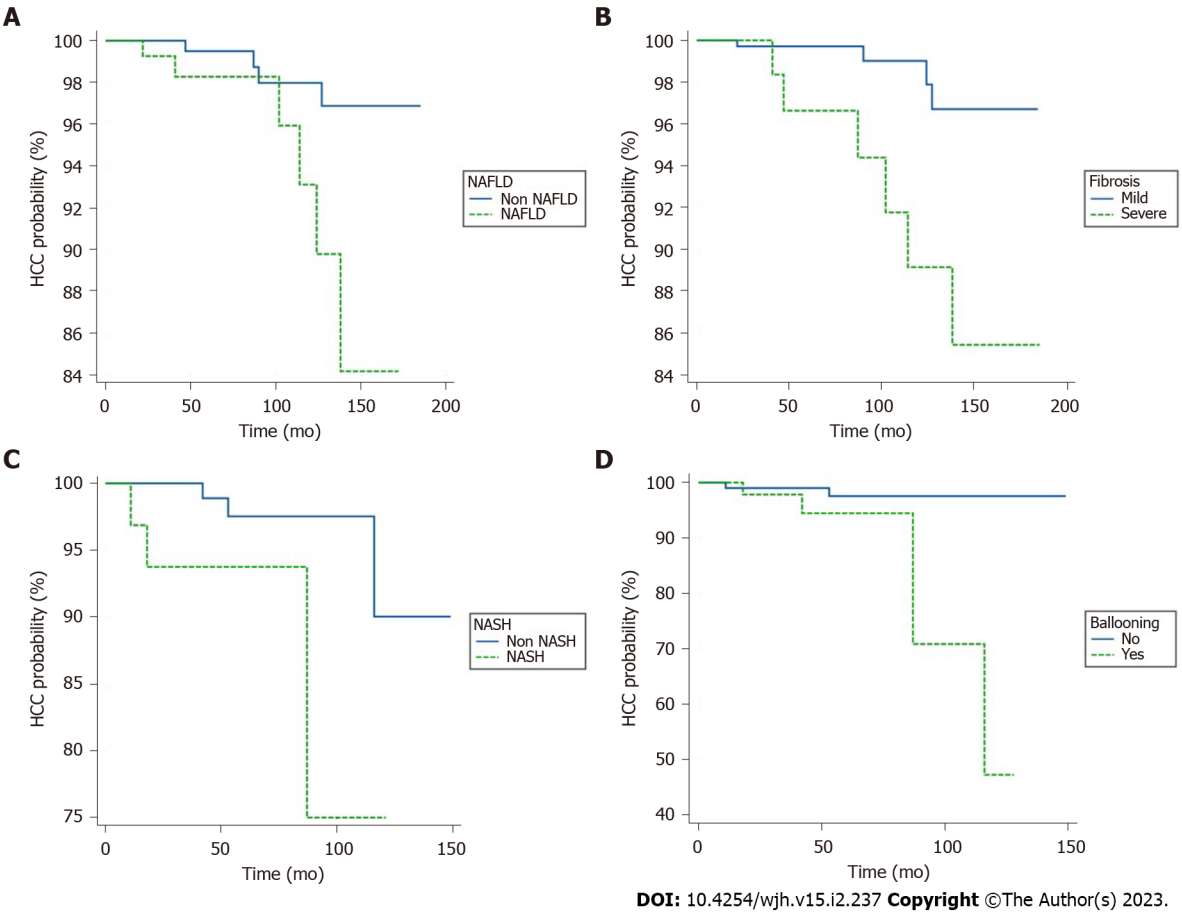
Table 8 Univariate and multivariate Cox regression analysis of hepatocellular carcinoma occurrence

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

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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

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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

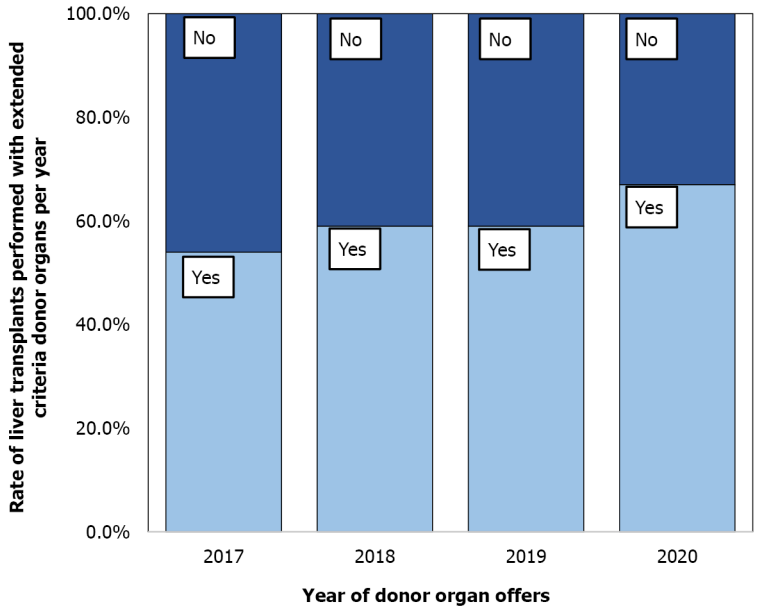
ICU: Intensive care unit; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

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.

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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

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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

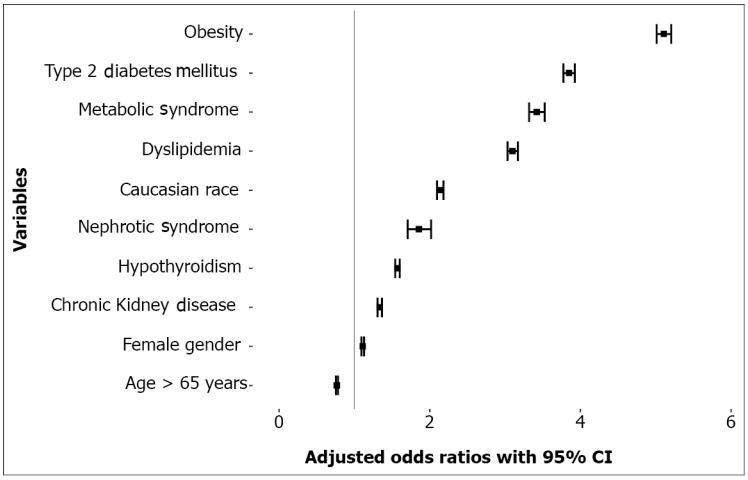
Parameters	NAFLD, *n* (%)	No NAFLD, *n* (%)	*P* value
Age, yr			< 0.00001
Adults 18-65	30980 (62.33)	56486180 (71.79)	
Seniors > 65	18720 (37.67)	22198870 (28.21)	
Gender			< 0.00001
Male	20640 (41.53)	35921730 (45.65)	
Female	29060 (58.47)	42763320 (54.35)	
Race			< 0.00001
Caucasian	39420 (79.32)	40569460 (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.



DOI: 10.4254/wjh.v15.i2.265 **Copyright** ©The Author(s) 2023.

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

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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%).

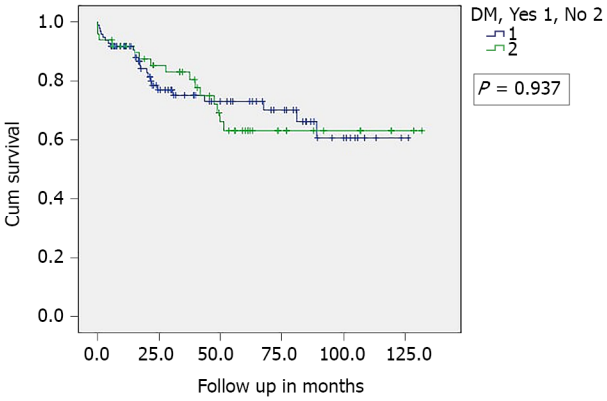
There was a similar median follow-up of 33.5 mo for both diabetic and non-diabetic groups, with a one-year survival rate of 89% (Figure 1). ACR arose in 20 (13.5%) of the total study population (DM = 13, 13.3% and non-DM = 7, 14%; P = 0.902). With regard to ICU readmission, the DM rate was 11 (11.2%) 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.

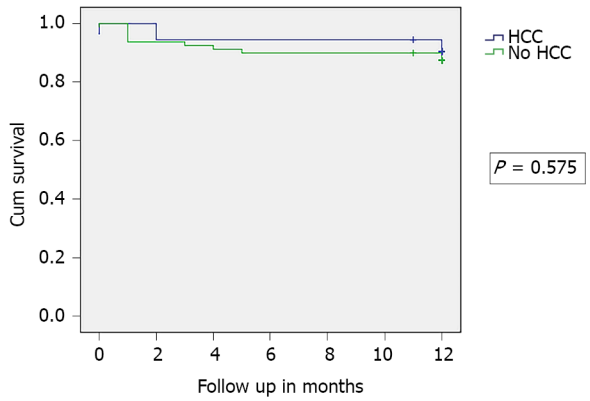


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Figure 1 One-year survival in our sample. DM: Diabetes mellitus.

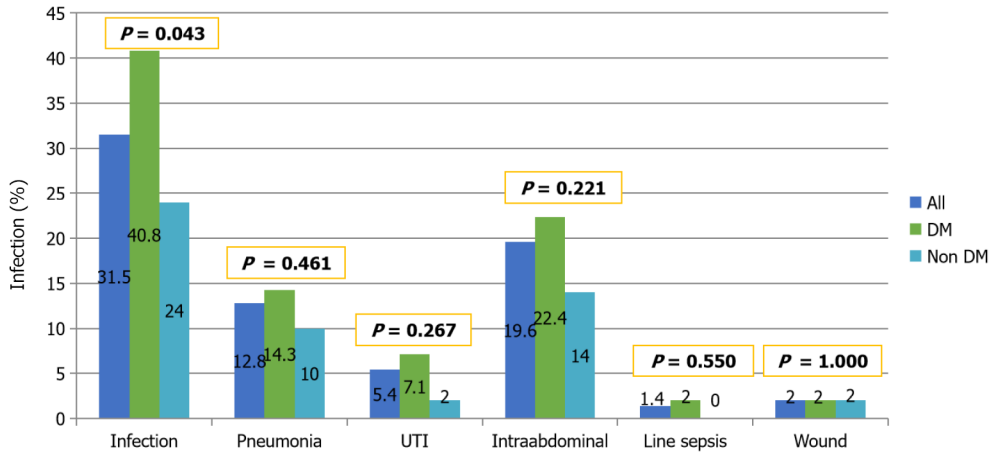
DISCUSSION

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

John Patterson Campbell, Vinay Jahagirdar, Adel Muhanna, Kevin F Kennedy, John H Helzberg

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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

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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* (%)

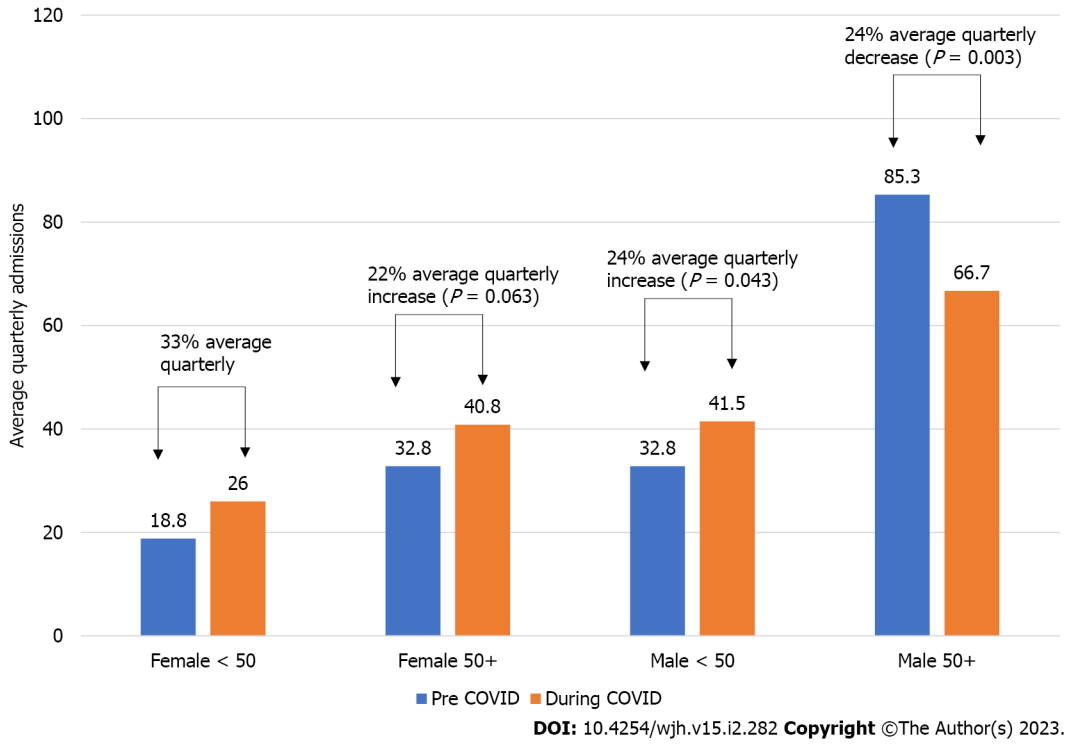
Variable	Total (*n* = 1378)	COVID-19 period (*n* = 700)	Pre-COVID-19 period (*n* = 678)	*P* value
Age (yr)	53.9 ± 13.1	53.1 ± 12.9	54.7 ± 13.2	0.025
Gender				0.002
Female	473 (34.3%)	267 (38.1%)	206 (30.4%)	
Male	905 (65.7%)	433 (61.9%)	472 (69.6%)	
Race				0.628
Black or African American	168 (12.2%)	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.

Institutional review board statement: The study was reviewed and approved by the Saint Luke's Health System Institutional Review Board (Approval No. SLHS-21-057).

Informed consent statement: Informed consent was not obtained from each patient evaluated since this project was completely retrospective and performed with an IRB exemption.

Conflict-of-interest statement: The authors declare no conflicts of interest for this article.

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

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

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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.

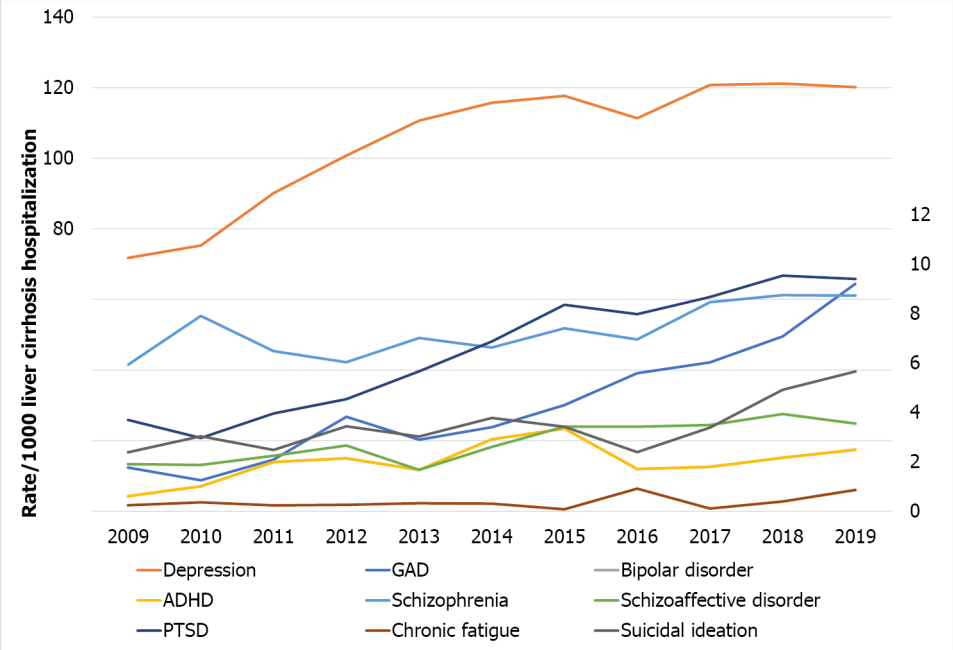
Psychiatric conditions based on etiology of cirrhosis

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.

Conflict-of-interest statement: There is no conflict of interest associated with publication of this manuscript.

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ORIGINAL ARTICLE

Observational Study

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

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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

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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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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

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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

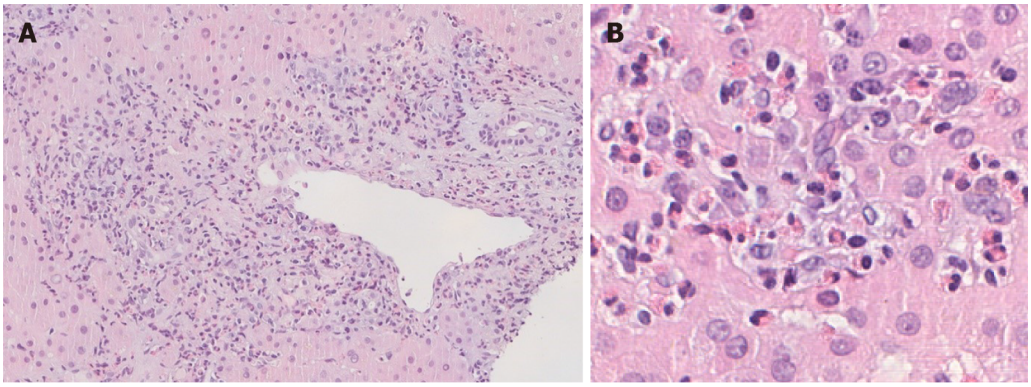
The patient denied any history that can suggest viral prodrome, sick contacts, recent travel, medication ingestion (comprising herbal or over-the-counter), exposure to well water, exposure to recreational drugs, tattoos, alcohol, high-risk sexual behavior or blood transfusions.

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

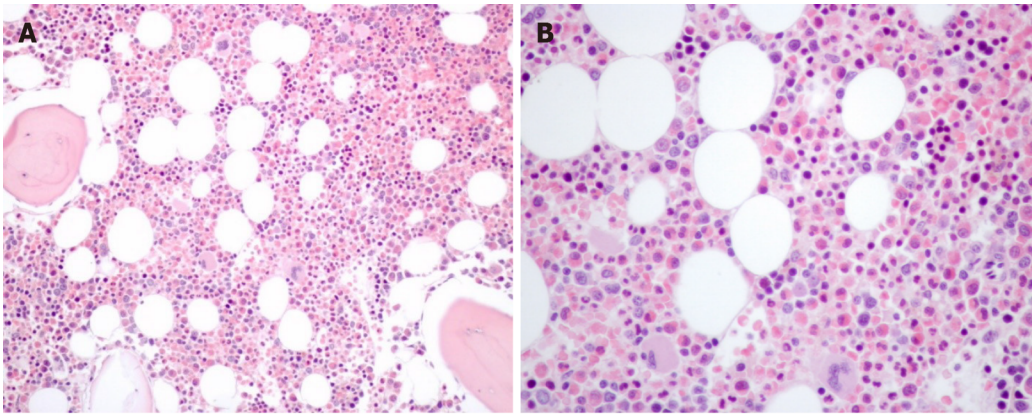
He started treatment with prednisone at 40 mg/d. Cytocholestasis (Figure 3A) and eosinophilia (Figure 3B) progressively improved. The corticosteroid dose was gradually titrated and azathioprine 2 mg/Kg was then added.

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.

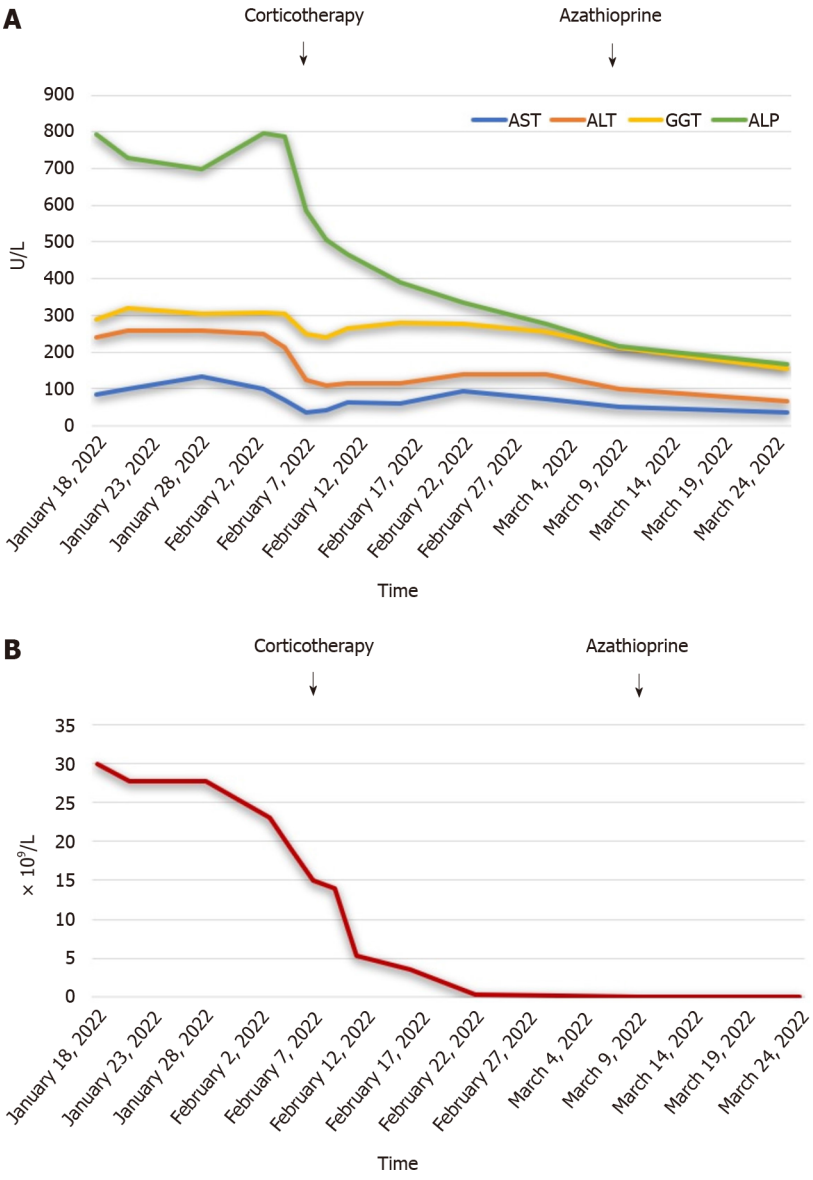
DISCUSSION

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

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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.

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REVIEW

Main factors influencing long-term outcomes of liver transplantation in 2022

Elisa Fuochi, Lorenzo Anastasio, Erica Nicola Lynch, Claudia Campani, Gabriele Dragoni, Stefano Milani, Andrea Galli, Tommaso Innocenti

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Abstract

Liver transplant (LT) outcomes have markedly improved in the recent decades, even if long-term morbidity and mortality are still considerable. Most of late deaths are independent from graft function and different comorbidities, including complications of metabolic syndrome and *de novo* neoplasms, seem to play a key role in determining long-term outcomes in LT recipients. This review discusses the main factors associated with late mortality and suggests possible strategies to improve long-term management and follow-up after liver transplantation. In particular, the reduction of drug toxicity, the use of tools to identify high-risk patients, and setting up a multidisciplinary team also for long-term management of LT recipients may further improve survival after liver transplantation.

Key Words: Alcohol; Liver transplantation; Long term survival; Metabolic syndrome; Renal dysfunction; Therapy adherence

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Core Tip: Survival after liver transplantation has increased in the last decades due to an improvement in early post-transplantation outcomes, underlining the need to shift the focus towards long-term outcomes. We herein discuss the main factors related to long-term morbidity and mortality in liver transplant recipients and outline the main management suggestions and recommendations to improve long-term outcomes.

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INTRODUCTION

Liver transplantation (LT) is the only lifesaving treatment option for patients with end-stage liver disease and acute liver failure, and for selected patients with hepatocellular carcinoma in whom other curative treatment options have failed or are not suitable[1]. Over the past decades, early post-transplantation outcomes have significantly improved[2], while the 20-year survival rate still remains only approximately 50%[3]. In fact, long-term survivors have an increased morbidity risk, not only related to "classical" transplant-related complications, such as graft dysfunction, rejection, or liver disease recurrence, but also to factors that are not strictly related to the graft[4]. Metabolic complications, cardiovascular disease, renal dysfunction, and extrahepatic malignancies play a major role in long-term morbidity and mortality of LT patients[1]. Long-term post-transplant management is complex and requires a close follow-up to recognize, manage, and prevent medical complications and comorbidities[1] (Figure 1). The purpose of this review is to discuss the factors associated with long-term morbidity and mortality after LT, describing current recommendations and suggesting possible strategies to improve the management and the follow-up of these patients.

BIBLIOGRAPHIC SEARCH

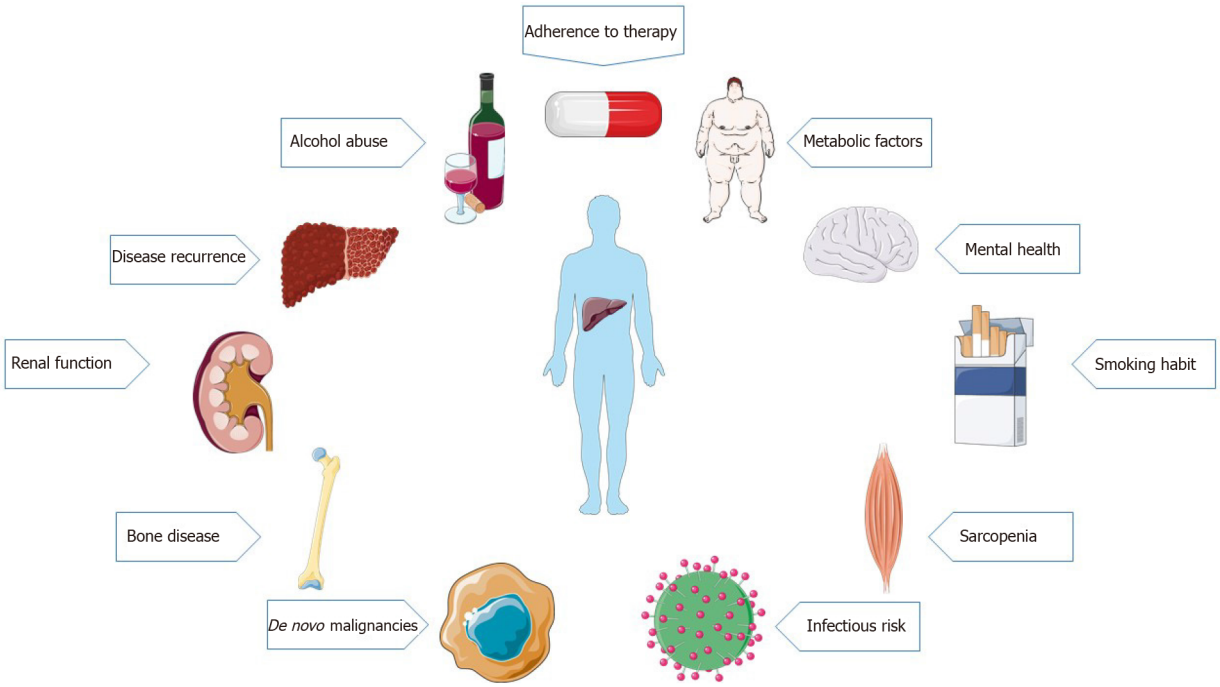
A bibliographic search was conducted by two Authors (Fuochi E and Anastasio L) using PubMed and EMBASE databases, with the following terms: "liver transplant", "liver transplantation", and "orthotopic liver transplantation". Searches of the databases were run on September 13th, 2022. Only papers written in English language were considered. After exclusion of duplicates, the search results were double-blind screened by two reviewers (TI and ENL), and abstracts assessed for eligibility. Reviews, conference abstracts and book chapters were excluded. Articles were declared not relevant by consensus.

METABOLIC FACTORS

Metabolic syndrome (MetS) is generally defined as the presence of three of five risk factors among elevated fasting glucose, reduced high-density lipoprotein cholesterol, elevated triglycerides, obesity, and hypertension[5]. It has been estimated that approximately a quarter of the world population is affected by this condition and its prevalence is still increasing[6].

There is a two-way correlation between metabolic syndrome and LT: On one hand, non-alcoholic steatohepatitis (NASH) is currently the second leading cause for LT waitlist registration/LT in general population and the first leading cause in females, at least in Western countries[7]; on the other hand, the majority of patients who have undergone transplantation develop diabetes mellitus, hyperlipidemia, and arterial hypertension[8]. It has been established that 50%–60% of these patients fulfill the criteria of metabolic syndrome[2]. In the United States, obesity is observed in 30%-40% of LT recipients within the first 5 years after transplantation[9]. Moreover, about 30% of patients suffer from diabetes after transplantation, and pretransplant diabetes is a predisposing factor[10]. Post-transplant development of MetS is due to multiple factors, such as reversal of cirrhosis, increased appetite, use of steroids, and may partly be due to the dysmetabolism of fats and sugars deriving from the use of immunosuppressants [11].

It is widely recognized that there is a strong association between metabolic syndrome and cardiovascular events. Since cardiovascular diseases are listed as the third cause of late mortality in patients who underwent LT[12], estimating cardiovascular risk and managing cardiovascular risk factors are central elements in the management of these patients. In all transplant candidates a cardiac evaluation is mandatory, although there is no ideal way to assess it. Several efforts have been made to



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Figure 1 Main factors affecting long-term morbidity and mortality after liver transplantation.

find a more complete scoring system capable of accurately evaluating cardiovascular risk in these patients. For example, VanWagner has recently proposed the CAR-OLT score, based on age, sex, race, working status, education, liver pathology, and comorbidities to evaluate the coronary risk at one year after transplantation[13]. In 2021, Rachwan *et al*[14] elaborated another score, The coronary artery disease (CAD)-LT score and algorithm, that stratified significant CAD risk as low (≤ 2%), intermediate (3% to 9%), and high ≥ 10%). The score seemed to identify 97% of all significant CAD and potentially avoided unnecessary testing such as cardiac catheterization in low-risk patients[14]. However, none of these scores has been validated yet and it is uncertain whether they are able to predict long-term cardiovascular outcomes after LT.

In the post-transplant setting, European Association for the Study of the Liver guidelines recommend a continuous cardiovascular risk stratification and an aggressive management of metabolic syndrome, with a prompt detection and treatment of modifiable risk factors by means of lifestyle changes, pharmacological therapies, and modifications of the immunosuppression in order to prevent serious cardiovascular complications[1]. American Association for the Study of Liver Diseases (AASLD) Guidelines also recommend dietary counseling for all LT patients to avoid obesity. For patients who fail behavioral weight-loss programs, bariatric surgery may be considered[4]. There is still a debate about the ideal timing of bariatric surgery with respect to transplantation. A recent metanalysis compared the outcomes of bariatric surgery performed before, during, and after LT in a large cohort of obese patients. In all the analyzed groups, the 30-d mortality after surgery was 0%, although patients who underwent bariatric surgery after LT had a higher mortality rate beyond 30 d (7%). The graft survival rate after 1 year was 70% in patients operated before LT, while it rose to 100% for patients who underwent bariatric surgery during LT. Thirty-day minor and major complication rates were 4% and 1%, respectively, if bariatric surgery was performed before transplantation[15]. Further studies are required to define the optimal bariatric procedure and its timing with respect to transplantation[4].

SARCOPENIA

Sarcopenia is defined as the loss of skeletal muscle mass, quality, and function[16].

The overall prevalence of sarcopenia among patients with cirrhosis is 37.5% with an estimated higher prevalence in males, alcohol-related liver disease, and greater severity of cirrhosis[17,18]. Moreover, sarcopenia not only can be present before transplant but may also develop after surgery. This condition can be related to multiple factors such as infections, renal dysfunction, lack of specific nutritional diets, and specific medications[19].

To investigate the correlation between post-transplant sarcopenia and long-term outcome, a study on a population of 382 adult LT recipients has been recently performed in the Netherlands. Stam *et al*[20] measured post-transplant urinary creatinine 24 h excretion rate (24-h CER, a noninvasive marker of total

body muscle mass) one year post-transplantation and found that low CER was associated with increased 10-year mortality and graft failure risk, independently of age, sex, and body surface area. Similarly, patients within the lowest tertile of CER values had worst outcomes in terms of mortality and graft failure, compared to transplant recipients in the highest tertile[20]. It must be noted that, although the 24-h CER index is an established method for assessing skeletal muscle mass, computed tomography (CT) or magnet resonance studies are currently considered the gold standard to assess sarcopenia[21]. However, urinary CER might be an inexpensive and accessible sarcopenia marker, without the need for costly exams or exposure to radiation[22].

In a recent Chinese study, sarcopenia was assessed by measuring psoas muscle index from tomography images obtained within 1 mo after transplantation in 70 male patients. Sarcopenia was identified as being significantly associated with worse post-transplant overall survival (OS) for an average of 63.3 mo of follow-up. Interestingly, sarcopenic patients seem to suffer from higher rates of hepatocarcinoma recurrence, although this difference did not reach statistical significance[23].

Another Asian study also found that sarcopenia, assessed as height-normalized psoas muscle thickness on computed tomography within 2 mo before surgery, was associated with a higher risk of tumor recurrence after transplantation for hepatocellular carcinoma. Authors hypothesized that sarcopenia may promote tumor progression by decreasing levels of certain cytokines (myokines and adipokines) and increasing others, such as tumor necrosis factor (TNF)-α[24]. Further studies are required to confirm these results.

It is important to underline that sarcopenia can also be found in obese people. In a German meta-analysis on 1515 patients, pre-transplant sarcopenic obesity (SO), assessed with different methods, was found to increase overall mortality compared to non-SO at 1, 3 and 5-years follow-up[25]. Unfortunately, using sarcopenia as a predictor of post-transplant survival is still limited by the significant heterogeneity among studies[26]. Many questions remain, including the best modality for assessing muscle mass, the optimal cut-off values for sarcopenia, the ideal timing and frequency of muscle mass assessment, and how to best incorporate the concept of sarcopenia into clinical decision making[27]. In our opinion, sarcopenia should be evaluated before transplantation, for example using CT scan, which is generally easily available as it is required for the global evaluation of the patient before transplantation. Then, the evaluation should be repeated one year after transplantation, using the same method and possibly the same CT machine, in order to compare results.

Treatment of sarcopenia is based on lifestyle modifications. Even if there are no standardized exercise programs, Tandon *et al*[28] recommend 150 min of mild aerobic activity divided in 3-5 d per week and more than two days per week of resistance training in cirrhotic patients. Nutritional intervention prior to transplantation may also play an important role although, to date, studies have been unable to identify strategies that offers convincing benefits. Furthermore, given that sarcopenia can also develop after transplantation, dietary advice by a nutritionist may help to improve patient prognosis. Nutritional supplementations may also play a role in this condition. For example, a recent Italian randomized pilot study reported that a 12-wk supplementation after LT with β-hydroxy-β-methyl-butyrate, an active metabolite of leucine with anabolic effect that inhibits muscle proteolysis, seems to significantly improve muscle mass values in sarcopenic LT patients[29]. However, these supplementations are usually expensive and further studies with larger cohort of patients are needed to confirm these results.

BONE DISEASES

Osteoporosis was defined by the World Health Organization in 1994 as a bone mineral density of less than 2.5 standard deviations below the sex-specific young adult mean[30]. Reduced bone density leads to decreased mechanical strength, thus making the skeleton more prone to fractures[31]. Many studies have reported how fragility fractures cause a significant morbidity and mortality burden in the general population[32], with hip fractures being the most serious, with a 33% cumulative mortality rate in the 12 mo after fracture[33].

One-third of LT recipients have a bone mineral density below the fracture threshold[34] and the fracture rate in these patients has been reported to be as high as 24%–65%[35]. Up to 55% of waitlisted patients might already have osteoporosis, especially women. In this setting osteopenia can be related to different factors such as malnutrition, physical inactivity, malabsorption of vitamin D in cholestatic liver disease, steroid use in patients with autoimmune hepatitis, and direct toxicity in alcohol-related liver damage[36]. Older age, female sex, and low body mass index (BMI) are also risk factors for osteoporosis in the general population. Furthermore, patients with end-stage liver disease present with decreased bone density compared with the age-matched control population[1].

It has been established that low bone mineral density before LT is a risk factor for developing osteoporosis after transplantation[37]. Bone loss often peaks at 6 mo after transplantation, resulting in a high fracture risk[38], even if this trend tends to reverse in the following period, with no deterioration afterward[34]. Multiple factors contribute to increased bone loss after transplantation, including use of corticosteroids, poor nutritional status, vitamin D deficiency, immobility, sarcopenia, hypogonadism, smoking, and alcohol abuse[39].

Current guidelines recommend regular measurement of bone mineral density pre- and post- LT. If osteopenic bone disease is confirmed or if atraumatic fractures are present, patients should be assessed for risk factors for bone loss; in particular, this should include an assessment of calcium intake and 25-hydroxy-vitamin D levels, an evaluation of gonadal and thyroid function, a full medication history, and thoracolumbar radiography[4]. The management of osteopenia and osteoporosis in transplant recipients correlates with recommendations for the general population and involves calcium and vitamin D replacement (if deficient) and weight-bearing exercise (whenever possible). Bisphosphonate therapy must be considered for patients with osteoporosis and/or recurrent fractures[1]. In particular, a recent multicenter randomized double-blind controlled trial evaluated the efficacy of neridronate (an amino-bisphosphonate) in patients with reduced bone mass after transplantation of the heart, liver, or lung. Neridronate, at the dose of 25 mg i.m./mo for 12 mo, significantly increased lumbar bone mineral density in these patients, with a good safety profile, even in case of minor renal impairment[40].

PSYCHOLOGICAL ASPECTS AND QUALITY OF LIFE

The World Health Organization defines quality of life (QoL) as "the individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns"[41]. Several studies have highlighted the importance of considering as determinants of successful transplantation not only mere survival rates, but also functional recovery and health-related QoL[42].

Few data are available on long-term QoL perception in transplant recipients. In a single-center cross-sectional study performed in England, QoL perception 10 and 30 years post-transplantation was found to be generally good, being reduced only in older individuals[43]. An American multicenter longitudinal study of 381 patients also reported that the general health perception declined over time in LT patients. There was also a general and progressive worsening of the distress or emotional burden related to physical symptoms such as fatigue, muscle weakness, headaches, and backaches[44]. These results could be explained by the normal age-related general health perception decline. Immunosuppression-related side effects could be at least partly responsible for the worsening of long-term QoL perception[45]. There is no clear association between gender or etiology of liver disease and QoL perception[46,47], although female sex and hepatitis C virus (HCV) related cirrhosis could be associated with worse QoL and higher levels of anxiety[48,49].

Moreover, in the post-transplant setting, patients tend to experience more clinically relevant symptoms of anxiety, depression, and post-traumatic stress compared to the general population[50].

Depressive symptoms appear to determine worse outcomes[51]. DiMartini *et al*[52] divided a cohort of 167 patients transplanted for alcoholic cirrhosis in three groups, according to the evolution of depressive symptoms within the first post-surgery year (consistently low depression levels at all time points; depression levels that rose over time; consistently high depression levels). The Authors found that recipients with increasing depression or persisting depression were more than twice as likely to die for all-cause mortality within the subsequent years. At 10 years follow-up, post-survival rate were significantly lower for the increasing-depression and high-depression groups compared with the low-depression one[52]. In another prospective cohort study, 134 LT patients were assessed for depressive symptoms using a validated questionnaire administrated 3 mo after surgery. Depressive symptoms were significantly associated with a higher 5-year mortality rate. Moreover, the questionnaire score correlated with the mortality rate in this population[53].

Interestingly, some Authors reported how patients receiving appropriate pharmacotherapy for early post-transplant depression had similar long-term survival rates to non-depressed liver-transplant recipients[54]. In addition, a recent Italian study reported how a personalized aerobic and strength training program not only improved metabolic aspects, but also the QoL perception in LT patients, so lifestyle modifications should probably be considered part of mental health management in these patients[55].

European Guidelines suggest that clinical physicians should identify depressive symptoms in the early post-transplantation period and treat them accordingly when present[1]. Unfortunately, the assessment of QoL in LT recipients has not yet been studied thoroughly and is not standardized[56]. More studies are needed to find effective strategies to manage psychological problems in this specific population. In our opinion, studies on larger cohorts of LT patients should be performed to compare different QoL and depressive symptoms questionnaires (for example, questionnaires that are already been validated in other populations such as elderly people or oncologic patients) in order to select the ones that better correlate with long term outcomes after LT.

RENAL DYSFUNCTION

Most of liver-transplanted patients develop impaired kidney function with a variable degree of severity. Within the first 10 years post-surgery, 30%-80% of patients develop chronic kidney disease stages 3-4

[57] and 25–5% of patients require dialysis[58].

Renal function impairment may already be present before LT or develop or worsen after surgery. In LT candidates, renal dysfunction can be related to cirrhosis itself but also to other coexisting conditions such as diabetes, glomerulosclerosis, or IgA nephropathy[59]. Hepatorenal syndrome (HRS)-related kidney injury deriving from intense renal vasoconstriction secondary to complex circulatory changes in cirrhotic patients may not be fully reversible after transplantation[60]. Liver transplantation is considered the definitive treatment for HRS because renal failure is functional and liver disease is the actual cause of the renal impairment[61]. Patients with hepato-renal syndrome seem to have worse survival expectancy than other patients with cirrhosis for any given value of model for end-stage liver disease (MELD) score, which suggests that HRS may be considered a poor-prognosis factor after LT [62]. However, a recent meta-analysis demonstrated that about 83% of HRS patients achieved HRS reversal after LT[63] and HRS-non acute kidney injury seem to have worse outcomes compared to HRS-acute kidney injury[64]. Living-donor LT results in identical long-term outcome when compared with deceased-donor LT in patients with HRS[65]. Many factors may contribute to the development or worsening of kidney failure after transplantation, including perioperative acute kidney injury, hypertension, diabetes mellitus, atherosclerosis and, most importantly, exposure to calcineurin inhibitors (CNI)-based immunosuppressive regimens, especially when it comes to long-term therapies [1]. CNIs might be responsible for more than 70% of chronic kidney injury in post-transplant setting[57].

Many studies have reported an increased risk of death, myocardial infarction, stroke, and major bleeding in chronic kidney disease, especially in its most severe stages[66]; thus, preventing renal deterioration and preserving its function may be a key element in the management of transplanted patients.

Continuous monitoring of renal function is recommended to detect and treat kidney disease at an early stage. Not only serum creatinine, but also an estimating equation to evaluate the glomerular filtration rate should routinary be used. Urinary protein quantification using the concentration ratio of protein to creatinine in a spot urine specimen should be evaluated at least once yearly[4]. Sufficient treatment of potential risk factors such as diabetes and hypertension and avoiding nephrotoxic drugs is recommended and should be started immediately after transplantation[1]. Adjustment of the immunosuppression (IS) (usually on an individual level, especially in patients with impaired kidney function), is mandatory. In particular, reduction or withdrawal of CNI associated-immunosuppression or alternative CNI-free protocols should be considered as soon as possible in patients with impaired renal function[1]. For example, a recent meta-analysis on 769 patients has detected higher estimated glomerular filtration rates (eGFR) at one, 3, and 5 years post transplantation in patients on everolimus therapy (EVR) compared to those receiving CNI standard therapy[67]. In 2019, the observational CERTITUDE study, following patients who had completed the SIMCER trial, found that patients starting EVR therapy at month 1 after transplant with stepwise tacrolimus (TAC) withdrawal had a mean eGFR which was significantly higher compared to patients that were on standard tacrolimus-based regimen at 24 mo after transplant[68]. A phase 2, multicenter, randomized, open-label trial has evaluated the safety and efficacy of EVR initiation even earlier than 1 mo after LT[69]. In this study, patients treated with corticosteroids, TAC, and basiliximab were randomized to receive EVR (1.5 mg twice daily) from the eighth day post-surgery and to gradually minimize or withdraw TAC when EVR was stable at > 5 ng/mL or to continue TAC at 6-12 ng/mL (control group). eGFR was significantly higher in the EVR group, as early as 2 wk after randomization, with similar efficacy rates in the two groups at 3 mo follow up. These studies suggest that EVR based IS, started early after transplantation, might be a valid alternative to CNI-based therapies in patients with renal dysfunction. Despite its positive effect on renal function, early switch to EVR has been associated with higher biopsy-proven acute rejection at 6 mo follow up[70], so that the choice of IS treatment should always be personalized and made weighing up the risks and benefits of the different therapeutic strategies.

Kidney transplantation from deceased or living donors is beneficial in improving survival and should be considered the optimal therapy for LT recipients who develop end-stage renal disease[4].

INFECTION RISK AND VACCINATION

Solid organ transplant recipients are at an increased risk of infection because of the IS required to prevent graft rejection[71]. Vaccination is considered an important strategy to prevent infectious risk not only in the general population but also in transplanted patients[1]. As immunodepression can reduce immune response to vaccines[72] and live attenuated vaccines are not recommended in immunocompromised patient[73], guidelines suggest to perform HAV, HBV, Varicella, *Pneumococcus*, influenza, and tetanus vaccinations prior to transplantation, if possible[1]. Many national guidelines recommend annual influenza vaccination of immunocompromised patients, although the decision to vaccinate is usually at clinical discretion[74]. A meta-analysis conducted on 209 studies has found that transplanted patients, together with HIV and cancer patients, are those who benefit most from the annual boost as it significantly decreases the rate of laboratory-confirmed influenza cases in these patients[75].

Influenza is a considerable public health issue due to its dissemination and contagiousness, causing annually about 4 million severe infection cases and about half of million deaths each year[76]. The

precise epidemiology of influenza in the transplant population is not well known because little data are available describing the incidence of influenza in multi-season and multicenter prospective cohorts, in particular for recipients of allografts other than lung[77]. However, influenza seems to be more common among solid organ transplant recipients compared to the general population, as showed in a 10-year longitudinal study with an incidence of 4.3 cases per 1000 person years[78]. In patients with impaired immunity, influenza is more likely to lead to a lower respiratory infection and can also have unusual manifestations such as rhabdomyolysis and myocarditis[78,79].

A recent multicenter prospective study including 606 transplanted patients from twenty centers in the U.S., Canada, and Spain showed that receiving vaccination for influenza is associated with a decrease in disease severity as determined by the presence of pneumonia and Intensive Care Unit admission[80]. Similar results have been found in another recent Italian study, in which vaccination was associated with fewer hospital admissions for infectious respiratory diseases compared to unvaccinated patients (9.7% *vs* 23.5%). The main reason for vaccination refusal was fear of adverse reaction, impaired health status, or low vaccine efficacy. Interestingly, receiving advice of Reference Center physicians was positively associated with influenza vaccination, highlighting the important role of the transplant hepatologist with regard to vaccine communication and recommendation for high-risk patients[81].

In addition, there is no consistent evidence suggesting an association between influenza vaccine and graft rejection, worsening of allograft function, or other serious adverse events in immunocompromised patients[75].

European and American Guidelines recommend influenza vaccination in transplanted candidates and annual influenza vaccination in liver transplanted patients[1,4]. The Infectious Disease Society of America guidelines suggest administering inactivated influenza vaccine starting from one month after transplantation during community influenza outbreaks[82].

Adherence to seasonal influenza vaccination is still low in immunocompromised patients, reaching a maximum of 50%-60% of patients[83,84]. Accurate counseling by the hepatologist may increase the percentage of vaccinated patients and therefore improve the long-term outcome of these patients[81].

A recent meta-analysis of randomized controlled trial has compared the clinical benefit of high dose trivalent influenza vaccine (TIV) *vs* standard dose in adult patients. One of the 10 analyzed studies also included immunocompromised patients. The meta-analysis found that laboratory-confirmed influenza A (H3N2) was significantly reduced with high-dose TIV, especially in older adults, even if no difference in mortality or hospitalizations was demonstrated[85]. Further studies are needed to compare the efficacy of high-dose *vs* standard-dose TIV in LT patients.

Another strategy to prevent influenza disease is the prophylaxis with neuraminidase inhibitor (Oseltamivir) during periods of local influenza circulation. A randomized controlled trial on 477 immunocompromised subjects, mostly solid organ transplant adult recipients, has found that Oseltamivir, given orally at the dosage of 75 mg daily, significantly reduced laboratory-confirmed influenza incidence in these patients and it was also well tolerated[86]. Also, Oseltamivir does not affect the steady-state pharmacokinetic characteristics of cyclosporine, mycophenolate, or tacrolimus, at least in adult renal transplant patients[87]. In our opinion, Oseltamivir prophylaxis may be considered as a strategy to prevent influenza disease in LT recipients along with vaccination. Oseltamivir is also indicated to prevent serious complications when influenza is established. A recent randomized, placebo-controlled, phase 3 trial found that a single dose of Baloxavir marboxil, a selective inhibitor of influenza cap-dependent endonuclease, has similar efficacy to Oseltamivir in improving influenza symptoms in high-risk healthy individuals[88]. To our knowledge, specific studies on LT population comparing these medications are lacking and further studies are needed to support the use of this drug in transplant patients.

DE NOVO NEOPLASMS

Besides cardiovascular diseases, *de novo* malignancies are the leading cause of mortality after the first post-LT year[1]. LT patients have an 11-fold higher risk of developing cancer compared to the general population[89]. The overall incidence of *de novo* malignancies is considered between 3.1 and 14.4%, with a cumulative risk gradually increasing with posttransplant graft survival, rising to 55% at 15 years[90]. The overall estimated survival rates for all types of neoplasms are reportedly 70, 48, and 39% after 1, 5, and 10 years, respectively[90]. Notably, the probability of survival is generally worse than for a non-transplanted patient with the same tumor at the same stage and location[91].

Recent data suggest that solid organ tumors are becoming the most frequent malignancy in these patients, followed by skin cancers and lymphoproliferative disorders. In particular, Rademacher *et al*[92] have analyzed 1616 LT patients and have found that solid organ tumors were responsible for more than 50% of all the novo malignancies after a mean follow-up of 28 years.

The major causes of *de novo* malignancies in the post-LT course are related not only to the loss of immunovigilance induced by immunosuppressive agents but also to other carcinogenesis risk factors that are shared with the general population[1].

For example, Epstein Barr virus seropositivity before transplantation and aggressive immunosuppressive regimens are considered risk factors for developing lymphoproliferative disorders after transplantation[93]. On the contrary, major risk factors for developing non-melanoma skin cancers in these patients are older age, chronic sun exposure and sunburn, fair skin, and a history of previous skin cancers[94]. Considering solid organ tumors, significantly higher rates of colorectal cancer have been reported in patients with primary sclerosing cholangitis (PSC) and inflammatory bowel disease (IBD), even after LT[95]. Metabolic syndrome, that is common in transplanted patients, as previously discussed[7,8], is associated with a higher risk of endometrial, pancreatic, breast, and colorectal cancer [96]. Human papilloma virus (HPV) infection is associated with various cancers and, especially, with cervical cancer in women[97]. Patients with alcoholic cirrhosis are of particularly have a higher risk of developing upper gastrointestinal, oropharyngeal-laryngeal, and lung cancers, especially if there is also a positive present or past smoking history[98].

Treating modifiable risk factors and thus preventing cancer onset must be part of the clinical management of LT recipients. Smoking cessation and alcohol withdrawal should be promoted to reduce lung and head-neck cancers incidence[99]. Metabolic syndrome, obesity, and diabetes should be managed in order not only to prevent cardiovascular disease, but also to reduce cancer burden in these patients[100]. Sunbed use and sun exposure without adequate protection should be avoided to prevent skin cancers[101]. HPV vaccination is safe in immunosuppressed patients and is indicated to prevent cervical cancer[102]. A recent study has compared the immunological response and tolerability of HPV vaccination in pediatric kidney transplant (KT) recipients *vs* KT non immunosuppressed candidates. The study established that antibody concentration against HPV and seroconversion rates were significantly lower in patients vaccinated after KT compared to those who had been vaccinated before KT. The vaccination was well tolerated in both groups. This study suggests the importance of advocating for HPV vaccination prior to transplantation and acknowledges its safety after transplantation[103]. To our knowledge, there are no specific studies comparing HPV vaccination prior and after LT. Also, future studies are needed to investigate the effect of a supplemental dose of HPV vaccine in transplant recipients who do not seroconvert and to evaluate the long-term persistence of antibodies post-transplantation.

European and American guidelines highlight the importance of cancer screening protocols after LT, especially in high-risk populations, in order to detect *de novo* tumors at an early and potentially curative stage[1,4]. Patients transplanted for alcoholic liver disease should undergo a more intensive surveillance protocol for the detection of upper gastrointestinal, oropharyngeal, laryngeal, and lung cancers[1]. Patients transplanted for PSC with associated IBD should undergo annual colonoscopy to allow early detection of colorectal cancers[1]. American guidelines suggest that all LT recipients should see a dermatologist after transplantation to assess cutaneous lesions. Then, annual evaluation should be performed at least 5 years after transplantation for skin cancer prevention[4]. More data, however, are needed to define the optimal surveillance protocol after LT with individualized emphasis laid on patients' particular risk profiles[1]. A proposal for a possible screening protocol is shown in Table 1.

Immunosuppressants also play a key role in promoting cancer development and progression, not only by inhibiting the body's immune surveillance, but also by several other mechanisms, including the induction of insulin resistance and direct carcinogenic effects[104,105]. A lower incidence of neoplastic disease has been reported in patients treated with mammalian target of rapamycin inhibitors (mTORi) with a gradual tapering of CNI, if compared with patients on standard-dose CNI[106,107]. For this reason many transplant centers frequently add mTORi to CNI or convert to an mTOR inhibitor IS regimen when there are risk factors for malignancy after transplantation, or when a tumor has been diagnosed[1]. Nevertheless, all immunosuppressant regimens could increase *de novo* neoplasms risk, including those based on mTORi[89], so it is advisable to keep IS levels as low as possible, when feasible [108].

SMOKING

The prevalence of patients with a lifetime history of smoking before LT varies between 47% and 60%, while that of active smokers at the time of LT ranges between 10%-12%, with a relapse rate of 7%-12% [109-111]. As expected, patients who quit smoking for a shorter time before LT are those with higher rates of relapse[109]. These figures are subject to variation depending on the considered population, as there are higher rates of smokers among patients who underwent LT for alcohol-associated liver disease (ALD)[112,113]. Cigarette smoke is in fact clearly linked to alcohol consumption[114], which is why smoking habits should be especially investigated in individuals with previous ALD or current known alcohol use.

Other risk factors associated with being an active smoker until and after LT are younger age, higher MELD score, comorbid substance use disorder, and six months or less of alcohol abstinence before LT; alcohol dependence awareness is a protective factor from smoking after LT, underlining the close relationship between the two habits[115].

Table 1 Screening protocols proposal for the surveillance of de novo neoplasms in liver transplantation population

Malignancy	Screening proposal
Skin cancer	Annual dermatological visit[4], shorter follow up interval for high risk patients (*i.e.* every six months)
Lung cancer	Annual thoracic X-Ray; CT-scan in active or past smokers[282]
Colorectal cancer	Perform baseline colonoscopy on patients > 50 years old; annual fecal occult blood test in younger patients or if colonoscopy is negative; annual colonoscopy if patient affected by PSC + inflammatory bowel disease[1]
Ear, nose, and throat cancers	Annual otolaryngological visit in patient with active or past alcohol and/or smoking habit[124]
Renal cancer	Annual abdominal ultrasound
Cervical cancer	Annual papanicolau-test; annual gynecological visit
Breast cancer	Annual mammography, ultrasound evaluation if needed
Prostate cancer	Annual PSA and PSA ratio evaluation

CT: Computed tomography; PSC: Primary sclerosing cholangitis; PSA: Prostate specific antigen.

The majority of studies investigating smoking habits of LT recipients uses self-reported instruments (*i.e.* questionnaires) which appear to be sufficiently reliable in LT candidates, with about 10% not disclosing their smoking habit. In this setting, the use of a biomarker such as serum cotinine can be helpful in detecting deceptive reporting[116].

Tobacco smoking hampers long-term survival[112,117], with a worse prognosis in ALD-transplanted recipients who are active smokers at the time of LT compared to former smokers[115]. Smokers have a 79% higher risk of dying compared to nonsmokers[115]. A history of smoking is not only a well-known risk factor leading to the major causes of death in the long-term post-LT, such as development of cardiovascular complications and *de novo* neoplasms, as showed by a recent meta-analysis[118], but it has also been associated with alcohol relapse in ALD-transplanted patients[119], recurrent viral-hepatitis[120], an augmented risk of IBD flare in PSC-transplanted patients[121], and with an increase in biliary complications[122]. On the other hand, an increased time from smoking cessation to transplantation seems to be a protective factor against developing biliary complications[123].

Undoubtedly, special attention during follow-up is warranted for patients with a history of smoking, by means of screening (annual chest CT and ear-nose-throat evaluation) for early detection of *de novo* malignancies[124] and by actively assessing their smoking status at each visit, focusing on those with particular risk of relapse, implementing tobacco cessation treatments, and, if needed, providing a referral to start behavioral and/or pharmacological treatment[115,116,125,126].

MAINTENANCE IMMUNOSUPPRESSION AND ADHERENCE TO THERAPY

The transplanted liver becomes partially tolerant to immune-mediated injury, so the need for IS declines after the first 90 d[4]. Since the liver is considered a privileged organ in terms of immunological interaction, the clinician's aim has switched from trying to achieve complete suppression of acute rejection to obtaining a reduction of IS-related side effects, as long-term direct and indirect side effects of immunosuppressive therapy are a major cause of morbidity and mortality[1].

Maintenance IS therapy after LT is mainly based on CNI, with TAC being favored over Cyclosporine, with a variable use of other two classes: Antimetabolites like Azathioprine and mycophenolate mofetil, and mTORi, such as sirolimus (SRL) and everolimus. Management of immunosuppressants should take into consideration recipient characteristics, etiology of primary liver disease, and magnitude of alloimmune activation[108].

Each of these drugs has adverse effects (Table 2); for this reason, given the tolerogenic aspect of LT, an immunosuppressant minimization strategy should be considered for each patient (except for those with a history of graft rejection or those transplanted for immune-mediated diseases), while waiting for further development of personalized therapies[108].

Even though IS complete withdrawal should only be limited to clinical trials[108] it always remains an interesting perspective. Recently, Levitsky *et al*[127] conducted a pilot clinical trial of SRL monotherapy withdrawal in 15 selected recipients, who were followed-up for 12 mo after complete IS withdrawal with serial peripheral blood and graft biomarker assessments: 8 (53%) patients were successfully withdrawn from SRL at a median of 18 wk. Interestingly the authors found higher percentages of tolerogenic dendritic cells (HLA-DR +CD11c+ILT3+ILT4+ DC) prior to and after successful SRL withdrawal, compared to those who failed withdrawal. Furthermore, the authors previously identified a real-time PCR based biopsy signature relating to iron metabolism that predicted

Table 2 Maintenance immunosuppressants main adverse effects

Drug class	Adverse effects
CNI	Nephrotoxicity[283], recurrence of HCC[188,284], risk of *de novo* neoplasia[285-287], new onset diabetes mellitus (TAC more than CyA)[288,289], hypertension (CyA more than TAC)[290], dyslipidemia[291] (CyA more than TAC)[292], neurotoxicty[293], weight gain[294,295]
Antimetabolites	Leukopenia, thrombocytopenia, gastrointestinal disturbances (MMF and AZA) diarrhea, CMV reactivation (MMF)[296], pancreatitis, hepatotoxicity, risk of *de novo* neoplasia (AZA)[296,297]
mTORi	Leukopenia, dyslipidemia[298,299], cutaneous and mucosal alterations[300], wound complications, lymphocele[301], hypertension[302]

CNI: Calcineurin inhibitors; HCC: Hepatocellular carcinoma; TAC: Tacrolimus; CyA: Cyclosporine; MMF: Mycophenolate mofetil; AZA: Azathioprine; mTORi: Mammalian target of Rapamicin inhibitors.

tolerance and found that this same signature on pre-weaning biopsy accurately predicted tolerance to withdrawal, with 88% sensitivity, 83% specificity, 88% positive predictive value and 83% negative predictive value.

On the other hand, it must be noted that poor adherence to therapy and/or low blood levels of immunosuppressant are associated with a higher number of acute rejection episodes[128,129], which has been linked to chronic rejection that may lead to re-transplantation or death[130].

Poor-adherence to therapy has been reported in up to 50% of LT recipients[131], even though there is a substantial heterogeneity in the definition of non-compliance[132], often causing difficult comparisons among study results.

Assessing adherence to therapy is also an issue. Electronic monitoring (*e.g.* using pill bottles with a special cap that contains microelectronics to register the time and date of every bottle opening) yields detailed and reliable data but it is time- and cost-consuming[133]. There is also debate for its use as a gold standard, since it may not be feasible in clinical practice[134,135]. Trough levels can be affected by a variety of conditions, such as graft function and the concomitant use of other drugs[136]. Self-reporting could be a reliable method[137], but it lacks of objectivity[138].

A study conducted in kidney transplant recipients showed how a composite score using self-reported non-adherence and/or collateral-reported non-adherence and/or non-therapeutic blood assay variability had the highest sensitivity in assessing non-adherence to therapy[139].

Factors associated with poor compliance to therapy are: Young age[140], divorce, history of substance or alcohol use, mental health disorders, missing clinic appointments[131], belief in alternative medications, high regimen complexity, poor knowledge about medications, and cost issues[137].

As showed by a recent meta-analysis of randomized controlled trials conducted on solid organ recipients (mainly kidney)[134], adherence-enhancing interventions can result in significant increases in total adherence, medication dosing, and timing adherence rates, and even if there is insufficient evidence to assess which type of intervention (mobile health, cognitive, or behavioral) may be maximally effective, probably a combination of multiple interventions led by a multidisciplinary team may improve the immunosuppressive therapy adherence rate for solid organ recipients.

In a review by Burra *et al*[136], the Authors underlined the need to adopt a multidisciplinary approach for LT patient management, where multidisciplinary measures are developed by professional educators, supported by psychologists, and coordinated by physicians.

DISEASE RECURRENCE

ALD

ALD is the main indication for LT in Europe[141] and the United States[142].

There is no standardized definition for relapse thus reported relapse rates vary greatly: from nearly 50% if relapse is intended as alcohol use of any measure[143], to 12% if relapse is intended as harmful alcohol consumption, starting as soon as 1 mo after LT[144]. Interestingly, the study from Faure *et al*[145] reports excessive alcohol consumption post-LT in about 10% of the patients who were not transplanted for ALD as a primary indication, but who reported excessive alcohol consumption before LT, and about 3% of patients who did not report excessive alcohol consumption before LT. For this reason, a thorough history and ongoing monitoring of alcohol consumption in all patients is of great importance during follow-up of LT patients.

Factors associated with alcohol relapse are psychiatric comorbidities, pre-transplant abstinence of less than 6 mo[146], smoking[147], alcohol consumption from an early age[148], noncompliance with appointments or medication[149], and the lack of social support, in particular the absence of a companion in life[150]. Satapathy *et al*[151] have proposed a "Harmful Alcohol Relapse after Liver Transplant" score, that included 4 variables (*i.e.* age at LT, alcohol abstinence measured in months, daily

alcohol use, and history of non-alcohol-related criminal history) to help identify ALD patients at high-risk for harmful alcohol relapse, with an Area Under the Curve (AUC) of 0.79 for predicting relapse after LT.

Lee *et al*[152] developed an artificial intelligence model to predict post-LT harmful alcohol consumption in patients who underwent early liver transplant for alcohol associated hepatitis, using variables generated through content analysis: These variables included the identification of a primary support person, the presence of young children or grandchildren living with the patient, being a home caregiver for children or elderly relatives, opioid abuse, and being religious; this model could predict harmful alcohol consumption in the external validation set with a positive predictive value of 0.82 (95%CI: 0.625–1.000) and a negative predictive value of 0.81 (95%CI: 0.803–0.819), with and AUC of 0.69, indicating potential for AI to assist in the discovery of novel predictors of post-LT hazardous alcohol use, which may be used as a tool to tailor therapies for alcohol use disorder based on a projected likelihood of relapse.

While transplantation for ALD has a favorable outcome even when compared to other etiologies, *de novo* malignancies and cardiovascular events are still more frequent in this category of patients[153]. Excessive alcohol consumption post-LT is associated with a further reduction of long-term survival, with cancer and cardiovascular events as the main causes of death[154,155]. It seems that an average of 5 years follow-up post-LT is needed to observe an increase in liver-related mortality in the excessive alcohol consumption group[156]. It is important to keep in mind that excessive alcohol consumption-related impact on long-term survival can be an issue for every LT recipient, no matter what the primary indication for LT was[145].

Early diagnosis and prevention of relapse is important, given the clinical influence of excessive alcohol consumption post LT. Self-reported alcohol use can lead to deceptive reporting, so that biomarkers can be a supportive tool, in particular liver function tests and metabolites of alcohol, such as urinary ethyl glucuronide[157,158].

A structured management of patients at risk of relapse by a multidisciplinary team, including transplant hepatologist, clinical psychologists, psychiatrists with expertise in alcoholism and social workers is an effective strategy to prevent relapse post-LT[159,160].

In the study by Addolorato *et al*[161] , follow up of LT-recipients by an alcohol addiction unit, formed by internists, physicians in training, and psychologists with expertise in alcoholism, hepatology, and neuroscience, providing multimodal treatment (clinical and medical management, including counseling and pharmacological treatment), proved to be effective in reducing alcohol recidivism and mortality.

To our knowledge, there is still no published RCT evaluating the best intervention to prevent alcohol relapse in LT recipients. However, there is evidence suggesting that a multidisciplinary team approach is an effective way to prevent relapse[159-161]. We still do not have an ideal tool to predict who will relapse after LT, but some risk factors have been identified[147-151], allowing the clinician to focus on specific psychosocial features. Ongoing monitoring for alcohol-relapse is necessary for ALD patients [162], but a thorough history of alcohol consumption and assessment use during follow-up is also important for non-ALD recipients, since excessive alcohol consumption cannot be excluded in this category, also many years after LT[163]. Referral to psychiatric treatment or counseling is recommended in case of relapse, and every patient who underwent LT for ALD should also be encouraged to undertake smoking cessation[1,4].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is an indication for LT[1,164] in the first stages of the neoplastic disease, or after downstaging[165]. Recurrence of the disease develops in 8-18% of the recipients after a median time of 12 mo[166,167]. It significantly affects survival, especially if it appears in the first two years after LT, with a median survival after LT of 4 years *vs* 12 years in those without recurrence[168]. Among the many pre- and post-operative risk factors associated with recurrence[169], we can find tumor stage[170,171], vascular invasion[172], high Alpha-fetoprotein (AFP) levels[171] and differentiation grade[173-175], size, and number of nodules[175].

Several prognostic models have been developed using some of these risk factors; these models can be divided in pre- and post-transplant. Among the pre-transplant prognostic models the Milan criteria (solitary HCC with diameter < 5 cm or up to 3 nodules with diameter ≤ 3 cm) represent the benchmark for the selection of HCC patients for LT and the basis for comparison with other proposed criteria[1]. Attempts to expand the Milan criteria usually occur at the expense of HCC recurrence[176]. Other pre-transplant criteria which have demonstrated comparable survival results are those relying on size/number of nodules alone, such as the University of California San Francisco (UCSF) criteria (single nodule ≤ 6.5 cm or 2–3 nodules ≤ 4.5 cm and total tumor diameter ≤ 8 cm)[177] and the Up-to-7 criteria (sum of the largest tumor size and number of lesions < 7)[178] and those including AFP beside the size/number of nodules, such as Metroticket 2.0 (AFP levels + tumor number and size of the largest tumor) which showed good results compared to the above-mentioned models[179]. Every nodule with an intermediate-to-high probability of harboring HCC according to LI-RADS protocol seems to contribute to tumor burden and should be entered in the Metroticket 2.0 calculator in order to grant appropriate performance[180]. It has also been demonstrated how incorporating the modified RECIST criteria in response to neoadjuvant therapies into the Metroticket 2.0 framework can improve its predictive ability

[181]. In any case, it is mandatory to evaluate not only preoperative but also postoperative predictors of recurrence as there may be a mismatch between radiological findings before surgery and postoperative pathological assessments[182].

Among the post-transplant criteria, Parfitt *et al*[183] developed a risk score to predict HCC recurrence, based on microvascular invasion, tumor size, satellitosis and giant/bizarre cells visible at low power which was subsequently externally validated[184], showing a sensitivity of 80%, specificity of 79%, and an area under the ROC curve (AUROC) of 0.80.

Mehta *et al*[185] developed a simple prognostic score (RETREAT score) involving patients transplanted within the Milan Criteria, using 3 variables: AFP levels, the presence of microvascular invasion, and the sum of the diameter of the largest viable tumor plus the number of viable tumors. The score was able to stratify 5-year HCC recurrence risks ranging from less than 3% in those with a risk score of 0 to higher than 75% with a risk score of 5 or higher.

In this setting, the choice of the IS regimen is extremely important since mTORi, primary SRL, seem to have a protective effect against HCC recurrence[186,187] while CNI therapy is associated with an increased risk of tumor recurrence[188], even though the current recommendation is to minimize IS [108], with no mention to a specific IS regimen. AASLD guidelines also suggest considering an IS regimen including SRL, started several weeks after transplantation, for patients undergoing transplantation for HCC[4].

Strategies for preventing HCC recurrence mainly rely on an adequate pre-transplant selection of candidates[176] and on optimizing IS regimen[108] since there currently is no approved adjuvant therapy that has demonstrated prolonged disease-free survival[189-191]. On this basis, early diagnosis of HCC recurrence gains a central role in the post-LT care but there is no consensus about screening for recurrence, translating in a significant variability in center practices. In fact, Aggarwal *et al*[192] recently conducted a survey among 48 American adult liver transplant centers: There was considerable variation in the duration of surveillance, with 48% of the reporting centers maintaining surveillance for 5 years, while 18% discontinued surveillance after 2 years; 38 out of 48 centers used a risk stratification method for disease recurrence post-LT, categorizing patients into high and low risk groups, mostly based on the presence of microvascular invasion, tumor differentiation grade, discrepancy between pretransplant radiologic tumor size or number and explant pathology, and serum AFP measured before LT or at the time of LT. As expected, AFP was the most commonly used biomarker for detecting recurrence and 13 centers used specific cutoff values for serum AFP (between 100 and 500 ng/mL). On the other hand, 21% of the reporting centers employed solely abdominal/pelvic imaging and only 5% including bone imaging. The most frequently used imaging monitoring routine was every 3–4 mo in the first year, followed by every 6 mo in the second year, and every 6–12 mo at 3 years or beyond. For patients who were thought to be at a higher risk for HCC recurrence, 21 of the 38 facilities that stratified HCC recurrence risk had a more stringent "high risk" surveillance protocol, with a significant variability among the centers: In the first five years after liver transplantation, imaging was most frequently reported every 3 to 6 mo. Only in a few centers surveillance was interrupted after two years of follow-up (14%).

A reasonable surveillance strategy should therefore include chest and abdomen imaging and serum AFP monitoring, and should be more rigorous for those patients with high risk features for recurrence, especially during the first year after LT[193]. Most authors report monitoring for HCC recurrence post-LT with thoracic CT, abdominal CT or MRI, and AFP levels with 3- to 6-mo intervals in the first 2 or 3 years, increasing the interval between exams after the 2 or 3-year timepoint[194].

Increased surveillance may improve post-recurrence survival, though optimal surveillance strategies have yet to be proven; currently, no surveillance guidelines exist in this setting[195,196].

In fact, it is not clear whether screening for recurrence is worthwhile at all, due to poor results of systemic treatment for recurrence after LT[197]. Thus, surveillance should be customized according to a known recurrence pattern (*i.e.* frequent time and space frame) in order to be more cost-effective[198], considering that recurrence beyond 5 year is less common and associated with better prognosis[199]. Recurrence of HCC can be intrahepatic and/or extrahepatic, with the lung and bones as the most common extrahepatic sites of recurrence[199,200].

The study from Ladabaum *et al*[201] suggests that the relatively small gains in life-expectancy that may be achieved by screening for recurrence after LT are likely to be associated with relatively high incremental costs per life-year gained, and that the greatest benefit of screening is more likely to be derived by screening patients whose explant pathology exceeded the Milan criteria and by limiting screening to the first two years after LT.

Lee *et al*[195] showed how increased surveillance, measured by cumulative exposure to surveillance (CETS-*i.e.* the cumulative sum of all the protected intervals that each surveillance test provides) is associated with improved post-recurrence survival and a higher probability of aggressive treatment: In particular 252 d of CETS in the first 24 mo after LT would yield the best sensitivity and specificity for identifying disease which can be treated with either resection or ablation.

The above mentioned RETREAT score underwent a subsequent validation by Mehta *et al*[202] who found that a higher score is associated with a shorter time to HCC recurrence; the authors proposed a cost-saving surveillance strategy in which no surveillance is needed for patients with a score of 0, surveillance every 6 mo for 2 years is warranted for those with a score of 1-3 and for 5 years for those

with a score of 4, while for those with a score > 5 surveillance is warranted every 3– 4 mo for 2 years then every 6 mo for 2-5 years.

Further studies addressing the optimal strategy, the survival benefit, and the cost-effectiveness of surveillance for HCC recurrence should be undertaken, given that not being amenable to a curative-intent treatment has been found to be a poor prognosis factor in recurrent HCC post-LT[203].

Regarding treatment options, European guidelines suggest that treatment of HCC recurrence should probably follow the same algorithms used for immunocompetent patients, and also considering re-transplantation in selected cases[1], while American guidelines state that resection or ablation is usually the treatment of choice for a solitary extrahepatic metastasis or intrahepatic recurrence of HCC and that ablation with radiofrequency (RFA) is the best treatment for small solitary recurrences[4]. It seems that patients undergoing surgical treatment have a better post-recurrence survival, for both in intra-[204] and extrahepatic[205] recurrence, compared to those not undergoing resection, with a reported OS of 20-27 mo after HCC recurrence, in those who underwent surgery, significantly superior to patients who received only nonsurgical therapy (9-10 mo) or best supportive care (2-4 mo)[204,206]. There is still a dearth of information regarding locoregional treatments for the management of HCC recurrence following LT, so further data is needed. Among the few studies evaluating locoregional treatment we can find a study by Huang *et al*[207], which was conducted on 78 patients who had recurrence of HCC post-LT and found no significant difference in terms of OS or recurrence-free survival between the group undergoing surgical resection and the group undergoing RFA.

In a retrospective study conducted on 28 patients with HCC recurrence, Zhou *et al*[208] compared the outcomes of 14 patients receiving chemoembolization to 14 matched control subjects not receiving chemoembolization: Patients who underwent chemoembolization had significantly longer OS after LT and after the diagnosis of HCC recurrence (median OS after LT 865 d, median OS after HCC recurrence 286 d) compared to those who did not (median OS after LT 228 days, median OS after HCC recurrence 85 d), respectively, with no severe complications, and 57% in the treatment group showing partial response. The development of new recurrence, both intra- and extrahepatic, was still high in both groups (86% in those receiving chemoembolization *vs* 93% in those who did not), implying that the improved survival in patients receiving chemoembolization is likely attributed to the control of established tumors instead of the prevention of new lesions.

Regarding systemic therapies, Mancuso *et al*[209] conducted a meta-analysis of studies on survival and safety of sorafenib for HCC recurrence after LT with the aim of estimating the 1-year rates of survival: Overall the median survival was 10.5 mo (range 5 to 21.3). The pooled estimate of the 1-year survival rate was 63% (range, 18%–90%) with a significant heterogeneity among studies ($P < 0.0001$). Studies on sorafenib have shown that systemic therapy improves survival when compared to optimal supportive care alone. Patients experienced considerable medication toxicity, along with poorly tolerated side effects[210,211]. Close monitoring is necessary and should be even closer if IS regimen includes mTOR inhibitors as well, as the association between Sorafenib and mTORi showed an increased frequency of dose reduction and discontinuation due to adverse events[212,213]. Regorafenib could be an option in patients progressing while on treatment with sorafenib[214].

Recently Iavarone *et al*[215] conducted an observational multicenter retrospective study on 81 LT patients with HCC recurrence who discontinued first-line sorafenib (36 treated subsequently with regorafenib and 45 undergoing BSC at sorafenib discontinuation): The median OS was significantly longer in the group treated with regorafenib than in the group undergoing BSC (13.1 mo *vs* 5.5 mo, $P < 0.01$); treatment with regorafenib was an independent predictor of reduced mortality (hazard ratio, 0.37, $P = 0.02$).

Scarce data is currently available for other tyrosine kinase inhibitors (lenvatinib and cabozantinib), which would allow to propose new treatment sequences for patients with HCC recurrence after LT[198].

Another promising option for post-transplant HCC recurrence includes immunotherapy: The combination of atezolizumab (an anti-PD-L1) and bevacizumab (anti-vascular endothelial growth factor monoclonal antibody) proved to be superior to sorafenib in non-LT setting[216].

It must be noted that there is evidence that immunotherapy can interfere with post-transplant immunological tolerance and lead to allograft rejection that is resistant to treatment[217].

Luo *et al*[218] recently conducted a pooled analysis of the published cases of post-LT immunotherapy-treated HCC, including 29 patients: The overall response rate (complete response and partial response) to immunotherapy was 31.3% , including 18.8% with complete response and 12.5% with partial response

In the immune checkpoint inhibitors subgroups, including 19 patients, rejection was experienced by 6 out of 19 patients (32%), including 5 receiving nivolumab and 1 receiving pembrolizumab; allograft rejection exhibited a tendency to occur shortly after immunotherapy initiation, at a median time of 12 d and patients who started immunotherapy shortly after LT seemed to be at a higher risk of rejection than those starting after a longer interval of time.

After a median follow-up of 3 mo 68% of patients died (13/19), but only 23% (3/13) of those deaths were due to early rejection; the authors concluded that allograft rejection can be lethal, but the possibility of rejection-related death justifies considering immunotherapy as a backup plan because disease progression invariably results in death.

Post-transplant HCC recurrence currently represents a diagnostic and therapeutic challenge, and as such, patients transplanted for HCC need a close surveillance and an individualized management discussed in a multidisciplinary team in case of recurrence.

Autoimmune diseases

Reported recurrence rates vary among the different autoimmune diseases: The prevalence of recurrent primary biliary cholangitis (PBC) ranges between 9% and 35% with mean time to recurrence between 1.6 and 6.5 years[219]. Autoimmune hepatitis (AIH) recurs in 8%-12% of patients within the first year after LT and 36%-68% after 5 years[220].

PSC recurs in about 20% of patients after a median time of 4.6 years[221] with a cumulative incidence up to 45% at 6 years[222].

Diagnosing recurrence of autoimmune disease can be challenging, leading to a substantial variation of the data reported in the literature[219], since there are many conditions in the transplanted liver that can mimic autoimmune diseases (*e.g.* ischemia related biliary insults, hepatic artery thrombosis and/or chronic ductopenic rejection, infectious cholangitis can mimic PSC; acute cellular rejection can mimic PBC and AIH)[223].

Recurrence of PBC has little impact on patient and graft survival, with a reported patient survival at 5 and 10 year of 96% and 83% in those with disease recurrence[224], with a proportion of graft lost to disease recurrence of about 5%[225].

Reported patient survival for AIH is approximately 79% and 70% at 5 and 10 years, while graft survival is 73% and 63% after 5 and 10 years of follow up. Compared to recurrence of PBC and PSC, AIH recurrence leads to an increased risk of death due to infection or graft rejection[226].

Patient survival for PSC recurrence is approximately 86% and 70% at 5 and 10 years whereas reported graft survival at 5 and 10 years is 79% and 60%, respectively[227]. There is some controversy about whether recurrence of the disease affects survival[219,221,227,228]. A recent study conducted analyzing the European Liver Transplant Registry[229] shows that PSC recurrence has a negative impact on both graft and patient survival, leading to higher number of re-transplantations and a 33% decrease in 10-year graft survival.

As showed by a recent meta-analysis, the only identified risk factor for PBC recurrence[230] is the use of tacrolimus while the use of preventive UDCA was a protective factor. UDCA is also an effective treatment for disease recurrence[231,232], while there is a lack of data on the use of Obeticholic Acid and Fibrates for the treatment of recurrent PBC.

Risk factors associated with recurrent AIH are younger age at LT, use of mycophenolate mofetil post-LT, sex mismatch and high IgG pre-LT[233], suboptimal IS, disease type and severity[234], histological findings of severe disease in native liver[235]; of note, long term use of low-dose corticosteroid after LT seems to reduce the incidence of recurrent disease with a good safety profile[233,236].

The choice of the best treatment for recurrent AIH depends on the severity of presentation: For mild recurrence, such as asymptomatic disease with minimal changes in liver biochemistry and histology, an adjustment of the IS regimen may be sufficient, while severe disease recurrence may require re-introducing or increasing the dose of corticosteroids, or adding another immunosuppressive drugs [237]. Although this strategy remains controversial[233]. Re-transplantation may be required for patients with recurrent AIH who present with liver failure and graft loss[237].

Another meta-analysis showed that identified risk factors for PSC recurrence[238] are intact colon before LT and IBD presence, cholangiocarcinoma, advanced donor age, higher MELD score, acute cellular rejection (ACR) and multiple episodes of ACR.

Autoimmune etiology is a risk factor for Late T-Cell mediated rejection, which is associated with reduced graft survival, that is why particular attention is warranted in the IS protocol, even though the optimal IS regimen has not been defined[108].

In this scenario, early diagnosis of recurrence gains particular importance, even though diagnosis could be challenging[223] and liver function tests alteration should be considered highly suspicious for disease recurrence[239], while also considering other risk factors[240].

Montano-Loza *et al*[240] proposed strategies to reduce the risk of autoimmune liver disease recurrence after LT, acting on the main risk factors for recurrence: Treatment of active cirrhosis and normalization of transaminases and IgG for AIH in the pre-transplantation period plus long term corticosteroid use after LT; use of preventive Ursodeoxycholic Acid for PBC; control of IBD and considering pre- or post-transplant colectomy in patients with PSC.

In summary, prevention of recurrence in the future will probably rely on identification of risk profiles starting from the pre-transplantation phase and on tailored IS protocols.

Non-alcoholic fatty liver disease /NASH

The proportion of transplants performed for NASH has increased significantly over time[241], outpacing HCV[142], and metabolic liver disease has become a top indication for LT worldwide[242]. Post-transplant outcomes of NASH patients are generally good, even though there is some controversy about the overall survival rates for patients transplanted for NASH cirrhosis or HCC compared to other etiologies; graft survival rates are comparable[241,243,244].

Pre-LT screening for MetS is mandatory given the high prevalence in this population[245].

The distinction between recurrent and *de novo* non-alcoholic fatty liver disease (NAFLD) after LT is made clinically by accurately identifying the preexisting liver disease. There are few data on NAFLD after LT and a broad range in the recurrence or *de novo* rate depending on which diagnostic criteria is used[246]. There are no histologic characteristics to distinguish recurrent from *de novo* NAFLD after LT and histological findings in recurrent or *de novo* NAFLD in the allograft are considered to be the same as in immune-competent native livers[246].

Recurrence of NAFLD is up to 100% in patients who were transplanted for NASH after 1 to 5 years of follow-up, while reported NASH recurrence rates over a comparable follow-up period are between 4%–57%, with 2%-5% demonstrating compensated cirrhosis. Over a similar time period, the incidence of *de novo* NAFLD ranged from 18%- 78%, whereas *de novo* NASH ranged from 13% to 17%, showing lower rates of *de novo* disease compared to recurrence[247-249]. A meta-analysis on the incidence and risk of NAFLD/NASH post-LT reported the rate of cirrhosis in recurrent NAFLD recipients to range from 1 to 11%, with one study reporting the rate as high as 29%, whereas the rate of cirrhosis in *de novo* NAFLD LT recipients was 14% at five years after LT[250]. Despite the lack of data, NAFLD/NASH cirrhosis post-LT is probably a rare cause of death or graft loss in the first years post-LT given the good 5-year graft survival rate[246], but further data is needed on the long term. A recent study has pointed at recent pre-LT cardiovascular history and a combined donor-recipient age of 135 as major prognostic factors [251].

Management recommendations for LT recipients are the same as those for other NAFLD/NASH patients[252].

Diet and lifestyle changes have in fact a main role in the treatment of fatty liver disease[253]. NASH can be resolved with a weight loss of at least 7% of total body weight, and fibrosis can be stabilized or can regress with a weight loss of at least 10% of total body weight; a lower target of weight loss of 3%-5% is advised for patients with lean NAFLD[254]. Patients with fatty liver disease should follow the Mediterranean diet, mainly constituted by fresh fruit, vegetables, legumes, whole grains, fish, olive oil, nuts, and seeds while limiting the consumption of red and processed meat as well as commercially produced fructose[254]. This type of diet has been showed to reduce liver steatosis[255] and improve liver stiffness[256]. Patients with NAFLD should also consider engaging in regular physical activity, aiming for 150–300 min of moderate–intensity aerobic exercise per week[254], since it has been found that exercise alone, even without dietary intervention, can significantly decrease liver fat. Both European[257] and American[252] guidelines recommend that pharmacological treatments, aimed primarily at improving liver disease, should be limited to those with biopsy-proven NASH and fibrosis. It is also recommended to consider pharmacological treatment for patients with less severe disease who are at high risk of disease progression (*i.e.* with diabetes, MetS, persistently increased ALT, high necroinflammation). As of today, no NASH drug has been approved by Food and Drugs Administration, European Medicines Agency, or any other leading regulatory agencies[258]. Bariatric surgery should be proposed in case of non-response to lifestyle changes and pharmacotherapy[257].

Regarding modification to the IS regimen, ILTS advises corticosteroids minimization where possible, since they carry a significant risk for all components of MetS, and CNI minimization to mitigate post-transplant weight gain and hypertension[108].

There are no societal or professional guidelines for post-transplant surveillance in NASH LT patients at the moment, nor a frequency of post-LT monitoring for recurrent or *de novo* NASH has been defined, given the low likelihood of clinically significant recurrence of NASH. Conversely, high-risk individuals identified during the pretransplant work-up, such as those with PNPLA3 polymorphism or hypopituitarism, definitely require closer surveillance[246].

Suggested strategies for NAFLD/NASH screening in LT patients include annual ultrasound and liver enzymes monitoring: If fatty liver disease is identified or suspected then the patient should undergo noninvasive tests for fibrosis assessment[259]. A metanalysis by Bhat *et al*[260] showed how transient elastography (TE) performed better that APRI and FIB-4 at diagnosing recurrent fibrosis in LT recipients, but none of the studies included in the analysis was specific for NAFLD/NASH. A recent study from Siddiqui *et al*[261] conducted on 99 patients who underwent LT, showed how TE can detect advanced fibrosis with an AUROC of 0.94 and exclude advanced fibrosis with a negative predictive value of 0.99 when a liver stiffness cutoff value of 10.5 kPa is used; furthermore a controlled attenuation parameter cutoff value of 270 dB/m can identify any hepatic steatosis with an AUROC of 0.88. Along with TE, also Magnetic Resonance Elastography is an accurate method for assessing liver fibrosis in LT recipients[262]. Singh *et al*[263] conducted a pooled analysis including 6 cohorts where the mean AUROC values for diagnosis of advanced fibrosis and cirrhosis were respectively 0.83 (0.61-0.88) and 0.96 (0-93-0.98), with a good diagnostic performance even after stratification based on sex, BMI and degree of inflammation.

Beyond being a way to perform a differential diagnosis with other potential causes for elevated liver enzymes, liver biopsy remains the gold standard for the diagnosis of post-LT NAFLD/NASH; patients who have an established diagnosis of recurrent or *de novo* NAFLD can probably be followed with serial noninvasive testing to diagnose advanced fibrotic disease, but given the lack of data regarding fibrosis monitoring and the presence of factors that can influence these tests, suspected fibrotic disease at noninvasive testing still needs confirmation with biopsy[259].

While *de novo* or recurrent NASH appears to have little effect on prognosis, infections and cardiovascular diseases are among the leading causes of mortality[241,264].

A multidisciplinary approach in the management of NAFLD/NASH transplanted patients, promoting increased physical activity, diet modifications, behavioral therapy, and pharmacological treatment, when necessary, should be explored[265,266].

Unfortunately, in many cases a full multidisciplinary team is not available for the patient due to limited resources; either way the active assistance of the physicians is essential since their advice to lose weight has favorable impacts on the likelihood that patients will adhere to the suggested lifestyle changes[253].

Viral hepatitis

HCV recurrence post-LT was an issue with a major impact on the prognosis in the pre-direct acting antiviral (DAA) era, given its shortened natural history in the LT setting (development of cirrhosis in 10-30% of patients after a median of 5 years)[267-269].

The advent of DAA therapy was a "game changer", with HCV recurrence as cause of death or re-transplantation decreasing from 5.89% in the Interferon era to 0.60% in DAA era over a three-year period[270].

LT may not remove HBV from a persistently infected host because HBV may reside in extra-hepatic sites and serve as a source of reactivation. As a result, in a chronically infected patient, after LT, prophylaxis is used to avoid reactivation rather than re-infection or recurrence of HBV. Because of this, lifetime antiviral prophylaxis is required[271]. Treatment with hepatitis B immunoglobulin (HBIG) and Nucleos(t)ide Analogues is an effective strategy to prevent HBV recurrence in most HBV-infected patients undergoing LT, showing very low recurrence rates[272]; monotherapy with entecavir or tenofovir is probably not sufficient to prevent graft reinfection but is considered sufficient to prevent disease recurrence[1].

Risk factors for HBV recurrence include a high HBV-DNA level at LT, presence of HBeAg positivity, HCC, anti-viral drug resistance, and HBIG monoprophylaxis[1,273,274].

Recurrence is defined by the presence of HBsAg in the serum and detectable quantities of DNA, and it is typically linked to clinical evidence of recurrent disease. The goal of treatment is to keep HBV replication under control throughout time to prevent graft loss, even though there is no standard follow-up protocol for early diagnosis[1,275]. Therapy with ETV and/or TDF seems to be efficient and safe when used for treatment of HBV recurrence after LT[276]. Treated recurrence is associated with good prognosis[277-279] but care should be taken in patients transplanted for HCC since HBV recurrence could be a signal of HCC recurrence[277,278].

CONCLUSION

With the dramatic improvement in short-term survival of LT recipients that occurred in recent years, the focus of the physician is shifting to the improvement of long-term outcomes[280]. The main causes of late mortality in this category are not liver related[2]. In this review, we described the main comorbidities and risk factors affecting LT recipients, which in most cases are preventable, can be treated, or are amenable of screening measures, even though there is a lack of consensus to define the best strategy for the follow-up and management of part of these factors, for which we reported some of the suggested approaches (Table 3).

The hepatologist's role in long-term management of LT recipients is becoming more complex with the increase of comorbidities/risk factors that can affect long-term outcomes. A multidisciplinary approach could help overcome this complexity. The importance of a multidisciplinary team is underlined by current guidelines[1,164] with regard to pre-LT evaluation, and its value is recognized in some particular settings such as the prevention and management of alcohol relapse or to improve adherence to therapy[1,161].

The availability of a "long-term management multidisciplinary team" dedicated to LT recipients, could handle or prevent the onset of the aforementioned comorbidities/risk factors and should be composed by psychiatrists, psychologists, cardiologists, general practitioners, nutritionists, dieticians, social workers, and should be coordinated by the transplant hepatologist. We believe this approach could improve the long-term outcomes of the LT recipients.

A body of the evidence is already available for the identification of high-risk patients[14,151,185] and such prognostic ability could allow healthcare providers to focus on those who could benefit the most from preventive measures in a cost-effective manner.

Furthermore, a special mention should go to maintenance immunosuppression, which has a strong impact on patient long-term survival: Although further studies are needed to propose IS withdrawal [108], which should therefore be limited to clinical trials, early minimization of IS (when feasible) seems a rational strategy to limit adverse events and improve long-term outcomes. A deeper understanding of the immunological pathways of rejection would allow to design more specific and safer drugs, in order to tailor therapy[281].

Table 3 Comorbidity/risk factors impairing long-term survival with suggested follow-up and management

Risk Factor/Comorbidity	Follow-up	Management
Metabolic syndrome	(1) Electrocardiogram and transthoracic echocardiography before transplantation. If patient older than 50 and has multiple cardiovascular risk factors, perform a cardiopulmonary exercise test. If coronary disease is suspected, coronary angiography should be executed[1]; and (2) Repeatedly perform a cardiovascular risk stratification after transplantation (for example, every 6 mo)[1].	(1) Aggressive and rapid management of metabolic risk factors in the form of lifestyle changes, pharmacological therapies and modifications of the IS[1]; (2) Dietary counseling for all LT patients[4]; and (3) Consider bariatric surgery in patients who failed behavioral weight-loss programs[4].
Sarcopenia	(1) Sarcopenia evaluation before LT (for example, measuring skeletal mass from CT scan, even required for other reasons)[23]; and (2) New assessment at 1 year after transplantation (possibly using the same method and the same CT machine, in order to compare results. Consider also 24-CER evaluation[20]).	(1) Manage sarcopenia with lifestyle modification and nutritional interventions both pre and post transplantation[1, 28]; (2) Consider nutritional counseling after transplantation; and (3) Consider nutritional supplementations (*e.g.* with HMB)[29].
Osteoporosis	(1) Regular measurement of bone mineral density pre- and post-LT, with a follow up timing dependent on the severity of the disease; and (2) If osteopenic bone disease is confirmed or if atraumatic fractures are present, assess calcium intake, vitamin D levels, gonadal and thyroid function, a full medication history, and thoracolumbar radiography[4].	(1) Manage osteoporosis with calcium and vitamin D replacement (if deficient), consider a weight-bearing exercise pre-operative program[1]; and (2) Consider bisphosphonate therapy (for example, Neridronate, at the dose of 25 mg i.m./mo for 12 mo)[40].
Psychological health and QoL	Actively look for depressive symptoms since the early post-transplantation period[1].	(1) Treat promptly depressive symptoms with adequate pharmacotherapy[54]; (2) Consider psychological support by a specialist if needed; and (3) Propose lifestyle modifications (in particular, personalized aerobic and strength training programs)[55].
Renal dysfunction	(1) Continuous monitoring of renal function with serum creatinine and glomerular filtration rate measurements[1]; and (2) Urinary protein quantification at least once yearly[4].	(1) Treat potential risk factors (diabetes, hypertension); (2) Avoid nephrotoxic drugs[1]; (3) Adjustment of the IS; reduction or withdrawal of CNI or use alternative CNI-free protocols as soon as possible in impaired renal function (for example, EVR combination regimens starting 1 mo after transplantation[67,68]; and (4) Consider kidney transplantation in end-stage renal disease[4].
Infectious risk	Hepatologist accurate counseling to increase the percentage of vaccinated patients[81].	(1) Perform HAV, HBV, varicella, Pneumococcus, influenza and tetanus vaccinations prior to transplantation; (2) Administer inactivated influenza vaccine starting one month after transplantation during community influenza outbreak; (3) Annual influenza vaccination in liver transplanted patients[4]; and (4) Consider Oseltamivir prophylaxis during periods of local influenza circulation[86].
De novo malignancies	Define and follow a surveillance protocol with individualized emphasis laid on patients' particular risk profiles[1].	(1) Treating modifiable risk factors: stop smoking, alcohol withdrawal[99], metabolic syndrome management[100], avoid sunbed use and sun exposure[101], promote HPV vaccination[102]; and (2) Use mTOR- based therapy if possible[1] or a CNI-mTOR combined therapy always at the lowest effective dose.
Smoking	(1) Assess smoking status at each visit, focusing on those with particular risk of relapse (ALD); and (2) Use of biomarkers (serum Cotinine)[116].	(1) Encourage to undertake smoking cessation[4]; and (2) Referral for behavioral/pharmacological therapy[125,126].
Adherence to therapy	Self- and collateral-reported non-adherence, trough levels (considering graft-function and concomitant use of other drugs)[136,139] with particular attention to younger patients and those missing clinic appointments, with a history of substance or alcohol use, mental health needs, divorced or high regimen complexity[131,137,140].	Multidisciplinary measures developed by professional educators, supported by psychologists, and coordinated by physicians[1,136].
Alcohol abuse relapse	(1) Assessing alcohol use at each clinic visit, with particular attention to those transplanted for ALD with pre-transplant abstinence of less than 6 mo, with psychiatric comorbidities, smoking, noncompliant with clinic appointment or medication and lacking social support[147,149,150]; and (2) Assessment by self-reported alcohol use, liver function tests and metabolites of alcohol, such as urinary ethyl glucuronide[157,158].	(1) Preventive structured management by a multidisciplinary team including transplant hepatologist, clinical psychologists and psychiatrists with expertise in alcoholism and social workers[159,160]; (2) Encouraging smoking cessation; and (3) Referral to psychiatric treatment or counselling in case of relapse[1,4].
HCC recurrence	(1) Thoracic CT – abdominal CT or MRI and AFP levels with 3- to 6-mo intervals in the first 2 or 3 years, increasing the interval between exams from that date[194]; (2) Selection of patients who needs a stricter follow-up using prognostic criteria such as: AFP levels, the presence of microvascular invasion, the diameter of the largest viable tumor and the number of viable tumors[202]; and (3) Particular attention to patients transplanted outside of Milan Criteria[176].	(1) Minimizing overall IS; consider adding mTORi[108]; (2) Individualized management of HCC discussed in a multidisciplinary team[303]; and (3) Surgical treatment when feasible[204,205].

Autoimmune disease recurrence	(1) Monitoring liver function tests[223,229] and performing liver biopsy and/or cholangiography when deemed necessary[1]; and (2) Exclude mimicking conditions (ischemia related biliary insults, hepatic artery thrombosis and/or chronic ductopenic rejection, infectious cholangitis for PSC, rejection histological mimicking for PBC and AIH)[223].	(1) AIH: Treatment of active cirrhosis and normalization of transaminases and IgG in the pre-transplantation period plus long-term corticosteroid use after LT; (2) PBC: Use of preventive Ursodeoxycholic Acid; and (3) PSC: Control of IBD and considering pre- or post-transplant colectomy in patients with difficult to control PSC[240].
NASH recurrence	(1) Early identification of Metabolic Syndrome components pre- and post-transplant[304]; (2) Annual screening with US and liver function tests; (3) Noninvasive testing and liver stiffness measurement in case of alterations at the annual screening; and (4) Biopsy in case of suspected fibrotic disease[259].	(1) Management as for other NAFLD/NASH patients (multidisciplinary approach with diet and lifestyle modification, pharmacological treatment and bariatric surgery when necessary[252,257]; (2) Treating metabolic syndrome components)[265,266]; and (3) Corticosteroids and CNI minimization when possible[108].
Viral hepatitis recurrence	(1) Liver function test monitoring; (2) HCV-RNA titres[111]; (3) HBV DNA and HBsAg monitoring[1,275]; (4) Regular assessment of graft damage[1]; and (5) Particular attention to patients transplanted for HCC with HBV recurrence/reactivation[277,278].	(1) Treating HCV before LT when possible; use of DAA[1, 164]; and (2) HBIG and NUCs to prevent HBV recurrence/reactivation[1,272].

LT: Liver transplantation; CT: Computed tomography; CNI: Calcineurin inhibitors; EVR: Everolimus; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HPV: Human papilloma virus; mTORi: Mammalian target of Rapamicin inhibitors; ALD: Alcohol-related liver disease; HCC: Hepatocellular carcinoma; AFP: Alpha fetoprotein; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cholangitis; AIH: Autoimmune hepatitis; NASH: Non-alcoholic steatohepatitis; HCV: Hepatitis C virus; HBIG: Hepatitis B immunoglobulin; NUCs: Nucleos(t)ide analogues.

The question "How can we improve long-term outcomes after liver transplantation?" has no clear and simple answer. The combination of reduction of drugs toxicity, the use of precise instruments that allow to detect high-risk patients and the presence of a multidisciplinary team coordinated by an hepatologist could probably be the key for the improvement of long-term outcomes after LT.

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FOOTNOTES

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MINIREVIEWS

COVID-19 and liver dysfunction in children: Current views and new hypotheses

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Abstract

Coronavirus disease 2019 (COVID-19) poses an extremely serious global impact on public healthcare for individuals of all ages, including children. Increasing evidence has shown that liver abnormalities are commonly found in children with COVID-19, and age-related features in innate and adaptive response have been demonstrated. However, there are few reports and studies on COVID-19 related liver injury in children, and the data are scattered. So that many contradictions have arose. This situation is not only due to the serious ethical issues in studying pediatric patients with COVID-19, but also because of the short duration and wide coverage of the COVID-19 epidemic, the severity and complexity of clinical cases varied, as did the inclusion criteria for case reporting and patient outcomes. Therefore, we totaled the incidences, characteristics and pathomechanism of liver injury in children since the COVID-19 outbreak. The etiology of COVID-19-related liver injury is divided into three categories: (1) The direct mechanism involves severe acute respiratory syndrome coronavirus 2 binding to angiotensin-converting enzyme 2 in the liver or bile duct to exert direct toxicity; (2) the indirect mechanisms include an inflammatory immune response and hypoxia; and (3) COVID-19-related treatments, such as mechanical ventilation and antiviral drugs, may cause liver injury. In summary, this minireview provides fundamental insights into COVID-19 and liver dysfunction in children.

Key Words: COVID-19; SARS-CoV-2; Children; Liver injury; Inflammatory immune response; Cytokine storm

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Core Tip: There are few cases of liver injury in children with coronavirus disease 2019 (COVID-19) and clinical reports are scarce. We collected reports on COVID-19-related liver injury (CRLI) in children over the last two years and divided the etiology of CRLI into three categories: (1) The direct mechanism involves severe acute respiratory syndrome coronavirus 2 binding to angiotensin-converting enzyme 2 in the liver or bile duct to exert direct toxicity; (2) the indirect mechanisms include an inflammatory immune response and hypoxia; and (3) COVID-19-related treatments, such as mechanical ventilation and antiviral drugs, may cause liver injury. We also discuss the current controversies regarding the pathophysiology of CRLI.

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INTRODUCTION

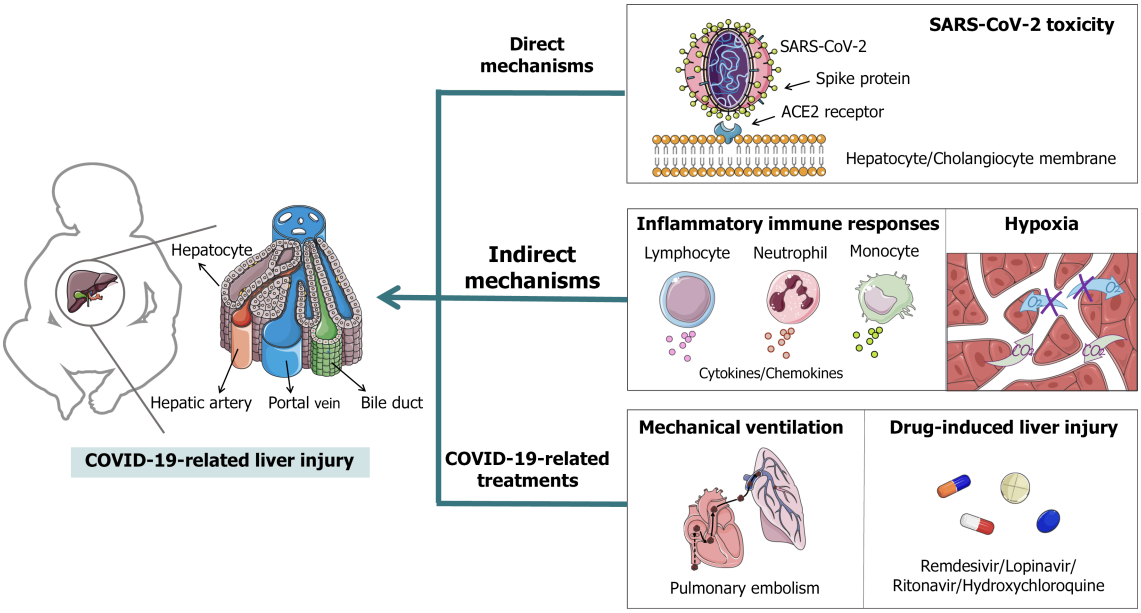
Coronavirus disease 2019 (COVID-19) is an infectious respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In response to this global health crisis, governments and medical institutions have been actively working to improve epidemic prevention measures, and diagnostic and treatment methods, all of which have significantly reduced the transmission rate and mortality rate[1-3]. However, this crisis is not yet over, and the physical damage caused by COVID-19 is gradually expanding from respiratory to systemic diseases. In addition to inducing acute respiratory distress syndrome (ARDS)[4,5], COVID-19 also causes damage to organs such as the liver, gastrointestinal tract, kidney, heart and nervous system[6-9]. The liver is an important center for regulating physiological processes such as nutrient and exogenous drug metabolism, immunity, endocrinology and blood volume[10]. Liver injury due to any cause (*e.g.*, viral infection, nutritional overload or tumor burden) is a global health problem. COVID-19-related liver injury (CRLI) is defined as any liver injury that occurs during the disease course and treatment in COVID-19 patients, regardless of the presence of prior liver disease[11]. A study showed that approximately 2–11% of patients had underlying chronic liver disease and 14%-53% developed liver injury during the course of COVID-19[12].

COVID-19 mainly occurs in the elderly and people with potential complications[13]. The lethality of infection increases logarithmically and linearly with age in those over 30 years, but children have a lower prevalence and tend to be asymptomatic or have mild to moderate disease[14]. Therefore, currently published case reports mainly describe adult patients, resulting in a lack of details in pediatric cases. However, most infections in children originate from family contacts, they play an important role in disease transmission and have become a key target population for epidemic prevention and control measures[15]. Meta-analyses have shown that liver injury is common in children, but is often overlooked[16]. Therefore, we focused on pediatric patients with CRLI and divided the pathogenesis of CRLI in children into three categories: direct, indirect and treatment-related pathogenesis (Figure 1).

FEATURES OF CRLI IN CHILDREN

COVID-19 can cause varying severity of liver injury, as evidenced by abnormal elevations in alanine aminotransferase (ALT) and aspartate aminotransferase (AST), accompanied by mild elevations in alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin (TBIL) and a reduction in albumin[17]. The abnormal liver enzyme levels in serum include: ALT > 40 U/L, AST > 40 U/L, GGT > 49 U/L, ALP > 135 U/L, TBIL > 17.1 μmol/L and albumin < 3 g/dL[18]. Recently, several studies have provided the results of abnormal liver tests in pediatric COVID-19. Alkan *et al*[19] found that 130 (44.2%) of 294 patients (age range: 14 d-18 years) with COVID-19 had abnormal liver function and most patients (33.3%) were characterized by elevated ALT, and other patients had elevated ALT (5.1%), ALP (6.6%), GGT (8.9%) and TBIL (3.8%). In addition, decreased albumin was also observed by Esmaeili *et al*[20] and Liu *et al*[21]. In their studies, the proportion of decreased albumin in pediatric patients was 16.7%[20] and 18.2%[21], respectively.

In general, the main manifestations of CRLI in children were mildly elevated ALT/AST and most research has confirmed this, for instance, Parri *et al*[22] reported on 130 children (age range: 0-17 years) with COVID-19 in Italy, and 8/68 (11.8%) children had elevated ALT and 11/60 (18.3%) had elevated AST. The analysis by Du *et al*[23] showed that ALT and AST increased in 9 (5.0%) and 24 (13.3%) of 180 subjects (age range: 0-15 years), respectively, and 11 (6.3%) of 174 subjects showed increased ALP levels. Thus, the elevation of liver enzymes in pediatric patients is not significant, which may be due to the fact that COVID-19 is mainly mild in children.



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Figure 1 The possible pathophysiological mechanisms of Coronavirus disease 2019-related liver injury in children. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2; COVID-19: Coronavirus disease 2019.

In addition, Sun *et al*[24] conducted a single center observational study of 8 children (age range: 2 mo-15 years) with severe COVID-19 and the results showed that ALT was increased in 4 (50.0%) cases but increased AST was not observed. It is possible that sometimes abnormally elevated ALT/AST is not a sufficient indicator of liver injury. The related studies on the features of CRLI in children are summarized in Table 1.

DIRECT PATHOPHYSIOLOGICAL MECHANISMS

Toxicity of SARS-CoV-2 to hepatocytes and cholangiocytes

Genome sequencing, and phylogenetic and structural analyses have confirmed that SARS-CoV-2 can bind to angiotensin-converting enzyme 2 (ACE2) of host cells depending on its spike protein, and this binding can mediate membrane fusion and viral invasion[25]. ACE2 is not only highly expressed in alveolar cells, but also distributed in various organs throughout the body, including the liver[26]. Thus, the direct pathological basis of CRLI is the viral virulence of SARS-CoV-2, and it can bind to ACE2 on liver endothelial cells and exert toxicity causing hepatocyte damage[27,28]. Unlike adults, children have milder symptoms of CRLI, possibly due to lower ACE2 expression, lower maturity, and weaker function (*e.g.*, binding to SARS-CoV-2). However, in contrast, ACE2 expression decreases with age; thus, ACE2 Levels are higher in children than in adults[29]. Moreover, one of the functions of ACE2 is to convert angiotensin (Ang) II to Ang(1-7), which has anti-inflammatory and anti-liver fibrosis effects[30,31]. Therefore, besides the ability to mediate viral infections, the distribution of ACE2 in different age groups and the "dual action" of it on organ damage require further investigation.

Interestingly, Chai *et al*[32] indicated that ACE2 is highly expressed on cholangiocytes compared to hepatocytes and that SARS-CoV-2 may prefer to bind directly to ACE2 on cholangiocytes. Cholangiocytes are epithelial cells that line the intrahepatic and extrahepatic bile ducts and play an important role in liver regeneration and immune response[33]. This suggests that the liver abnormalities in COVID-19 patients may not be directly caused by hepatocyte injury and that the potential damage to cholangiocytes by SARS-CoV-2 may have a profound effect on the liver.

In addition, CRLI can be classified into three categories according to the degree of liver enzymes exceeding the upper limit of normal (ULN) (Table 2). Patients were classified as hepatocyte injury type when they had raised ALT and/or AST more than 3×ULN; patients were classified as cholangiocyte injury type when they had raised ALP and/or GGT more than 2×ULN; when the first two requirements were met simultaneously, patients were considered to have mixed injury type[18,34]. There are obvious differences in CRLI types between adults and children. Cai *et al*[18] found that the number of liver injuries in 318 adult COVID-19 patients with abnormal liver test results was as follows: mixed type (43.4%) > cholestatic type (29.2%) > hepatocellular type (20.8%). Furthermore, elevation of ALP, a marker of bile duct injury, is less common than abnormal liver enzymes in adults[35]. With regard to children, Alkan *et al*[19] found that the number of liver injuries in 130 pediatric patients was as follows:

Table 1 Laboratory features of coronavirus disease 2019-related liver injury in children

Ref.	Number of patients	Age range	Abnormal liver function	E-ALT	E-AST	E-ALP	E-GGT	E-TBIL	D-albumi, *n* (%)
Alkan *et al*[19]	294	14 d-18 years	130 (44.2%)	15 (5.1%)	98 (33.3%)	19 (6.6%)	26 (8.9%)	11 (3.8%)	NA
Esmaeili *et al* [20]	18	3-10 years	6 (33.3%)	5 (27.8%)	7 (38.9%)	0	NA	3 (16.7%)	3 (16.7%)
Liu *et al*[21]	46	0-1 year	20 (43.5%)	11 (25.0%)	20 (45.5%)	NA	NA	6 (13.6%)	8 (18.2%)
Parri *et al*[22]	130	0-17 years	NA	8/68 (11.8%)	11/60 (18.3%)	NA	NA	NA	NA
Du *et al*[23]	182	0-15 years	NA	9/180 (5.0%)	24/180 (13.3%)	11/174 (6.3%)	NA	NA	NA
Sun *et al*[24]	8	2 mo-15 years	4 (50.0%)	4 (50.0%)	0	NA	NA	0	NA

E-ALT: Elevated alanine aminotransferase; E-AST: Elevated aspartate aminotransferase; E-ALP: Elevated alkaline phosphatase; E-GGT: Elevated gamma-glutamyl transferase; E-TBIL: Elevated total bilirubin; D-albumin: Decreased albumin; NA: Not available.

Table 2 The frequencies of different coronavirus disease 2019-related liver injury types in adults and children

CRLI types	Liver test parameters[18]	Adults[18]	Children[19]
Hepatocyte type	ALT/AST ≥ 3×ULN	66 (20.8%)	24 (18.5%)
Cholangiocyte type	ALP/GGT ≥ 2×ULN	93 (29.2%)	93 (71.5%)
Mixed type	ALT/AST ≥ 3×ULN and ALP/GGT ≥ 2×ULN	138 (43.4%)	13 (10.0%)

CRLI: Coronavirus disease 2019-related liver injury; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; ULN: Upper limit of normal.

cholestatic type (71.5%) > hepatocellular type (18.5%) > mixed type (10.0%). The rate of ALP elevation in pediatric patients was slightly more than ALT (Table 1). Cholestatic liver injury is especially common in children under 5 years of age, and this may be related to ACE2 being less distributed in hepatocytes and more distributed in cholangiocytes in younger children[19]. Therefore, we should be more concerned about cholestatic liver injury in pediatric patients with COVID-19.

INDIRECT PATHOPHYSIOLOGICAL MECHANISMS

Inflammatory immune response-mediated liver injury

Inflammatory response and immune response are inseparable systemic responses at the organ, tissue, cellular and molecular levels. A moderate inflammatory immune response (IIR) plays a crucial part in protecting the body from pathological damage in the internal and external environment. However, an excessive IIR is the pathological basis for the development of multiple systemic diseases. An increasing number of studies have shown that the systemic IIR induced by SARS-CoV-2 has an intricate pathophysiological link to liver injury.

Dysfunction of innate and adaptive immune responses: Natural killer (NK) cells and natural antibodies are key components of the innate immune system and can be the first to respond to new viruses[36,37]. The adaptive immune response then comes into play and produces highly specific memory T and B cells to clear the virus and prevent reinfection[38]. However, dysfunctional innate and adaptive immune responses mediate the host damage caused by SARS-CoV-2[39].

The innate immune system detects SARS-CoV-2 mainly by two strategies: first, the presence of SARS-CoV-2 can be detected using various pattern recognition receptors, such as plasmacytoid dendritic cells detecting incoming viral genomic RNA in the intranuclear body *via* toll-like receptor (TLR); second, during viral replication, double-stranded RNA intermediates can be recognized by the RIG-I like receptor (RLR), a cytoplasmic RNA sensor. Following the engagement of the TLR and RLR, downstream signaling activates the transcription of interferons (IFNs) and pro-inflammatory cytokines and chemokines[40]. SARS-CoV-2 can block innate immune recognition and signal transduction using the

expression of viral proteins[41]. Uncontrolled innate immune signaling may produce excess cytokines, which can trigger inflammation and worsen the condition.

With regard to the adaptive immune system, its three basic components are B cells, CD4+ T cells and CD8+ T cells. B cells can rapidly produce neutralizing antibodies after infection with SARS-CoV-2, and the antibody target is the spike protein of the virus[42]. T-cell responses were detected in almost all SARS-CoV-2 infections, and virus-specific CD4+ T cells can differentiate into Th1 cells and T follicular helper cells, which have antiviral activity through the production of IFN-γ and related cytokines[43]. The CD8+ T cells are essential for clearing virus by killing infected cells. In milder symptoms, SARS-CoV-2 specific CD4+ and CD8+ T cells can respond rapidly in the acute phase of COVID-19 and thus exert antiviral effects[44].

Recently, studies have revealed changes in the immune responses of COVID-19 pediatric patients (Table 3). Diao *et al*[45] retrospectively reviewed 522 patients (age range: 5 d-97 years) and demonstrated that more than 70% of the patients had a significant reduction in total T cells, CD4+ and CD8+ T cells, but the reduction was age-dependent as the younger patients had the least reduction in T cells. In addition, unlike in adults, CD4+ T cells were even increased in moderate pediatric cases[46]. Also, compared with mild cases, moderate cases had higher levels of IL-10, complement (C) 4 and NK cells, while neutrophils were significantly lower[46]. These findings suggested that the innate cells such as NK cells and neutrophils play a crucial role in the initial phase and the CD4+ T cells perform a function in the later phase of COVID-19. Studies have shown that CD4+ T cells and IL-10 Levels are positively correlated with CRLI biomarkers in pediatric patients[46], and although the IL-10 derived from CD4+ T cells plays an important anti-inflammatory role in mild patients, excess IL-10 may cause liver injury with COVID-19 progression.

Furthermore, Li *et al*[47] showed that in children with severe COVID-19, CD3+, CD3+CD4+ (helper T cells), and CD3+CD8+ (memory T cells) counts were decreased, and the pro-inflammatory cytokine IL-6 and immune regulatory cytokine IL-10 were increased. Other inflammatory cytokines such as IL-2, IL-4, IL-10, TNF-α and IFN-γ were also detected[47]. However, the inflammatory cytokines IL-2, IL-4, IL-6, TNF-α and IFN-γ were rarely increased in mild and moderate pediatric patients[46]. These results suggested that although the adaptive immunity of children is relatively weak, their innate immunity is less likely to be disordered after SARS-CoV-2 infection and the cytokine storm associated with inflammation is not severe in most pediatric patients. Therefore, the direct liver injury caused by cytokine storms in children may not be as severe as in adults. However, recent research found that CRLI is most common in children under the age of five years[19]. This illustrated that SARS-CoV-2 infection can still trigger a series of damages once it exceeds the threshold due to the weak adaptive immunity of younger children.

Chemokines are small molecule proteins that have the ability to induce targeted chemotaxis of immune cells during inflammation and they also play an important role in dealing with viral infections [48]. Several studies have shown that the main pro-inflammatory chemokines such as CXCL [chemokine (C-X-C motif) ligand] 8, CXCL9, CXCL10, CCL (CC chemokine ligand) 2, CCL3 and CCL5 were increased in patients with aggravation of SARS-CoV-2 infection[48]. However, the levels of CXCL8, CXCL10, and CCL2 were unchanged in pediatric COVID-19 according to Warner *et al*[49]. These findings also suggested that the IIR in pediatric patients with COVID-19 is less severe than in adults.

B cells play a key role in immune regulation and antibody secretion. Previous studies have indicated that total B cells in COVID-19 patients were induced[23,48]. Du *et al*[23] conducted an analysis of 182 pediatric COVID-19 patients with different severity and showed that the levels of immunoglobulin (Ig) E, IgG and IgA were generally in the normal range or were elevated in isolated cases among mild or moderate patients. However, IgG and IgM counts were induced in severe COVID-19 patients[23]. Wu *et al*[46] noted that Igs including IgG, IgA and IgM were negatively correlated with biomarkers in the liver of pediatric patients. This may be the reason why CRLI is more common in severe patients and AST is only slightly elevated in mild patients.

Multisystem inflammatory syndrome in children-mediated liver injury: Recently, clinical reports have shown that children infected with SARS-CoV-2 for several weeks may develop a characteristic complication: multisystem inflammatory syndrome in children (MIS-C). A national study initiated in 2020 at Boston Children's Hospital, Boston, MA, United States, with real-time monitoring of approximately 35 United States children's hospitals, reported that of 186 MIS-C cases, 131 were positive for SARS-CoV-2[50]. MIS-C presents with persistent acute fever, abdominal pain, diarrhea, rash, lymphadenopathy, and in severe cases appendicitis and peritonitis, which may progress to multiorgan dysfunction[51,52]. Different types of liver injury mediated by MIS-C are being reported successively. Giannattasio *et al*[53] reviewed 55 pediatric patients (mean age 6.5 ± 3.7 years) with MIS-C and showed that 16 patients had acute liver injury (ALI) at admission and 10 more patients developed ALI during observation, ALI was defined by the presence of ALT elevation > 40 U/L. Furthermore, a 14-year-old boy developed MIS-C after SARS-COV-2 infection which was followed by hepatic steatosis, and the researchers also found elevated levels of ALT, AST and indices of cholestasis[54]. Another 10-month-old boy developed fulminant acute liver failure due to MIS-C[55]. The pathophysiology of MIS-C-mediated liver injury may also be related to the IIR as described previously.

Table 3 Immune features in pediatric patients with varying degrees of coronavirus disease 2019

Biomarker	Severe	Moderate	Mild/asymptomatic
T lymphocyte[45-47]	CD3+↓; CD4+ ↓; CD8+ ↓; CD3+CD4+ ↓; CD3+CD8+ ↓	CD3+ →; CD4+ →/↑; CD8+ →	CD3+ →; CD4+ →; CD8+ →
B lymphocyte[23,46]	↓	→/↑	→/↑
Innate cell[23,46]	Monocytes ↑; Neutrophils ↑; NK cells ↓	Monocytes ↑; NK cells ↑; Neutrophils ↓	Monocytes →; Neutrophils →; NK cells →
Immunological parameters [23,46]	IgE ↑; IgG ↓; IgA ↑; IgM ↓; C3 ↑/↓; C4 ↑/↓	IgE →/↑; IgG →/↑; IgA →/↑; IgM →; C3 ↑/↓; C4 ↑	IgE →/↑; IgG →/↑; IgA →/↑; IgM →; C3 ↑/↓; C4 →
Inflammatory cytokine[23, 46,47]	IL-2 ↑; IL-4 ↑; IL-6 ↑; IL-10 ↑; IFN-γ ↑; TNF-α ↑	IL-2 →; IL-4 →; IL-6 →; IL-10 ↑; IFN-γ →; TNF-α →	IL-2 →; IL-4 →; IL-6 →; IL-10 →; IFN-γ →; TNF-α →
Chemokines[49]	CXCL10 →; CXCL8 →; CCL2 →	CXCL10 →; CXCL8 →; CCL2 →	CXCL10 →; CXCL8 →; CCL2 →

↑: Increased; ↓: Decreased; →: Unchanged.
NK: Nature killer; Ig: Immunoglobulin; C: Complement; IL: Interleukin; IFN: Interferon; TNF: Tumor necrosis factor; CXCL: Chemokine (C-X-C motif) ligand; CCL: CC chemokine ligand.

Complement dysfunction-mediated liver injury: A new pathological mechanism of CRLI is dysfunction of the complement system. Complement is also part of the immune system which provides innate defense against pathogens and mediates inflammatory reactions. However, during SARS-CoV-2 infection, an overactive complement response leads to systemic inflammation, and a negative complement response promotes viral replication and infection, thereby exacerbating disease and inducing damage to other organs[56]. Du *et al*[23] published a report on 183 pediatric patients with COVID-19, C3 was elevated and decreased in 12.4% and 18.6% of severe patients, respectively, and C4 was elevated and decreased in 3.7% and 4.3% of severe patients, respectively (Table 3). In addition, it has been shown that complement correlates with the coagulation cascade and dysregulated complement activation may also contribute to the hypercoagulable state in severe COVID-19 patients[57]. For example, a report by Antala *et al*[58] showed that of four children with CRLI, two had complement dysfunction and resulted in microangiopathy, one of which showed rapid improvement in liver function after treatment with eculizumab. All of these findings demonstrate that severe CRLI may be associated with complement dysfunction and microangiopathy features.

Hypoxia-mediated liver injury

The liver normally consumes 20% of whole body oxygen due to its dual blood flow system in the hepatic artery and portal system. In addition, the liver is able to extract 95% of blood-oxygen in order to maintain oxygen uptake[59]. It is well-known that ARDS is the most significant complication of COVID-19, which usually presents with respiratory distress, hypoxemia and acute respiratory failure[4,5]. All of these are important risk factors contributing to hypoxic hepatitis (HH). HH is characterized by a large and rapid increase in serum transaminases due to a decrease in oxygen delivery to the liver[60]. Furthermore, inflammatory cytokines may reduce the ability of hepatocytes to extract oxygen from the blood leading to hepatocyte death[61]. Thus, IIR caused by SARS-CoV-2 infection may promote the development of HH. Current studies suggest that HH is uncommon in patients with COVID-19, but has a very high mortality rate. For instance, Wu *et al*[62] identified 8 adult cases with HH among 3041 COVID-19 patients, and only 1 (12.5%) patient was discharged, and 7 (87.5%) died. Despite the lack of related reports on HH in pediatric patients, it is also a warning signal that we should be more concerned about the possibility of HH in children.

COVID-19-RELATED TREATMENT CAUSES LIVER INJURY

Mechanical ventilation-mediated liver injury

Approximately 23% of patients with SARS-CoV-2 infection developed pulmonary embolism[63]; therefore, some form of ventilation support, such as a high-flow nasal cannula, non-invasive and invasive mechanical ventilation, is required to prevent hemodynamic instability[64]. Woodruff *et al*[65] investigated COVID-19 -associated hospitalization surveillance network of 14 states in United States, they found that 691 (30.1%) patients required ICU admission and 122 (5.3%) patients needed invasive mechanical ventilation among 2293 hospitalized children (aged < 18 years). Moreover, other several researches also have showed 6% to 18% pediatric patients of COVID-19 required mechanical ventilation and 3% have died[66-71]. Current pediatric ventilation strategies are usually based on adult reports,

which may lead to increased pulmonary vascular resistance and thus reduced right ventricular (RV) activity[72]. RV dysfunction is a good predictor of heart failure[73]. As the liver is the largest visceral organ in the human body and receives up to 25% of the entire cardiac output, RV failure can not only aggravate liver injury by liver congestion attributed to elevated central venous pressure, but also ischemic hepatitis[59]. Additionally, a multivariate regression analysis showed a significant increase in the severity of COVID-19 among pediatric patients receiving mechanical ventilation[74]. Therefore, physicians should pay attention to the changes in cardiac function and the possibility of subsequent liver injury when mechanical ventilation is given to pediatric patients.

Drug-induced liver injury

Drugs are mainly metabolized by the liver. Drug-related liver injury (DRLI) remains an important focus in the monitoring of new drugs and drug repurposing. At present, the use of anti-SARS-CoV-2 drugs in pediatric patients is dependent on the evidence from adult clinical cases due to the emergency of COVID-19. The Italian Society of Infectious Pediatric Diseases recommends the use of remdesivir in pediatric patients with severe COVID-19 in whom renal and liver functions are normal, lopinavir/ritonavir should only be considered if remdesivir is incompatible or unavailable, dexamethasone and tocilizumab can be administered in patients with ARDS or MIS-C[75]. A medication guidance from a North American institution suggested using hydroxychloroquine as first-line treatment in children under 12 years and as second-line treatment in children above 12 years[76]. DRLI in pediatric cases is predominantly characterized by elevated liver enzymes as described by Goldman *et al*[77] in 77 children with severe COVID-19 treated with remdesivir, where 3 patients discontinued remdesivir due to elevated liver enzyme levels. The evaluation of other antiviral drugs in the pediatric population is uncommon. Although there are fewer pediatric patients with severe COVID-19, the use of antiviral drugs still deserves a separate discussion to develop a more appropriate therapy for children.

POINTS OF CONTENTION ON CRLI

The above conclusions are drawn from a limited number of pediatric cases and there are serious ethical questions about research on children with COVID-19. Therefore, many conflicting views remain to be further explored, for example: (1) "SARS-CoV-2 binds to ACE2 and exerts direct liver injury" *vs* "Ang(1-7) produced by ACE2 hydrolysis of Ang II has anti-inflammatory and anti-fibrotic effects on the liver"; (2) "The expression and function of ACE2 are weaker in children" *vs* "The expression of ACE2 decreases with age"; (3) "Cholestatic liver injury is more common in children" *vs* "The elevation of biliary injury marker ALP was not significant"; (4) "Cytokine storm can lead to inflammation and liver injury" *vs* "Cytokine storm is mild in pediatric patients"; and (5) "Inhibition of the complement system may aggravate viral infection and cause liver injury" *vs* "Excessive activation of the complement system may induce inflammation and cause liver injury".

It is normal for these contradictions to emerge. As the short duration and wide coverage of the COVID-19 epidemic, the severity and complexity of clinical cases vary, and the criteria for inclusion and the outcome of patients are also different among case reports. For the longer term future, we should continue to focus on CRLI to address these issues.

CONCLUSION

With the continuous progress of COVID-19, liver injury is becoming a research focus. We have divided the etiology of CRLI in children into three categories, and the possible pathophysiological mechanisms are discussed separately. Of these, the direct mechanism involves SARS-CoV-2 binding to ACE2 in the liver or bile duct to exert direct toxicity, the indirect mechanism includes IIR and hypoxia, and COVID-19-related treatments may also cause liver injury under some circumstances, such as the use of mechanical ventilation and antiviral drugs. In summary, children are characterized by strong innate immunity but weak adaptive immunity, and the IIR resulting from SARS-CoV-2 is still the main cause of liver injury. The evaluation of liver injury in pediatric patients with severe COVID-19, especially those with MIS-C, should be a focus. Another focus is the toxicity of SARS-CoV-2 to cholangiocytes, as children more commonly have cholestatic liver injury. In addition, hypoxia may promote liver injury due to the high incidence of ARDS complications. Finally, liver injury induced during COVID-19 treatment is often overlooked. Mechanical ventilation in children with respiratory distress can lead to the risk of RV dysfunction and subsequent liver injury, and the use of antiviral drugs in children may also lead to DRLI. In order to reach a consensus on the etiology of CRLI, more pediatric case reports, more detailed classifications and more in-depth studies are pending.

FOOTNOTES

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MINIREVIEWS

May 2022 acute hepatitis outbreak, is there a role for COVID-19 and other viruses?

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Abstract

There has been an increasing number of reported cases of acute hepatitis of unknown origin in previously healthy children since first reported on March 31, 2022. This clinical syndrome is identified by jaundice and markedly elevated liver enzymes with increased aspartate transaminase and/or alanine aminotransaminase (greater than 500 IU/L). We conducted an inclusive literature review with respect to acute hepatitis outbreaks in children using the search terms acute hepatitis, outbreak, children, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), coronavirus disease 2019 (COVID-19), and adenovirus. According to the cumulative data presented in four main studies, the median age is 4 years, with a male predominance (1.3:1). Jaundice was the most common clinical mani-

festation (69%), followed by vomiting (63%), anorexia (52.9%), diarrhea (47.2%), abdominal pain (39%), pyrexia (33.3%), pale stool (30%), and dark urine (30%). Coryza and lethargy were reported in 16.6%, while pruritus was reported in 2% of cases. Acute liver failure was observed in 25% of cases. The exact mechanism of this acute hepatitis outbreak is still not entirely clear. Adenoviruses and SARS-CoV-2 were detected in a significant number of patients. Coinfection with adenovirus and SARS-CoV-2 could be a possible underlying mechanism. However, other possible infections and mechanisms must be considered in the pathogenesis of this condition. Acute hepatitis of unknown origin in children has been a serious problem since the start of the COVID-19 pandemic but has not yet been sufficiently addressed. Many questions remain regarding the underlying mechanisms leading to acute liver failure in children, and it is likely that extensive future research is needed.

Key Words: Acute hepatitis of unknown origin; Children; Adenovirus; SARS-CoV-2; COVID-19; Hepatic failure

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Core Tip: There has been an increasing number of acute hepatitis of unknown origin in children since first reported on March 31, 2022. The exact mechanism of this acute hepatitis outbreak is still unclear. Still, the increased detection rate of adenoviruses and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) may imply a key role for these viruses in the pathogenesis of this childhood condition. Coinfection with adenovirus and SARS-CoV-2 could also play a role, but comprehensive research is still needed to reach an exact mechanism. Until an aetiology is uncovered, the focus should be placed on the prevention of this syndrome in children *via* the use of proper hygiene.

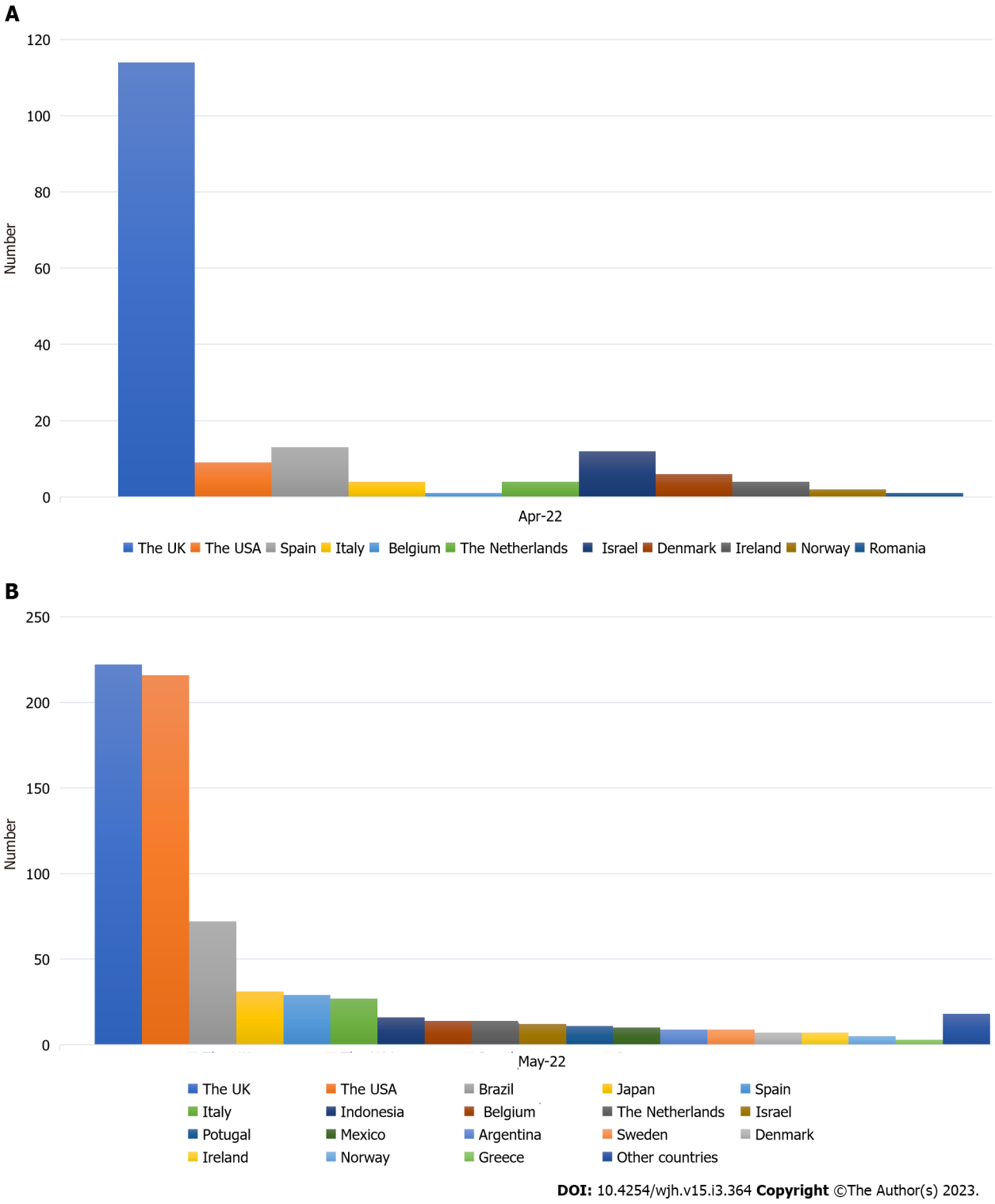
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INTRODUCTION

Since it was first reported on March 31, 2022, in Scotland, and with the recent increase in the reported cases since April 15, 2022, in the United Kingdom, a reason for acute hepatitis of unknown origin in previously healthy children has not been clearly defined. There is speculation as to whether this phenomenon represents a true increase in the number of cases or if it is an inflated statistic due to increased awareness and reporting. By the last week of April 2022, there were 169 cases of acute hepatitis of unknown origin in children aged 1 mo to 16 years reported from 11 countries, including The United Kingdom, Spain, Israel, The United States, Denmark, Ireland, Netherlands, Italy, Norway, France, Romania, and Belgium[1]. By the end of May 2022, the number of reported cases increased to 746, reported from 36 countries in 4 continents, mainly Europe and America (Figure 1)[2].

The clinical syndrome caused by acute hepatitis is identified by jaundice and markedly elevated liver enzymes, with increases in aspartate transaminase (AST) and/or alanine aminotransaminase (ALT) to greater than 500 IU/L. These findings may be preceded by gastrointestinal symptoms such as abdominal pain, vomiting, and diarrhoea. Fever is also reported in a few cases, but most affected children are afebrile. Furthermore, many patients experience more severe complications, such as liver failure[3]. Most hepatotropic viruses that cause acute hepatitis, such as hepatitis A, B, C, D, and E viruses, are not detected in acute hepatitis of unknown origin. About 10% of cases require liver transplantation, with 1 fatal case being reported. Despite most cases being reported from Europe, Israel, and The United States, there is no link between traveling to any specific country and developing the syndrome[4].

The exact mechanism of this type of acute hepatitis outbreak is still not known. However, adenovirus is reported in 74 cases, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 20 cases, and coinfection in 19 cases. Meanwhile, data from The United Kingdom and the Netherlands show an increase in adenovirus infection in the community concurrently with the rise in the number of cases of acute hepatitis of unknown origin[5]. Although there is some evidence for the role of adenovirus with or without SARS-CoV-2 coinfection in the aetiology of this syndrome, other factors, such as immunopathogenesis and non-infection-related factors, could play a role. This review aims to shed light on the understanding of this syndrome[6].

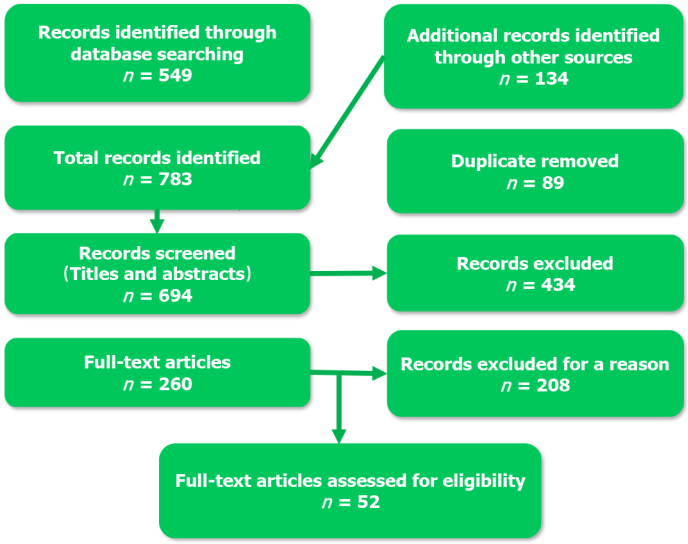


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Figure 1 Reported cases of acute hepatitis syndrome of unknown aetiology in children. A: April 2022; B: May 2022. Apr: April; The UK: The United Kingdom; The USA: The United States of America.

METHODOLOGY

We conducted an inclusive literature review by searching various electronic databases for reports on acute hepatitis outbreaks in children. Databases searched included PubMed, Embase, Cumulative Index to Nursing and Allied Health Literature, Web of Science, Cochrane Library, Scopus, Library and Information Science Abstracts, Google search, and the National Library of Medicine catalogue. The search included reports published before August 31, 2022. Search terms utilized included acute hepatitis, outbreak, children, SARS-CoV-2, coronavirus disease 2019 (COVID-19), and adenovirus. Reference lists were inspected, and citation searches were also done on the included studies. We included open access papers published in English. Figure 2 shows a flow chart of the reviewed articles.



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Figure 2 Flow chart of studies included in this review.

We reviewed 260 articles concerned with acute hepatitis of unknown aetiology outbreaks in children; information from 52 was included in this review.

CLINICAL SPECTRUM OF ACUTE HEPATITIS OF UNKNOWN CAUSE IN CHILDREN

The World Health Organization (WHO) has classified cases of severe acute hepatitis of unknown origin in children occurring during the current outbreak into three categories: (1) Confirmed; (2) Probable; and (3) Epi-linked. As there are no well-defined diagnostic criteria for this clinical entity, the probable case definition is the most widely used. Probable cases are those which present with manifestations of acute hepatitis with elevated serum aminotransferase levels of greater than 500 IU/L and without evidence of infection with hepatitis A-E viruses. The patient population is defined as children aged 16 years or younger as of October 1, 2021, with the youngest reported patient being 1 mo old. An epi-linked case is defined as a patient of any age presenting with acute hepatitis who has had a history of close contact with a probable case since October 1, 2021, and has no evidence of hepatitis A-E virus infection[7]. Three-quarters of reported cases in European countries are younger than age 5, as many studies have been focused on children younger than 10 years[8]. According to cumulative data presented in 4 main studies (2 from The United Kingdom and 2 from The United States), the median age of presentation is 4 years, with a male predominance (1.3:1). Jaundice is the commonlyommon reported clinical manifestation (69%), followed by vomiting (63%), anorexia (52.9%), diarrhoea (47.2%), abdominal pain (39%), pyrexia (33.3%), pale stool (30%), and dark urine (30%). Coryza and lethargy are reported in 16.6%, while pruritus is reported in 2% of cases. Acute liver failure is reported in 25% of these cases. See Table 1 for an overview of these data[9-12].

Kelgeri *et al*[9] found that in 44 cases of childhood acute hepatitis of unknown origin, hepatitis most commonly resolved. However, progression to fulminant liver failure requiring liver transplantation is reported in 14% of these cases. This finding underscores the severity of this condition and emphasizes the importance of recognizing its potential outcomes. In affected children, a prodromal phase is typically indicated by biochemical testing. Ultrasound findings of gallbladder wall thickening, pericholecystic fluid, mesenteric lymphadenopathy, and mild hepatosplenomegaly indicate a potential viral aetiology. If these findings are present, an extensive viral workup is required, especially if infection with an adenovirus is suspected. Laboratory tests essential to the diagnosis of suspected acute hepatitis of unknown origin are summarized in Table 2.

GUT-LIVER AXIS AND HEPATIC DISEASES

The gut microbiome affects various essential processes, including immunological, structural, metabolic, and neurological functions. For this reason, gut microbiome status can considerably impact physical and mental health. The gut is colonized by over 1000 microbial species, a process that starts *in utero* and continues after birth in an ongoing, complex, dynamic manner to promote gut maturation and development[13]. Although the number of microbial species in the gut microbiota of children and

Table 1 Clinical data from various studies, *n* (%)

Ref.	Kelgeri *et al*[9], 2022	Cates *et al*[10], 2022	Marsh *et al*[11], 2022	Baker *et al*[12], 2022	Cumulative data
Demographic data					
Country	UK	USA	Scotland, UK	Alabama, USA	
Number of patients	44	296	13	9	362
Age (yr), median (range)	4 (1-7)	2.2 (0–9.7)	3.9 (3-5)	2 (1.66-5.7)	3 (0-9.7)
Male/female ratio	0.83:1	1.42:1	1.2:1	0.28:1	1.3:1
Clinical findings					
Jaundice	41 (93)	71/123 (57.7)	8/9 (88.9)	8/9 (88.9)	128/185 (69)
Vomiting	24 (54)	76/123 (61.8)	4/4 (100)	7/9 (77.8)	113/180 (63)
Diarrhoea	14 (32)	61/123 (49.6)	4/4 (100)	6/9 (66.7)	85/180 (47.2)
Pale stools	13 (30)	/	/	/	13/44 (30)
Abdominal pain	12 (27)	48/123 (39.0)	7/9 (77.8)	/	69/176 (39)
Lethargy	10 (23)	15/123 (12.2)	4/4 (100)	1/9 (11.1)	30/180 (16.6)
Dark urine	6 (14)	44/123 (35.8)	/	/	50/167 (30.0)
Coryza	6 (14)	20/123 (16.3)	/	3/9 (33.3)	29/176 (16.5)
Pyrexia	4 (9)	51/123 (41.5)	0/4 (0)	5/9 (55.6)	60/180 (33.3)
Pruritus	1 (2)	/	/	/	1/44 (2.0)
Anorexia		65/123 (52.9)	/	/	65/123 (52.9)
Acute live failure	6 (14)	37/123 (30.1)	/	1/9 (11.1)	44/176 (25)

UK: The United Kingdom; USA: The United States of America.

adolescents mirrors that of adult, the relative abundance of species varies. In children and adolescents, there are more abundant *Faecalibacterium spp.*, *Bifidobacterium spp.*, and subspecies of *Lachnospiraceae*[14]. In addition, fungi and viruses are also present in the gut. Virobiota of the gut include bacteriophages that can infect prokaryotic cells, viruses that can infect eukaryotic host cells, and virus-derived genetic particles embedded in host chromosomes; the term "virome" refers to the entire complement of viral genetic elements found in the human genome[15].

In the oro- and nasopharyngeal areas, bacteriophages, coronaviruses, herpes viruses, adenoviruses, respiratory syncytial viruses, picornaviruses, influenza A viruses, and other uncharacterized eukaryotic viruses are frequently encountered. Common gastrointestinal viruses include bacteriophages, adenoviruses, caliciviruses, parvoviruses, picornaviruses, papillomaviruses, astroviruses, plant viruses, and other uncharacterized eukaryotic viruses[16]. Some eukaryotic DNA viruses, such as anelloviruses, herpesviruses, human bocavirus, and adenoviruses, and some RNA viruses, such as picobirnaviruses and parechoviruses can continue shedding for months. For this reason, these viruses form a significant fraction of the typical human virome due to their capacity for persistent infection[17]. Unfortunately, human adenoviruses in the gut can be reactivated and cause persistent infection, leading to serious morbidity and mortality, especially in immunosuppressed patients (*e.g.*, children with hematopoietic disorders)[18].

The liver-gut microbiome axis, which also includes virobiota, is a bidirectional pathway in which portal veins transport gut-derived products directly from the gut to the liver, and bile and antibodies produced in the liver are transported back to the gut (Figure 3). Gut microbiome products preserve the immune homeostasis of the intestine and liver. Conversely, some microbial-derived metabolites such as ethanol, trimethylamine, short-chain fatty acids, and secondary bile acids may play a role in liver disease. Meanwhile, liver diseases such as cirrhosis can induce significant changes to the gut microbiome due to impairment of the vascular, epithelial, and immune barriers of the intestine[19]. Accordingly, gut dysbiosis can induce an abnormal mucosal immune response and lead to homeostatic imbalance. This resulting imbalance causes microbes and immune cells to migrate to the liver, provoking inflammation and associated hepatic injury, and may also influence neoplastic processes[20, 21].

Table 2 Suggested workup in the diagnosis of acute hepatitis of unknown aetiology

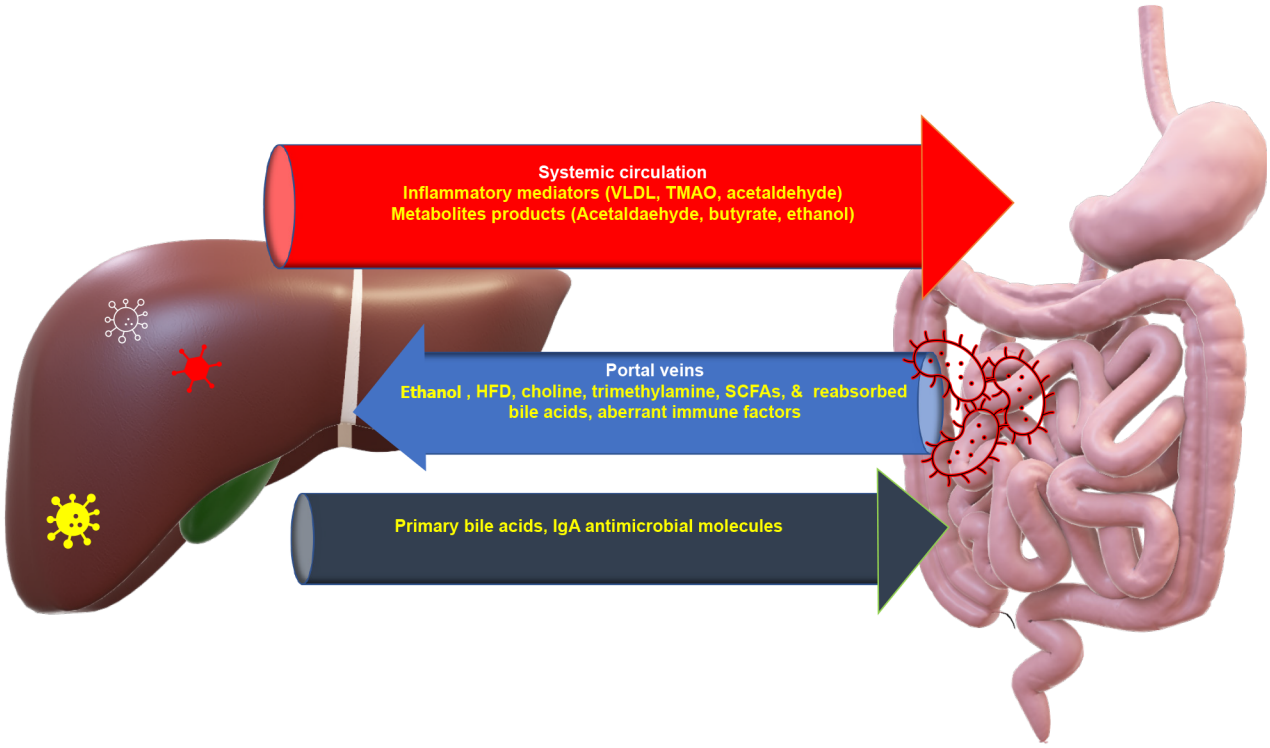
Item		Sample test
History		To be taken according to WHO for case definition, probable or confirmed case. History of traveling to high-risk areas endemic to hepatitis viruses, exposure to a local outbreak, household contact, sharing personal items with an infected person, attendance at day-cares, history of transfusion-dependent illness, or exposure to tattoos and/or body piercing using nonsterile techniques
Clinical examination		Low-grade fever, fatigue, anorexia, nausea, vomiting, enlarged and tender liver with/without splenomegaly, jaundice, abdominal pain, dark urine, pale or clay-coloured stool
Liver Functions		Total bilirubin, conjugated bilirubin, liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and gamma-glutamyl transferase), albumin, and prothrombin time
Liver biopsy		Staining with haematoxylin and eosin in selected cases
Imaging studies		Usually not required; may be needed to rule out biliary obstruction and other aetiologies for elevated liver enzymes and to exclude complications such as cirrhosis and hepatocellular carcinoma. Abdominal ultrasound: Shows enlarged liver with decreased (acute) or increased (chronic) echogenicity, brighter portal vein, periportal oedema, gallbladder wall thickening, and ascites. CT findings of acute hepatitis are nonspecific: Hepatomegaly, gallbladder wall thickening, periportal oedema, and ascites
Tests for autoimmune hepatitis		Autoantibodies such as ANAs and anti-SMAs
Detecting viral causes of hepatitis	Serology	Antibodies against Hepatitis A-E, Epstein-Barr virus, cytomegalovirus, HIV, varicella, adenovirus, SARS-CoV-2 (anti-S and anti-N antibodies)
	Culture	Blood: Adenovirus, cytomegalovirus, Epstein-Barr virus, herpes simplex virus, influenza viruses. Rectal Swab: Adenovirus, rotavirus, enteroviruses
	PCR	Blood: For hepatitis A, C, E, adenovirus, cytomegalovirus, enteroviruses, Epstein-Bar virus, Parechovirus, Herpes simplex virus, HHV 6 and 7. Throat Swab: Respiratory virus screening by multiplex assay (including Adenovirus, Influenza, Parainfluenza, Respiratory Syncytial Virus, Rhinovirus, Human bocavirus 1-3, Human metapneumovirus, Enteroviruses, SARS-CoV-2, *etc.*). Stool: For enteric viruses screening by multiplex assay (including Norovirus, Enteroviruses, Rotavirus, Astrovirus, Sapovirus)
Detecting bacterial causes of hepatitis	Serology	Antibodies against: *Brucella spp., Bartonella henselae, Borrelia burgdorferi* (when epidemiologically appropriate)
	Culture	Blood: Routine procedures for bacterial pathogens, when clinically applicable. Throat Swab: *Streptococcus* group A. Stool: *Salmonella, Shigella, Campylobacter, E. coli* 0157. Urine: Routine procedures for bacterial pathogens, when clinically applicable
	PCR	Stool or rectal swab: Enteric bacterial pathogens. Urine: *Leptospira spp*
Toxicological screening		Blood and urine by different methods, including mass spectrometry: Drugs (*e.g.*, acetaminophen, antibiotics, antiepileptics, herbal medicines) or toxins (*e.g.*, carbon tetrachloride)
Metabolic work-up		Ceruloplasmin; 24 h of urinary copper excretion; Celiac disease screening; Urine organic acid profile; Plasma amino acids; Plasma acylcarnitine; Whole exome and mitochondrial gene examination to rule out other inborn metabolic disorders that can cause liver injury; Other metabolic work-up according to the clinical scenario

ANA: Antinuclear antibody; CT: Computerized tomography; HIV: Human immunodeficiency virus; HHV: Human herpesvirus; PCR: Polymerase chain reaction; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; SMA: Smooth muscle antibody; WHO: World Health Organization.

ADENOVIRUS HEPATOTROPIC EFFECTS

Adenoviruses are medium-sized, nonenveloped, double-stranded DNA viruses. They are named for their first isolation from the adenoid in 1953. Adenoviruses are widespread viruses that classically trigger mild cold- or flu-like disease, pneumonia, conjunctivitis, and acute gastroenteritis in all age groups throughout the year[22]. Adenoviruses are not typically hepatotropic in immunocompetent children. However, they can still cause hepatitis in children with liver stem cell transplantation, immunosuppressed children (*e.g.*, with severe combined immune deficiency), and children receiving chemotherapy for solid malignant neoplasms.

The species C adenoviruses are the most commonly implicated in adenovirus-associated hepatitis, with type 5 being the most frequently encountered[23]. Furthermore, human species F adenoviruses (*e.g.*, types 40 and 41) are well-known causes of paediatric gastroenteritis. Chhabra *et al*[24] showed that F adenovirus type 41 is more widespread than type 40 in the setting of viral gastroenteritis in children younger than 5 years. Despite mainly causing respiratory infections, adenoviruses can produce transient nonspecific "reactive hepatitis" findings in children during an active infection, with AST and ALT levels used as markers of hepatitis severity[25]. Adenovirus infection can be diagnosed by direct antigen detection (in blood, stool, or respiratory samples), polymerase chain reaction (PCR) amplification, virus culture and isolation, and serology. Specimens are ideally collected within 1 week of symptom onset. Positive serology is expected in most children by the age of 4 years, but a 4-fold or more increase in the titre of adenovirus-specific antibodies is considered evidence of a recent infection. Adenovirus typing



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Figure 3 Gut-liver axis. Mutual effects of the gut and liver through systemic and portal circulation and biliary enterohepatic circulation. HFD: High-fat diet; IgA: Immunoglobulin A; SCFAs: Short-chain fatty acids; TMAO: Trimethylamine N-oxide; VLDL: Very-low-density lipoprotein.

can be performed with molecular tests, and is essential from an epidemiological point of view[26]. When performing adenovirus molecular testing in suspected cases of acute hepatitis of unknown cause, whole blood samples instead of serum or plasma should be used as it has a higher viral yield[12].

Out of 74 cases of acute hepatitis of unknown aetiology with detected adenovirus, 18 patients were identified by molecular tests as serotype F41, and a few others were serotype F40[27]. This finding may indicate that enteric adenoviral infection may be related to hepatic infection by means of the gut-liver axis, a topic that warrants further research[28]. The low viral load in the clinical samples necessitated PCR amplification of part of the viral hexon gene followed by Sanger sequencing for the detection of adenoviruses. However, notably, children presenting with acute hepatic failure had a high viral load. Importantly, there are also intratypic genetic variations in adenoviruses of serotype F type 41[29]. A study at an Alabama hospital in August 2022 showed three different strains of adenovirus serotype F41, observed in 5 patients with acute hepatitis of unknown aetiology. This finding may indicate a low probability with regard to an outbreak being caused by a specific adenovirus serotype[30].

Meanwhile, serotyping data obtained from 4 adenovirus-positive patients in the European cohort showed 2 with serotype F41, 1 with serotype F40, and 1 with a serotype of "other." This supports the potential of adenovirus to negatively affect the liver after reaching it through the gut-liver axis. This adenovirus pathogenicity could be attributed to the development of mutations that promote hepatotropism, but this hypothesis needs to be confirmed by whole genome sequencing to detect any such mutation[28]. Despite being isolated from a significant number of children with acute hepatitis of unknown origin, the exact role of adenoviruses in the pathogenicity of this condition still needs to be confirmed. Infection with adenoviruses is usually mild and resolves spontaneously. However, the infection can be associated with high morbidity and mortality in immunocompromised children, particularly those with allogeneic stem cell transplants. Although adenoviruses have been widely studied, there is currently no anti-adenoviral treatment approved by the United States Food and Drug Administration. As of this review, cidofovir and ribavirin are the only antiviral drugs used as first-line therapy to treat adenoviral infections. Brincidofovir has no nephrotoxicity and has better bioavailability than cidofovir, but this drug is no longer manufactured[31,32].

CORONAVIRUSES HEPATOTROPIC EFFECTS

Despite pulmonary symptoms being the dominant finding in the clinical presentation of COVID-19, SARS-CoV-2 may also affect other organs such as the liver[33]. The liver is affected by 14%–53% of SARS-CoV-2 infections, regardless of preexisting liver disease[34]. SARS-CoV-2 accesses the liver *via*

binding angiotensin-converting enzyme-2 receptors, which are strongly expressed on cholangiocytes, minimally expressed on hepatocytes, and absent on Kupffer cells[35]. COVID-19-associated liver injury could be related to immune-mediated damage with a severe inflammatory response to SARS-CoV-2 infection, direct cytotoxicity due to active viral replication inside the liver cells (Figure 4), COVID-19-associated anoxic liver damage, drug-associated liver injury, or reactivation of preexisting liver infections (*e.g.*, Hepatitis B)[30]. SARS-CoV-2 infection can also activate autoimmune hepatitis *via* systemic immune hyperstimulation, molecular mimicry, or both[31].

Meanwhile, few cases of autoimmune hepatitis have been reported after SARS-CoV-2 vaccination, and those that have been reported all showed complete remission with steroid therapy[36]. Crisan *et al* [37] showed that patients who presented with elevated liver enzymes and abnormal chemistries on arrival were more likely to have worse disease and poorer outcome. The presence of fibrosis in hospitalized patients with COVID-19 is associated with increased mortality. Therefore, regular monitoring of liver function should be standard for all COVID-19 patients, and serological testing for specific hepatotropic viruses (*e.g.*, Hepatitis B or C according to the local epidemiological status) should be strongly considered[38].

Figure 4 shows the effects of COVID-19 infection on the liver, which is first evidenced by increased liver enzymes. The virus reaches the liver from the gut-liver-lung axis, which may be re-shed back to the gut through the bile. These effects are mediated through the impact of hypoxia, systemic venous congestion, immune-mediated hepatic damage by inflammatory mediators induced by SARS-CoV-2 infection, the direct hepatic cytopathic effect of SARS-CoV-2, and the hepatotoxic effects of some medications used to treat SARS-CoV-2 infection such as azithromycin, chloroquine, lopinavir, ritonavir, and tocilizumab. Hepatic damage can also result from SARS-CoV-2 reactivation of pre-existing liver diseases such as hepatitis B or C[39,40].

SYNERGISM BETWEEN SARS-COV-2 AND ADENOVIRUS

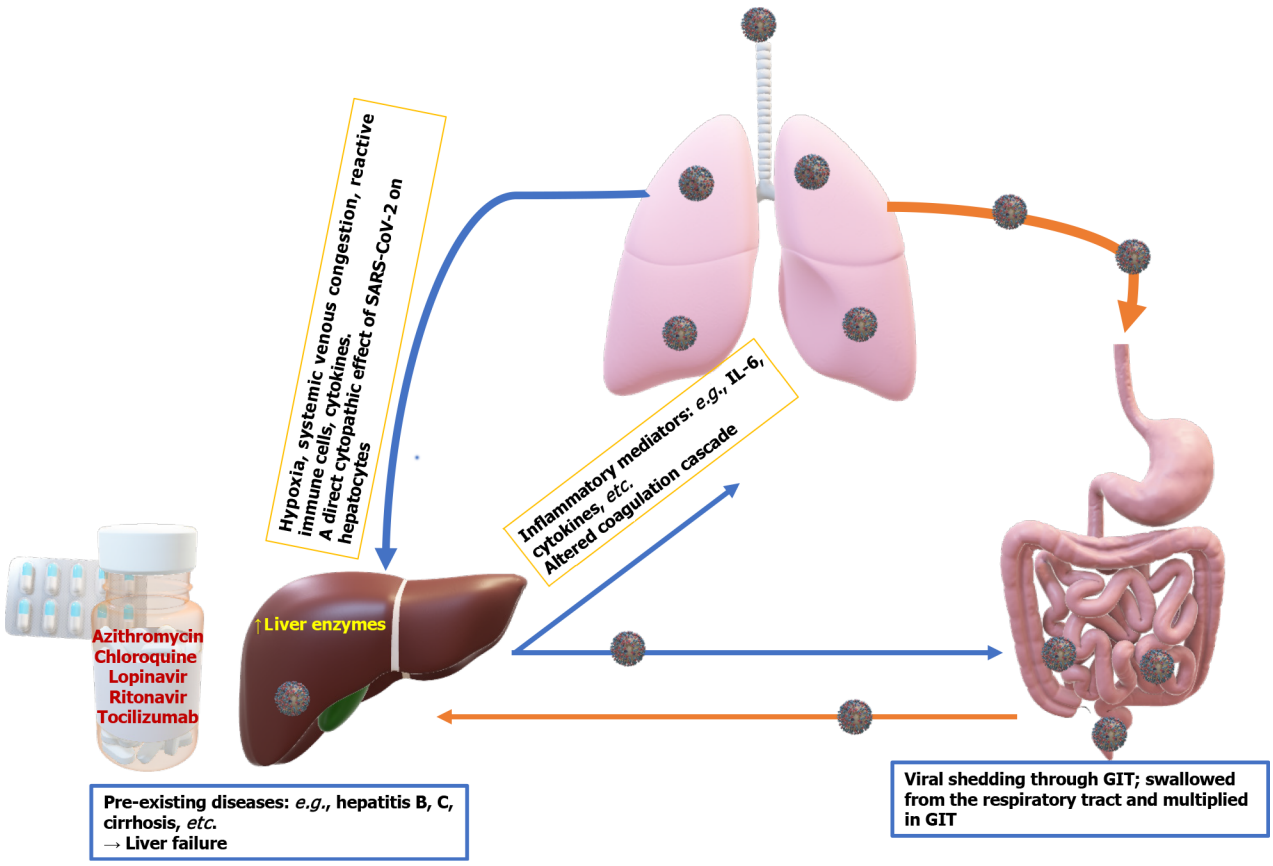
Many studies have shown an increased incidence of adenovirus among children infected with SARS-CoV-2. Coinfection with SARS-CoV-2 and adenovirus may also occur. Mohammadi *et al*[41] showed that the rate of SARS-CoV-2 and adenovirus coinfection is 1.1%, and all cases had mild respiratory disease. Another study from The United States showed a 0.4% rate of SARS-CoV-2 and adenovirus coinfection, being the third most common coinfection after rhinovirus/enterovirus and influenza A viruses[42]. Another study from the United Kingdom showed a 2% rate of coinfection with SARS-CoV-2 and adenovirus which increased the odds of death by 1.22[43]. Finally, a study from China found a slightly higher coinfection rate of 2.8%, associated with a worse diagnosis than bacterial coinfection[44].

EPSTEIN-BARR VIRUS AS A POSSIBLE CAUSE

Epstein-Barr virus (EBV) is a member of the herpesvirus family. It causes a heterogeneous group of infections in children and adults with a classic presentation (infectious mononucleosis) or other atypical presentations. Baker *et al*[12] showed that EBV was identified in 6 out of 9 children with acute hepatitis of unknown cause, verified using molecular methods. However, due to the absence of IgM, these cases could represent the reactivation of an old EBV infection and not a primary infection. However, EBV was reported to cause acute hepatitis in adults as well. García-Martínez *et al*[45] reported coinfection of SARS-CoV-2 and EBV in a 19-year-old woman who presented with pyrexia and bilateral eyelid and hemifacial swelling and was found to have splenomegaly, cervical lymphadenopathy, and elevated AST and ALT. In addition, Nadeem *et al*[46] described the reactivation of an EBV infection in a 62-year-old man attributed to coinfection with SARS-CoV-2. This patient was also found to have elevated AST and ALT. Despite these cases, the role of EBV in the pathogenesis of hepatitis is unclear, as many other confounding factors were present.

QUESTIONS NEED TO BE ANSWERED

Although both adenoviruses and SARS-CoV-2 are not typically hepatotropic viruses and rarely cause acute hepatitis in immunocompetent patients, coinfection with both viruses may produce significant effects on the liver and induce an acute hepatitis-like syndrome. Many questions remain, and further research may lead to key information regarding acute hepatitis of unknown origin. As there is an increased rate of autoimmune diseases after COVID-19 and its vaccines[47], could acute hepatitis of unknown cause be a COVID-19 immune-triggered reaction? Could this syndrome be caused by new variants of either adenovirus or SARS-CoV-2? Could coinfection with SARS-CoV-2 and adenoviruses trigger aggravated inflammatory responses affecting a sensitized liver and consequently induce acute hepatitis? Could acute hepatitis be a local form of the multisystem inflammatory syndrome, as



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Figure 4 Effect of coronavirus disease 2019 infection on the liver as indicated by increased liver enzymes. The virus reaches the liver from the gut-liver-lung axis and may be re-shed back to the gut through the bile. These effects are mediated through the impact of hypoxia, systemic venous congestion, immune-mediated hepatic damage by inflammatory mediators induced by severe acute respiratory syndrome coronavirus-2 infection (SARS-CoV-2), the direct hepatic cytopathic effect of SARS-CoV-2, and the hepatotoxic effects of some medications used to treat SARS-CoV-2 infection such as azithromycin, chloroquine, lopinavir, ritonavir, and tocilizumab. GIT: Gastrointestinal tract; IL-6: Interleukin 6.

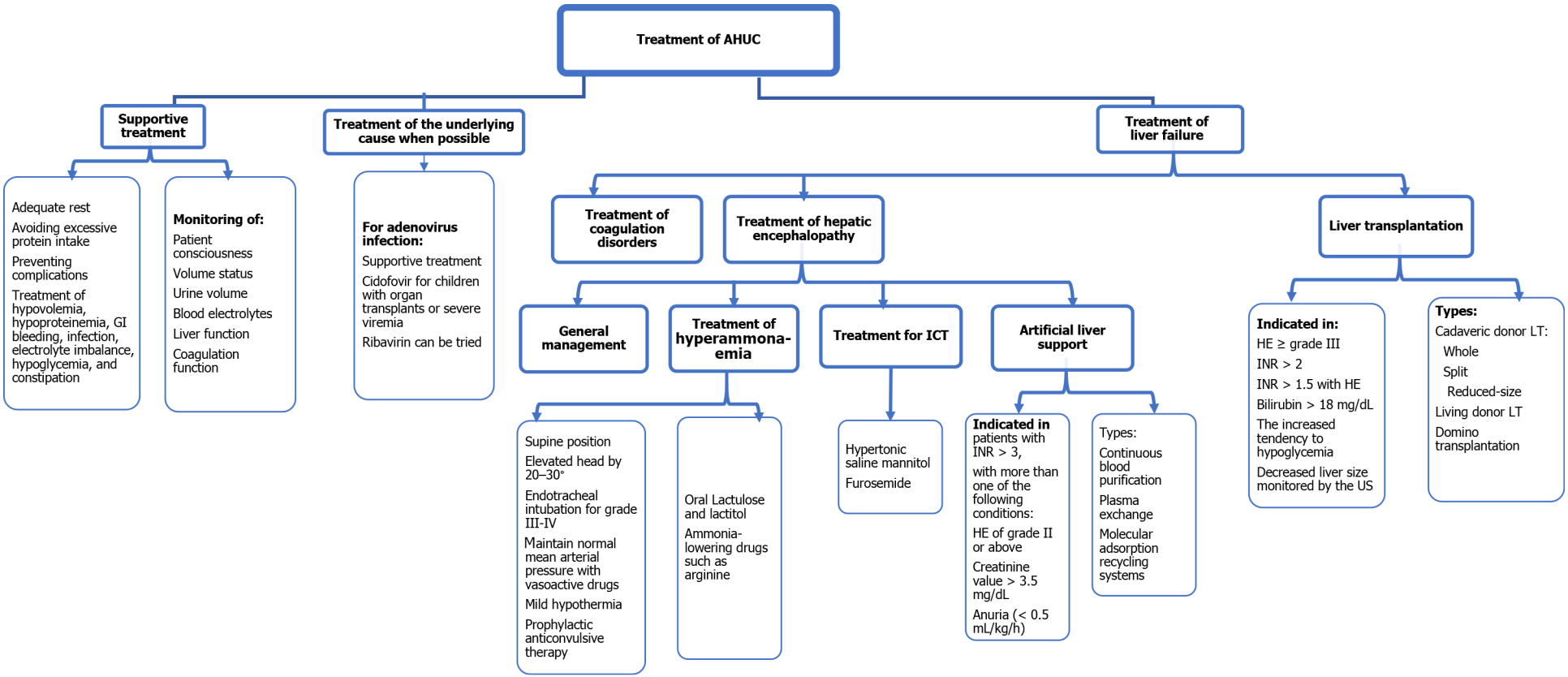
described by Cantor *et al*[47]? Could adenoviruses serve as a vector for SARS-CoV-2, easing the entry of SARS-CoV-2 in hepatocytes? Could this syndrome of acute hepatitis be related to other undiscovered microbial or non-microbial agents? Other aetiologies cannot be ignored. Despite being isolated from cases with acute hepatitis of unknown cause, the role of adenoviruses in the pathogenesis of this syndrome is not yet proven. We must strive to answer these questions and better define the correlation between SARS-CoV-2 and adenovirus infection and the development of this syndrome.

TREATMENT

Treatment of acute hepatitis of unknown cause in children is mainly symptom-based, supporting the recovery of liver function and treating complications as they arise. Cidofovir can be used when adenovirus infection is suspected, particularly in children with an organ transplant or severe viremia[48,49]. When multisystem inflammatory syndrome in children (MIS-C) is suspected to cause acute hepatitis, the treatment protocol is the same as the management of MIS-C[47]. Liver transplantation is indicated in children with acute fulminant hepatic failure refractory to aggressive therapy[50]. A summary of the treatment of acute hepatitis of unknown cause is illustrated in Figure 5.

PREVENTION

As SARS-CoV-2, adenoviruses, EBV, and other viruses are strongly suspected as potential mediators of acute hepatitis of unknown cause, appropriate hand hygiene and regular surface disinfection are essential to reduce viral spread. Hand and respiratory hygiene manoeuvres can reduce the spread of nonenveloped viruses such as adenoviruses[51]. Moreover, it is key that healthcare professionals know the signs and symptoms of hepatitis in children. In suspected cases, clinicians should order serum ALT



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Figure 5 Summary of the treatment of acute hepatitis of unknown cause. AHUC: Acute hepatitis of unknown cause; GI: Gastrointestinal; HE: Hepatic encephalopathy; ICT: Increased intracranial tension.

and AST transaminase testing to ensure efficient detection of cases as early as possible.

CONCLUSION

There has been an increasing number of acute hepatitis of unknown origin in children since first reported on March 31, 2022. The exact aetiology of this condition in children, which was observed to increase in prevalence during the COVID-19 pandemic, is still unclear. Despite adenoviruses and SARS-CoV-2 being isolated from some patients with acute hepatitis of unknown cause, the role of these viruses in the pathogenesis of this syndrome is not yet proven. Coinfection with SARS-CoV-2 and other

viruses may relate to the pathogenesis of this condition. However, many questions remain and will require comprehensive research to better understand this correlation. Until a better understanding is reached, emphasis must be placed on preventing the development of acute hepatitis in children by using proper hygiene (*e.g.* hand washing, frequent surface disinfection) to reduce viral spread. Treatment of acute hepatitis of unknown cause in children is mainly symptom-based, supporting liver recovery and treating complications as they arise.

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MINIREVIEWS

Challenges and recommendations when selecting empirical antibiotics in patients with cirrhosis

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Abstract

There is abundant evidence that bacterial infections are severe complications in patients with cirrhosis, being the most frequent trigger of acute-on-chronic liver failure and causing death in one of every four patients during hospitalization. For these reasons, early diagnosis and effective treatment of infections are mandatory to improve patient outcomes. However, treating physicians are challenged in daily practice since diagnosing bacterial infections is not always straightforward. This situation might lead to delayed antibiotic initiation or prescription of ineffective regimens, which are associated with poor outcomes. On the other hand, prescribing broad-spectrum antibiotics to all patients suspected of bacterial infections might favor bacterial resistance development. This is a significant concern given the alarming number of infections caused by multidrug-resistant microorganisms worldwide. Therefore, it is paramount to know the local epidemiology to propose tailored guidelines for empirical antibiotic selection in patients with cirrhosis in whom bacterial infections are suspected or confirmed. In this article, we will revise current knowledge in this area and highlight the importance of surveillance programs.

Key Words: Bacterial infections; Cirrhosis; Multidrug resistance; Antibiotic prophylaxis; Antibiotic stewardship

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Core Tip: Practitioners who participate in caring for patients with cirrhosis are challenged when using antibiotics rationally. On one side, bacterial infections are frequent, severe, and not always straightforward to diagnose; on the other, scant granular data is publicly available about the causal microorganisms and their susceptibility patterns. According to experts, empiric antibiotic treatments should cover 80% of the common pathogens in stable patients and 90% in critically ill patients with suspected infections. Therefore, it is necessary to know the microorganisms expected to be involved in the most frequent bacterial infections and their susceptibility patterns to develop evidence-based guidelines. This opens a window of opportunity for research because bacterial infections and multidrug resistance are global health issues expected to grow over the following decades.

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INTRODUCTION

Impact of bacterial infections in patients with cirrhosis

Bacterial infections are extremely frequent in patients with cirrhosis, present in about 25%-46% of those hospitalized for an acute decompensating event. In two third of cases, infections are diagnosed at admission, whereas the remaining patients develop nosocomial infections[1,2]. The commonest infections in patients with cirrhosis include spontaneous bacterial peritonitis (SBP), urinary tract infection, pneumonia, spontaneous bacteremia, and skin and soft tissue infections[3]. Although gram-negative enteric organisms were the primary pathogens involved, gram-positive infections are increasing in prevalence. This situation might be favored by antibiotic prophylaxis, medical procedures, and prior hospitalizations, among other risk factors[2,4].

Bacterial infections are currently recognized as a surrogate for the final stage of chronic liver disease [5,6]. Even though any type of decompensation in patients with cirrhosis is associated with worsening survival, not all decompensating events carry the same weight in patients' prognosis. The relevance of bacterial infections as a prognostic factor has been clearly stated in a meta-analysis that found that they increase mortality four-fold in this population, considering 30% of patients die within one month and another 30% die one year after these infections are diagnosed[7].

Factors associated with an increased risk of infection are poor liver function, variceal bleeding, low ascitic fluid protein levels, prior SBP, and hospitalization[8]. In addition, bacterial infections have also been defined in the large prospective cohort study CANONIC as the most frequent trigger of acute-on-chronic liver failure (ACLF), negatively impacting patients' prognosis irrespective of the resolution of the infection[5]. In fact, infections as precipitant or complications arise in 50% of patients with ACLF and 70% of patients with three or more organ failures[9].

Challenges in timely diagnosis and treatment of bacterial infections

Early diagnosis of bacterial infections is crucial for the rapid initiation of antibiotic treatment[8]. However, this poses a challenge since they are often oligo-symptomatic. For example, only one-half of patients with cirrhosis and bacterial infections develop fever, and most do not present leukocytosis or systemic inflammatory response criteria[10]. This is why high clinical suspicion is critical; in fact, the European Association for the Study of the Liver (EASL) position paper on bacterial infections recommends that all patients with cirrhosis admitted to the hospital should be considered infected until proven otherwise[8]. Furthermore, it should also be considered in patients with cirrhosis that deteriorate their clinical status while admitted to the hospital[10].

A rapid evaluation, including physical examination, ascitic and/or hydrothorax evaluation, and a chest X-ray, might rule in or out some of the most frequent infections in patients with cirrhosis, such as SBP, spontaneous bacterial empyema, pneumonia, and skin and soft tissue infections. However, urinary tract infection and spontaneous bacteriemia, representing more than 40% of the infections[3], are not easy to approach because their diagnosis is mainly based on cultures, which are usually available 24 to 48 h after the initial evaluation. In practice, the difficulty of ruling out these two infections might lead to unnecessary empiric antibiotic prescriptions.

Several biomarkers have been assessed to aid in promptly diagnosing bacterial infections. C-reactive protein, ferritin, or leukocyte count lack specificity for bacterial infections[11]. Furthermore, they can be influenced by immune dysfunction and hypersplenism, presenting lower values than expected[10,11]. Procalcitonin has been proposed as a more specific marker for bacterial infection. Nearly all tissues produce this biomarker in response to endotoxin or mediators released during bacterial infections, such as interleukin (IL)-1β, tumor necrosis factor-alpha, and IL-6. It has been proposed that it highly

correlates with the severity of bacterial infections and may help distinguish bacterial from viral infections or non-infectious inflammatory syndromes[8,11]. In a meta-analysis of more than 1000 patients with infections and cirrhosis, procalcitonin and C-reactive protein had acceptable accuracy for diagnosing bacterial infection among patients with cirrhosis compared with patients with normal liver function; however, their suggested applications differ. Procalcitonin was suggested as a rule-in tool [positive likelihood ratio = 7.38, 95% confidence interval (CI): 4.70-11.58], whereas C-reactive protein was suggested as a rule-out tool (negative likelihood ratio = 0.23, 95%CI: 0.13-0.41)[12]. Ultra-sensitive procalcitonin has been suggested more recently as a valuable tool for bacterial infection diagnosis, with a sensitivity of 97% and a negative predictive value of 98%, considering a cutoff value of 0.098 ng/mL [13]. Despite these promising data, these tools have yet to be integrated into everyday clinical practice.

Due to all these limitations, other auxiliary tools have been proposed and validated in this population to diagnose sepsis. One of these is the Sepsis-3 score, which defines sepsis as a Sequential/Sepsis-related Organ Failure Assessment (SOFA) score of at least two points at intensive care unit (ICU) admission or an increase in the SOFA score during ICU hospitalization and suspected infection[14,15]. This updated clinical score aims to achieve greater consistency for future trials and ease earlier diagnosis and management of patients with sepsis or at its risk[15]. Similarly, the qSOFA score considers a surrogate of poor prognosis the presence of at least two of the following: Respiratory rate of 22 breaths per minute or greater, altered mental status, or systolic blood pressure of 100 mmHg or lower[16]. This simplified score had a greater predictive validity for in-hospital mortality than SOFA and systemic inflammatory response syndrome when used outside of an ICU setting[17]. However, these scores must be broadly validated to be used as the standard of care.

When a bacterial infection is suspected in patients with cirrhosis, the immediate initiation of antibiotics is crucial in improving the prognosis. Similarly, to the scores mentioned above, the recommendation derives from studies and guidelines considering the general population. In the Surviving Sepsis Campaign 2021, the initiation of antimicrobials is considered an emergency in patients with sepsis or septic shock. In this latter group, for each hour of delay upon administration of antimicrobials, there is a 4%-13% increase in the odds of in-hospital mortality[14]. Similar findings have been reported in patients with cirrhosis and septic shock, where each hour of delay in using appropriate antimicrobials was associated with higher mortality[18,19].

Challenges in the selection of antibiotic prophylaxis or empiric treatment in the multidrug-resistant era

It has been stated in a consensus conference regarding infections in patients with cirrhosis that randomized clinical trials on antibiotic prophylaxis are affected by several methodological pitfalls: The majority of them were under-powered, considered short follow-up periods, had methodological flaws, and were conducted more than two decades ago, in a completely different epidemiological context than the one faced today[20]. Current recommendations are based on the results of these studies, which were performed in an epidemiological setting where microorganisms responsible for infections were rarely multidrug-resistant and when gram-negative bacilli predominated over gram-positive cocci. This has changed radically in the last 20 years, with an increasing prevalence of multidrug-resistant microorganisms (MDRO), especially in patients with decompensated cirrhosis prone to hospitalizations, prolonged antibiotic prophylaxis, and invasive procedures[21]. In fact, in a recent worldwide prospective multicenter study performed by Piano *et al*[3], the global prevalence of MDRO reached 34%. These findings differed significantly by country, with a prevalence higher than 70% in India, between 20%-30% in Argentina, Canada, and several western European countries, and lower than 20% in the United States and Russia. The consequences are not trivial: Infections caused by MDRO were associated with a lower efficacy of empirical antibiotic treatment, a longer duration of antibiotic therapy, a lower rate of resolution of the infection, and a higher incidence of septic shock than those with non-MDRO infections. Most importantly, mortality was significantly higher in patients with MDRO infections[3].

Rectal colonization by MDRO may guide empirical antibiotic therapy. A recently published study showed that MDRO rectal colonization is prevalent in critically ill patients with cirrhosis (up to 47% at admission) and is associated with an increased risk of infections caused by the MDRO colonizing strains [22]. Furthermore, colonization by MDRO has also been associated with higher mortality in the liver transplant waiting list[23] and higher mortality in patients with cirrhosis and hepatocellular carcinoma [24]. All in all, the frequency of rectal colonization surveillance and its interpretation when selecting empirical therapy is yet to be defined[25].

According to experts, empiric antibiotic treatment should effectively cover approximately 80% of expected bacteria in non-critically ill patients and 90% in critically ill patients[26]. However, in the scenario mentioned above in which infections by gram-positive bacteria and multidrug organisms are increasing, prior recommendations may need to be revised. Thus, the current challenge is whether we can still safely choose antibiotic prophylaxis and treatment based on the current practice guidelines or whether these general recommendations should be regularly updated and tailored according to local epidemiological information.

Antibiotic prophylaxis in patients with cirrhosis

Antibiotic prophylaxis should be prescribed in specific clinical situations where there is a high risk for bacterial infections and when the benefit of their use outweighs the risk for adverse events and the development of antibiotic resistance[10].

Antibiotic prophylaxis in patients with acute gastrointestinal bleeding: There is broad consensus regarding prescribing antibiotic prophylaxis in acute gastrointestinal bleeding in patients with cirrhosis. This is mainly based on their high rate of bacterial infections without antibiotic use (up to 50% during the first days of hospitalization) and on the efficacy of prophylaxis in preventing infections, re-bleeding, and death[27]. Furthermore, the proposed duration of treatment is of only seven days. Thus, the risk of inducing multidrug resistance is lower than in more extended prophylaxis strategies. Regarding the choice of antimicrobial agent, a meta-analysis reports several antibiotics regimens that have a beneficial effect, with cephalosporins, quinolones, and quinolones plus beta-lactams having a more substantial protective effect than other antibiotics. Notably, no significant difference between quinolones and cephalosporins was observed[28]. However, due to the emergence of quinolone-resistant organisms, most international guidelines recommend ceftriaxone as the antibiotic of choice[27,29-31]. In countries such as the United States, where norfloxacin has been discontinued, ceftriaxone is the only recommended option[32]. The EASL 2013 position paper suggests oral norfloxacin twice daily in patients with preserved liver function as the regimen of choice, endorsing ceftriaxone in patients with decompensated cirrhosis (those with at least two of the following findings: Ascites, severe malnutrition, encephalopathy, or jaundice). Additionally, oral nitrofurantoin or ertapenem is recommended in patients with infections caused by extended-spectrum b-lactamase-producing *Enterobacteriaceae* in the last three to six months[8]. However, in a more recent publication, this scientific society endorses the use of ceftriaxone 1 g/24 h for up to seven days not only in patients with advanced cirrhosis but also in those on quinolone prophylaxis and hospital settings with a high prevalence of quinolone-resistant bacterial infections, recommending oral quinolones only for the remaining patients. They stress these recommendations should be evaluated and cross-checked from the perspective of local resistance patterns[33].

When assessing the effectiveness of current antibiotic prophylaxis strategies, a recent large multicenter study of patients with cirrhosis and variceal bleeding found that almost 20% of patients developed a bacterial infection despite using the recommendations mentioned above[34]. On the other hand, the need for routine antibiotic prophylaxis has been questioned in less severely ill patients (Child-Pugh A) due to their lower risk of infections and death[35].

Despite an acceptable consensus regarding the use of ceftriaxone as the prophylaxis of choice, this should be adapted considering the growing worldwide prevalence of MDRO, the severity of the underlying liver disease, and/or the setting of the bleeding episode (community-onset *vs* nosocomial). For example, antibiotic prophylaxis should not be the same in a patient admitted for variceal bleeding as in a patient who bleeds while in the ICU receiving antibiotics for a prior bacterial infection.

Long-term primary and secondary prophylaxis of SBP: Primary prophylaxis is proposed for patients with ascites and severe impairment of liver function, without a prior episode of SBP. The criteria used differs slightly according to different guidelines. The EASL guidelines recommend primary prophylaxis should be started on patients with low protein concentration in ascites (< 1.5 g/L), liver failure (Child-Turcotte-Pugh score > 9 and bilirubin > 3 mg/dL), and either renal dysfunction or hyponatremia[33]. In contrast, the American Association for the Study of Liver Diseases (AASLD) 2021 practice guidelines suggest primary prophylaxis could be considered in patients with the same threshold of ascitic protein accompanied by liver failure (Child-Turcotte-Pugh score > 9 and bilirubin > 3 mg/dL), renal dysfunction or hyponatremia[31]. In the latter guideline, primary prophylaxis is left to each physician's discretion since available studies are considered of variable quality and thus insufficient to support a consensus guidance recommendation. The impact of primary prophylaxis on overall survival, and not only on SBP occurrence, is a topic of ongoing research. Recently, the effect of long-term (six months) primary prophylaxis with norfloxacin has been evaluated in a randomized controlled trial that included 291 Child C patients. The risk of death at six months was significantly lower in patients with ascites fluid protein concentrations < 1.5 g/L, whereas it had no effect in patients with higher ascites protein count. Interestingly, norfloxacin significantly decreased any gram-negative bacterial infection without increasing infections caused by Clostridium difficile or MDROs[36]. Further data regarding the efficacy and safety of primary prophylaxis of SBP is expected from the ASEPTIC trial, which aims to evaluate the impact of cotrimoxazole treatment *vs* placebo during 18 mo of therapy in overall survival SBP incidence, and antimicrobial resistance, among other objectives[37].

Secondary prophylaxis (*i.e.*, in patients with at least one prior episode of SBP) rationale is based on the high risk of SBP recurrence, and the significant impact antibiotic prophylaxis has on reducing its incidence. In a trial performed more than 30 years ago, secondary prophylaxis with norfloxacin significantly reduced the probability of SBP recurrence compared to placebo (20% *vs* 68%, respectively)[38]. However, the current benefit of secondary prophylaxis with norfloxacin has recently been challenged due to the growing prevalence of quinolone-resistant bacteria and heterogeneous results in observational studies[39,40]. Several alternative strategies have been proposed to norfloxacin, using

other antimicrobials such as ciprofloxacin, rifaximin, ceftriaxone, or cotrimoxazole with different frequencies of administration (daily, five days a week, weekly). Interestingly, in a recently published meta-analysis, only daily rifaximin significantly reduced SBP recurrence compared to other antibiotics or placebo[41]. However, due to methodological concerns affecting available trials, rifaximin is not considered the standard of care for prophylaxis of SBP[42]. This poses a challenge for the treating physician when facing a patients who are under rifaximin treatment for hepatic encephalopathy that need to start prophylaxis for SBP: The aforementioned EASL guidelines state that no recommendation can be provided to guide the choice of antimicrobial among patients already on rifaximin[33]: Choosing either antibiotic or both becomes a personalized choice.

Rational selection of empiric antibiotics: Easier said than done

In daily practice, various forces drive the decision to start empiric antibiotic treatment. Given the high incidence and severe impact of bacterial infections in patients with cirrhosis, it is likely that antibiotics are overused in this population. In fact, a recent sub-analysis of the ATTIRE clinical trial suggested that half of the antibiotics prescribed to hospitalized patients with decompensated cirrhosis might not be necessary[43].

That said, the next step after confirmation or suspicion of sepsis is to start an empiric antibiotic treatment, which will be selected taking into account the site of the infection (SBP, urinary tract infection, *etc.*), the type of infection (community-acquired, health-care-associated, or nosocomial), and the pattern of resistance according to the local epidemiology. However, it is also important to consider the degree of liver failure, renal function, and potential allergies, among other variables. Another critical factor that has to be taken into account is the severity of the infection, which might be explored by evaluating the presence and number of organ failures or by calculating scores like CLIF-C AD, CLIF-C ACLF, and quick SOFA, among others[33], as was previously discussed.

Several models to predict the risk of infection by multidrug-resistance organisms were published to refine the selection of the empirical antibiotic treatment. Unfortunately, none were developed or validated in patients with cirrhosis, and their performance was moderate[44,45]. The most desirable tool to guide the selection of antibiotics would be real-time techniques that inform on the involved microorganisms and their antibiotic susceptibility pattern. Gram stain preparation is the only widely available and straightforward approach, but it provides limited information. However, in the future, other rapid molecular tests still under development or validation could give this information in minutes or hours and might help select empirical treatments in patients with cirrhosis[46].

Guidelines for antibiotic selection and protocols for rapid evaluation of patients with suspicion of sepsis are very helpful[47]. However, the need for knowledge about the expected local microorganisms and their susceptibility patterns are some of the barriers to developing these guidelines. Therefore, the World Health Assembly proposed a plan for antimicrobial resistance in 2015, which enhances surveillance of antimicrobial susceptibility patterns to generate evidence-based empiric antibiotic recommendations. Surveillance can be performed at different levels, from single institutions to states or countries. But ideally, each institution should count on sufficient granular data to generate its recommendations which would guide the treating physician to select the shortest treatment duration with the lowest-spectrum antibiotic, which will cover 80%-90% of the anticipated microorganisms using an adequate dose and route of administration[3,48].

It is known that keeping an active surveillance program that performs periodic reports and recommendations requires a multidisciplinary expert team, is time-consuming, and is costly[49]. Therefore, scientific societies or governmental organizations should implement and lead these programs and report their results at different levels. For example, Argentina and Uruguay launched a surveillance program for bacterial infections in patients with cirrhosis in October 2018, which hepatologists, infectious diseases, and epidemiologists lead and aims to serve as a platform to perform evidence-based recommendations regarding empirical antibiotic selection in this population[50].

The most recently published recommendations for empiric antibiotic treatment in patients with cirrhosis can be found in the AASLD and EASL guidelines for managing patients with decompensated cirrhosis (Table 1)[31,33]. These recommendations should be adopted with caution after revisiting the epidemiological particularities that a given center or region might have and discussing them with infectious disease specialists and microbiologists.

For example, for the case of empirical treatment of SBP, guidelines suggest using a third-generation cephalosporin or piperacillin-tazobactam. However, it should be noted that there are essential differences among third-generation cephalosporins. Ceftazidime, ceftriaxone, and cefepime are mainly used to treat community-acquired SBP, but their spectrum varies. Generally speaking, cefepime and ceftriaxone cover most gram-negative and gram-positive bacteria, which are expected to cause community-acquired SBP. However, ceftazidime does not cover gram-positive bacteria, like *Streptococcus spp*, which are known to be highly prevalent in some regions in patients with community-acquired infections, like SBP and spontaneous bacteremia[39,51]. Similarly, these guidelines recommend using fluoroquinolones (ciprofloxacin or levofloxacin) in patients with community-acquired urinary tract infection, which might offer inadequate coverage in regions where the prevalence of resistance of community uropathogens to fluoroquinolones is known or expected to be high.

Table 1 Empiric antibiotic recommendations in patients with cirrhosis, according to source, severity and type of infection

Infection	AASLD	EASL
Spontaneous infections (peritonitis, bacteremia[1], empyema)	Community acquired: Third-generation cephalosporins	Community acquired: Third-generation cephalosporins or piperacillin/tazobactam Healthcare-associated: Area dependent: Like nosocomial infections if high prevalence of MDRO or sepsis
	Nosocomial: Piperacillin/tazobactam and daptomycin (if known VRE in past or evidence of GI colonization) or meropenem if known to harbor MDR gram-negative organisms	Nosocomial: Carbapenems alone or carbapenems and daptomycin, vancomycin or linezolid if high prevalence of MDR gram-positive bacteria or sepsis
Pyelonephritis/urinary tract infection	Uncomplicated pyelonephritis: Fluoroquinolones (ciprofloxacin or levofloxacin). Severe pyelonephritis: Third-generation cephalosporins (*e.g.*, ceftriaxone). If recent antibiotic exposure: Piperacilin/tazobactam or carbapenem	Community acquired: Uncomplicated: Ciprofloxacin or cotrimoxazole. If sepsis: Third-generation cephalosporins or piperacillin/tazobactam. Healthcare-associated: Area dependent: Like nosocomial infections if high prevalence of MDROs or if sepsis. Nosocomial: Uncomplicated: Fosfomycin or nitrofurantoin. If sepsis: Meropenem and teicoplanin or vancomycin
Pneumonia	Community acquired: (1) Non-severe: B-lactam and macrolide or respiratory fluoroquinolones; and (2) Severe: B-lactam and macrolide or B-lactam and fluoroquinolones. Vancomycin can be added if patient has prior respiratory isolation of MRSA. Hospital acquired (not ventilator associated): (1) Non-severe (not septic, not intubated): One of the following: Piperacillin/tazobactam or cefepime or levofloxacin. Vancomycin can be added if MRSA was isolated in the last 90 d or if antibiotics were used in the last 90 d; and (2) Severe (presence of sepsis or requiring intubation). One of the following: Piperacilin tazobactam or cefepime or meropenem and levofloxacin. Vancomycin can be added if MRSA was isolated in the last 90 d or if antibiotics were used in the last 90 d. Pseudomonas coverage: If there is prior respiratory isolation of pseudomonas of recent use of parenteral antibiotics or hospitalization	Community acquired: Piperacillin/tazobactam or ceftriaxone and macrolide or levofloxacin or moxifloxacin. Healthcare-associated: Area dependent: Like nosocomial infections if high prevalence of MDROs or if sepsis. Nosocomial: Ceftazidime or meropenem and levofloxacin ± glycopeptides or linezolid
Cellulitis	Moderate (with systemic signs of infection): Penicillin or ceftriaxone or cefazolin or clindamycin. Severe (failed antibiotics, presence of sepsis): Vancomycin and piperacillin/tazobactam	Community acquired: Piperacillin/tazobactam or third-generation cephalosporins and oxacillin. Healthcare-associated: Area dependent: Like nosocomial infections if high prevalence of MDROs or if sepsis. Nosocomial: Third-generation cephalosporin or meropenem and oxacillin or glycopeptides or daptomycin or linezolid

[1]European Association for the Study of the Liver refers only to spontaneous bacterial peritonitis and spontaneous bacterial empyema.
AASLD: American Association for the Study of the Liver; EASL: European Association for the Study of the Liver; GI: Gastrointestinal; MDR: Multidrug-resistant; MDROs: Multidrug-resistant microorganisms; MRSA: Methicillin-resistant *Staphylococcus aureus*; VRE: Vancomycin-resistant enterococcus.

Final thoughts

There is an evident conflict between ensuring adequate antibiotic prophylaxis or empiric treatment and rationalizing broad-spectrum antibiotics in patients with cirrhosis. After reviewing the literature in search of information that may be useful to guide the rational use of antibiotics in this population, several shortcomings emerge. There is insufficient granular data on the susceptibility patterns of the microorganisms involved in bacterial infections. This should stimulate research and publications of descriptive studies that serve as a platform for developing evidence-based guidelines. Many centers worldwide likely have valuable information that needs to be published. Part of the complexity of this type of research is that the microorganisms involved and their susceptibility patterns change over time. Therefore, it is necessary to have sustained surveillance programs and not just short-term studies.

CONCLUSION

Since the World Health Organization anticipates that drug resistance will have a catastrophic impact on health systems and the global economy by 2050, all healthcare professionals that participate at different levels in the care of patients with cirrhosis should advocate for the rational use of antibiotics.

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. NAFLD comprises a continuum of liver abnormalities from nonalcoholic fatty liver to nonalcoholic steatohepatitis, and can even lead to cirrhosis and liver cancer. However, a well-established treatment for NAFLD has yet to be identified. Exosomes have become an ideal drug delivery tool because of their high transmissibility, low immunogenicity, easy accessibility and targeting. Exosomes with specific modifications, known as engineered exosomes, have the potential to treat a variety of diseases. Here, we review the treatment of NAFLD with engineered exosomes and the potential use of exosomes as biomarkers and therapeutic targets for NAFLD.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Exosome; Engineered exosome; Targeted therapy

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Core Tip: Nonalcoholic fatty liver disease (NAFLD) is the fastest growing chronic disease in the world. As the disease progresses, NAFLD can lead to liver fibrosis, cirrhosis and even liver cancer. However, a well-established treatment for NAFLD has yet to be identified. Exosomes are small extracellular vesicles secreted by cells. Owing to their high delivery efficiency and biocompatibility, exosomes are expected to become a new means of drug delivery and precise treatment for a variety of diseases, including NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a metabolic disease that is prevalent worldwide affecting at least a quarter of the population[1]. NAFLD is a continuum of liver abnormalities from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) that can even lead to cirrhosis and liver cancer. NAFL is reversible, whereas NASH with cirrhosis is difficult to reverse[2]. Therefore, it is critical to explore the pathogenesis of NAFLD and identify therapeutic targets to treat or prevent its development. Exosomes are extracellular vesicles with a particle size of 30-150 nm that play a crucial role in communication between cells[3]. Some macromolecules such as RNA or proteins in exosomes are associated with the occurrence and development of liver-related diseases and can be used as potential molecular markers in the diagnosis of NAFLD[4]. Processed and modified exosomes (known as engineered exosomes) may also facilitate the study of NAFLD and the development of new therapeutic strategies[5]. In this review, the mechanism and function of engineered exosomes in the development of NAFLD are reviewed (Figure 1).

ENGINEERED EXOSOMES AND LIPID METABOLISM

The liver is the largest metabolic organ and a hub of lipid metabolism. Abnormal changes in lipid metabolism in the liver lead to the development of metabolic diseases[6]. A research team found that the release of exosomes in cultured astrocytes from apolipoprotein E knockout mice was significantly reduced compared to wild-type controls, and a PI3K inhibitor (LY294002) rescued the release of exosomes. They confirmed that the release of exosomes was regulated by cellular cholesterol through stimulation of the PI3K/Akt signalling pathway[7].

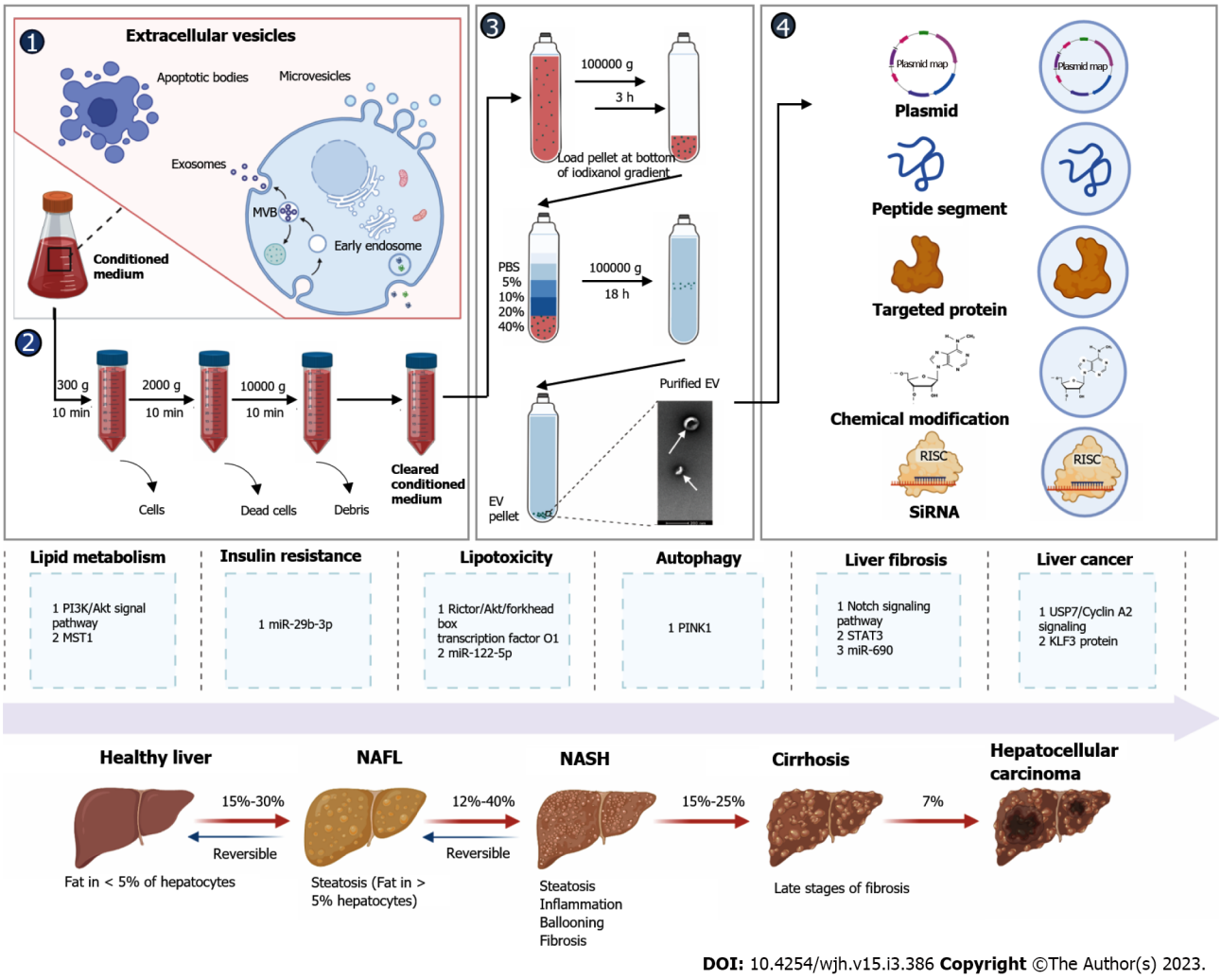
Li *et al*[8] systematically screened for microRNA expression using high-throughput small RNA sequencing and found that miR-199a-5p was significantly upregulated in adipose tissue in a mouse model of high-fat diet (HFD). Further studies confirmed that exosomal miR-199a-5p promoted lipid accumulation in the liver through induction of macrophage stimulating 1 (MST1) expression and fatty acid metabolism. Cheng *et al*[9] found that exosomal miR-627-5p reversed insulin resistance, prevented liver injury, normalized glucose and lipid metabolism and reduced lipid deposition in a rat model of NAFLD.

Brown adipose tissue (BAT) strongly promotes energy expenditure and shows good potential in the treatment of obesity. Zhou *et al*[10] treated HFD-fed mice with engineered exosomes derived from the serum of young healthy mice or from BAT. They found that treatment with BAT exosomes significantly promoted oxygen consumption in recipient cells, thus alleviating metabolic syndrome in HFD-fed mice.

Li *et al*[11] used a low-density lipoprotein receptor-deficient mouse (Ldlr mouse) as a model for hypercholesterolemia. Ldlr mRNA was encapsulated into exosomes by overexpression of Ldlr in donor AML12 mouse hepatocytes. The authors found that engineered exosomes loaded with Ldlr mRNA could restore the expression of Ldlr in the livers of Ldlr-deficient mice and rescue hypercholesterolemia. This study suggests that engineered exosomes may be an effective therapy for patients with hypercholesterolemia.

ENGINEERED EXOSOMES AND INSULIN RESISTANCE

Insulin resistance is now believed to play a key role in the onset and progression of NAFLD[12]. A HFD reduces insulin sensitivity. Kumar *et al*[13] found that feeding a HFD changed the lipid composition of intestinal exosomes. These exosomes were found to be absorbed by macrophages and hepatocytes, resulting in inhibition of the insulin signalling pathway. Castaño *et al*[14] found that obesity can alter the



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Figure 1 Diagram shows the correlation between nonalcoholic fatty liver disease and engineered exosome. MVB: Multivesicular body; EV: Extracellular vesicles; NASH: Nonalcoholic steatohepatitis; NAFL: Nonalcoholic fatty liver; STAT3: Signal transducer and activator of transcription 3; RISC: RNA-induced silencing complex; MST1: Mammalian STE20-like kinase 1; USP7: Ubiquitin specific peptidase 7; KLF3: Kruppel-like factor 3; PINK: PETN induced kinase 1; PI3K: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; Akt: Protein kinase B.

expression and composition of miRNAs in mouse plasma exosomes. Ying *et al*[15] found that miR-690, an exosome-derived miRNA from M2-polarized macrophages, improved insulin sensitivity in obese mice. Su *et al*[16] found that exosomes derived from the bone marrow mesenchymal stem cells (BM-MSCs) of aged mice could be ingested by fat, muscle and liver cells, leading to insulin resistance *in vivo* and *in vitro*. The authors found that the amount of miR-29b-3p in exosomes released by BM-MSCs was significantly increased in aged mice. Furthermore, they found that inhibition of miR-29b-3p with an aptamer-mediated nanocomposite delivery system improved insulin resistance in aged mice.

ENGINEERED EXOSOMES AND LIPOTOXICITY

Lipotoxicity promotes proinflammatory M1 polarization of liver macrophages during the development of NAFLD[17,18]. Liu *et al*[19] found that miR-192-5p-rich hepatocyte-exosomes induced by lipotoxic injury promoted macrophage M1 polarization and liver inflammation through Rictor/Akt/forkhead box transcription factor O1 signalling. Zhao *et al*[20] found that cholesterol-induced lysosomal dysfunction increased exosome release from hepatocytes, leading to M1 polarization and macrophage-induced inflammation in a miR-122-5p-dependent manner. Human umbilical cord mesenchymal stem cells (HUC-MSCs) are increasingly being studied in clinical trials of end-stage liver disease due to their excellent tissue repair and anti-inflammatory effects. Shi *et al*[21] found that HUC-MSC-derived exosomes could protect against methionine- and choline-deficient L-amino acid diet (MCD)-induced NASH.

Lipotoxicity can damage mitochondria and induce oxidative stress during the progression of NAFLD [22,23]. Studies have shown that adipocytes respond to mitochondrial stress by rapidly and vigorously

releasing exosomes[24]. Similarly, exosomes derived from chemically induced human hepatic progenitors inhibit cell death induced by oxidative stress[25].

ENGINEERED EXOSOMES AND AUTOPHAGY

Autophagy is a process in which cells degrade and metabolize their own damaged organelles or protein aggregates that plays a key role in maintaining liver homeostasis[26]. Increasing evidence suggests that autophagy plays a very important role in lipid metabolism. Autophagy mainly protects cells and regulates inflammation in NAFLD[26]. Because autophagy and exosomal biogenesis share common elements, some studies have found that plasma exosomal levels are higher in NAFLD patients than in healthy controls[27]. Luo *et al*[28] found that miR-27a inhibited mitochondrial autophagy and promoted NAFLD-associated liver fibrosis by negatively regulating PINK1 expression *via* lipotoxic hepatocyte exosomes. A research team established a model of hepatocyte injury and apoptosis induced by D-galactosamine and lipopolysaccharide (D-GalN/LPS) to study the protective effect of bone marrow mesenchymal stem cell (BMSC)-derived exosomes on liver injury. They found that BMSC-derived exosomes attenuated D-GaIN/LPS-induced hepatocyte apoptosis by activating autophagy *in vitro*[29]. Similar studies have shown that upregulation of miR-96-5p in BMSCs and their exosomes ameliorated NASH *via* caspase-2[30].

ENGINEERED EXOSOMES AND LIVER FIBROSIS

It is generally believed that during the development of NAFLD, liver-related cells are replaced by fibrotic scar tissue, giving rise to liver fibrosis or cirrhosis, which are associated with poor prognosis and mortality in patients with NASH[2]. The Notch signalling pathway is a key mediator of cellular differentiation, proliferation and apoptosis[31]. We designed hairpin-type decoy oligodeoxynucleotides (ODNs) for RBP-J to inhibit the activation of Notch signalling. ODNs were loaded into HEK293T-derived exosomes by electroporation. Furthermore, we observed that tail vein-injected exosomes were mainly taken up by hepatic macrophages in mice with hepatic fibrosis. RBP-J decoy ODNs delivered by exosomes efficiently inhibited Notch signalling in macrophages and ameliorated liver fibrosis in mice [32].

Hou *et al*[33] found that myeloid cell-specific IL-6 signalling promoted miR-223-enriched exosome production and attenuated NAFLD-associated fibrosis. Tang *et al*[34] found that exosomes embedded with siRNAs or antisense oligonucleotides targeting signal transducer and activator of transcription 3 (STAT3) could attenuate liver fibrosis. Gao *et al*[35] showed that Kupffer cells produced endogenous miR-690 and shuttled this miRNA to other hepatocytes through exosomal secretion. Treatment with miR-690 inhibitors reduced fibrosis and steatosis in a NASH model. Wang *et al*[36] found that miR-6766-3p-rich 3D human embryonic stem cell (hESC) exosomes could ameliorate liver fibrosis by targeting the TGFβ RII-SMADS pathway in hepatic stellate cells. Ji *et al*[37] developed an exosome-liposome hybrid loaded with clodronate-nintedanib that impaired hepatic fibrosis by reducing the activation of Kupffer cells.

CRISPR-Cas9 gene editing has become a powerful therapeutic technology. However, there is a lack of safe and effective *in vivo* delivery systems for CRISPR-Cas9, especially for tissue-specific vectors[38]. Luo *et al*[39] used exosome-mediated CRISPR/dCas9-VP64 delivery to reprogram hepatic stellate cells to construct engineered exosomes for the treatment of liver fibrosis. Similarly, Wan *et al*[40] delivered exosome-mediated Cas9 ribonucleoprotein complexes for tissue-specific gene therapy in liver disease.

ENGINEERED EXOSOMES AND LIVER CANCER

Without timely intervention, NAFLD inevitably results in liver cancer[41]. Liver cancer is the fourth leading cause of cancer-related death worldwide and occurs in patients with various chronic liver diseases[42]. To date, the exact pathogenesis of NAFLD-induced liver cancer is not fully understood, but may involve DNA damage responses, inflammation, autophagy, and disruption of the gut microbiota [41].

Adipose tissue is known to play a role in energy storage and metabolic regulation by secreting adipokines[43]. Studies have demonstrated that exosomal circRNA secreted by adipocytes promotes tumour growth by inhibiting miR-34a and activating the USP7/Cyclin A2 signalling pathway[44].

An acidic microenvironment has been shown to promote the release of exosomes, which are considered to be cell-to-cell communication agents involved in cancer progression and metastasis[45]. Tian *et al*[46] found that exosomal miR-21 and miR-10b induced by the acidic microenvironment in liver cancer could promote cancer cell proliferation and metastasis and be used as prognostic molecular markers and therapeutic targets for liver cancer.

Macrophage-derived exosomes play multiple roles in cancer initiation and progression[47]. Zhang *et al*[48] found that exosomes derived from RBP-J overexpressing macrophages inhibited the progression of liver cancer by miR-499b-5p/JAM3. M2 macrophages can influence tumour development by secreting various cytokines, including exosomes. Some studies suggest that M2 macrophage-derived exosomes modified by miR-660-5p-related oligonucleotides enhanced the development of hepatocellular carcinoma by regulating KLF3[49].

ENGINEERED EXOSOMES INVOLVED IN THE DIAGNOSIS OF NAFLD

Exosomes can be derived from healthy and stressed cells to provide a snapshot of the cell of origin under physiological and pathological conditions. Hepatocyte-derived exosomes released from stressed/injured hepatocytes have been identified as a partial cause of liver disease progression and liver injury, so circulating exosomes may serve as biomarkers of NAFLD. Nanopasmon-enhanced scattering of gold nanoparticles coupled with hepatocyte-specific antibodies was used to identify hepatocyte-derived exosomes[50]. Furthermore, microarray analysis of exosomal miRNAs isolated from the serum of 41 patients with NAFLD (diagnosed using liver biopsy) suggested that serum exosomal miRNAs could be used to assess the severity of NAFLD and identify potential targets for NAFLD treatment[33]. One of the determinants of liver degeneration in the progression of NAFLD is Wnt/frizzled (FZD) signalling; for example, FZD7 delivered by plasma-derived exosomes is a good candidate for a novel and effective biomarker for the diagnosis and prognosis of NAFLD[51].

CONCLUSION

The incidence of NAFLD is rapidly increasing with changes in lifestyle and dietary habits[1]. Exosomes not only mediate communication between cells but can also be engineered to deliver specific substances. Engineered exosomes have shown some effects on NAFLD in animal experiments. Owing to their low immunogenicity and liver targeting[52,53], engineered exosomes have great potential to treat NAFLD.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

mRNA transcriptome profiling of human hepatocellular carcinoma cells HepG2 treated with *Catharanthus roseus*-silver nanoparticles

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Abstract

BACKGROUND

The demand for the development of cancer nanomedicine has increased due to its great therapeutic value that can overcome the limitations of conventional cancer therapy. However, the presence of various bioactive compounds in crude plant extracts used for the synthesis of silver nanoparticles (AgNPs) makes its precise mechanisms of action unclear.

AIM

To assessed the mRNA transcriptome profiling of human HepG2 cells exposed to *Catharanthus roseus* G. Don (*C. roseus*)-AgNPs.

METHODS

The proliferative activity of hepatocellular carcinoma (HepG2) and normal human liver (THLE3) cells treated with *C. roseus*AgNPs were measured using MTT assay. The RNA samples were extracted and sequenced using BGIseq500 platform. This is followed by data filtering, mapping, gene expression analysis, differentially expression genes analysis, Gene Ontology analysis, and pathway analysis.

RESULTS

The mean IC_{50} values of *C. roseus*AgNPs on HepG2 was 4.38 ± 1.59 μg/mL while on THLE3 cells was 800 ± 1.55 μg/mL. Transcriptome profiling revealed an alteration of 296 genes. *C. roseus*AgNPs induced the expression of stress-associated genes such as *MT*, *HSP* and *HMOX-1*. Cellular signalling pathways

were potentially activated through MAPK, TNF and TGF pathways that are responsible for apoptosis and cell cycle arrest. The alteration of *ARF6*, *EHD2, FGFR3, RhoA, EEA1, VPS28, VPS25, and TSG101* indicated the uptake of *C. roseus*-AgNPs *via* both clathrin-dependent and clathrin-independent endocytosis.

CONCLUSION

This study provides new insights into gene expression study of biosynthesised AgNPs on cancer cells. The cytotoxicity effect is mediated by the aberrant gene alteration, and more interestingly the unique selective antiproliferative properties indicate the *C. roseus*AgNPs as an ideal anticancer candidate.

Key Words: *Catharanthus roseus*; HepG2; Silver nanoparticles; Transcriptome; oxidative stress; Apoptosis; Cell cycle

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Core Tip: Despite the increased attention on cancer nanomedicine which is advantageous to overcome the limitations of conventional cancer treatment, the information on the selectivity and detailed mechanisms at the cellular and molecular level remain unclear. To evaluate its selectivity effects, the proliferative activity of both liver cancer cells HepG2 and normal liver cells THLE-3 in response to *Catharanthus roseus*-silver nanoparticles (*C. roseus*-AgNPs) was assessed. To determine the possible signalling pathways induced by the *C. roseus*-AgNPs, the mRNA transcriptome profiling of hepatocellular carcinoma cell line HepG2 was performed, highlighting the expression of genes associated with oxidative stress, apoptosis, and cell cycle arrest. The elucidation of its selectivity effects and detailed wide genome screening would enlighten the cellular and molecular signalling pathways and provide a strong basis towards the development of *C. roseus*-AgNPs as an anticancer drug for liver cancer.

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INTRODUCTION

Nanoparticles are materials or discrete clusters of atoms having dimensions within 1-100 nm[1]. Having a large surface area-to-volume ratio with unique biological properties, nanoparticles have gained immense usage in the early diagnosis and treatment of cancer, the application of which is termed nano oncology[2,3]. Nanoparticles can offer an alternative to the current conventional chemotherapeutic agents which although exhibit high efficacy in killing cancer cells, still suffer from significant drawbacks due to the poor specificity in causing severe damage to healthy cells[4]. Amongst various nanoparticles, silver nanoparticles (AgNPs) have been reported to demonstrate a significant biological effect, particularly in the healthcare industry[5,6]. Concurrently, the market demand for eco-friendly, hazard-free, and cost-effective synthesis of AgNPs was higher as many of the common nanoparticle production methods involved hazardous chemicals and high energy- consumption[7]. One of the most effective biogenic approaches is to use plant extract that contains metabolites, which can enhance the reduction of silver ions. Plant extract-mediated silver nanoparticle synthesis is found to have a promising anticancer property. Plant extract-based synthesis is largely favoured due to the lower degree of adverse effect as well as the low cost of synthesis that enables large-scale production. Moreover, biologically active ingredients or phytomolecules in the plant extract act as reducing agents to promote the synthesis of AgNPs[8]. A previous study has corroborated the anticancer property of AgNPs, for example, biosynthesised AgNPs using *Acalypha Indica*, which exhibited anti-cancer activity against human breast cancer cell line MDA-MB-231[9]. In another study, AgNPs synthesised using leaf extract of *Tropaeolum majus* L. also demonstrated anti-cancer properties on the MCF7 cell line[10]. These findings cumulatively proved the anti-cancer property of the biogenic AgNPs.

Previously, an herbal plant *Catharanthus roseus* (*C. roseus*) G. Don has demonstrated its ability as a reducing agent to synthesise AgNPs. This plant is commonly known as periwinkle which belongs to the Apocynaceae family[11]. This plant is very synonymous with its content, indolomonoterpenic alkaloids vincristine and vinblastine[12]. These compounds are commonly used in the treatment of several

malignant conditions, such as Hodgkin's and non-Hodgkin's lymphomas, acute lymphoblastic leukaemia, neuroblastoma and breast carcinoma[13]. These alkaloids may be responsible for the reduction of the silver ions to AgNPs and at the same time exert their function by disrupting the mitotic spindle apparatus of microtubules through tubulin interaction, thus blocking the mitosis process, and arresting the cancer cells during metaphase[14].

An understanding of the anti-cancer mechanisms of AgNPs at the molecular level would provide detailed insight into various physiological processes involved. This is achievable *via* transcriptome analysis, a holistic view of gene expression. An overview or snapshot of the gene expression landscape could reveal the intricate molecular network that underlies the myriad of biological processes in a cell. As compared to hybridisation-based RNA quantification methods such as microarray analysis, this sequencing-based transcriptome detection can perform well within a wide range of circumstances, where this method could quantify gene expression with low background, high accuracy, and high reproducibility levels with significant dynamic range transcriptome analysis can detect subtle changes in gene expression, mutations, splice variants and fusion genes that cannot be identified by microarrays [15].

Fuelled by the intriguing capacity of the transcriptome analysis, in this study, we endeavoured to carry out an mRNA transcriptome profiling of the human hepatocellular carcinoma cell (HepG2) treated with AgNPs synthesised using an aqueous extract of *C. roseus* G. Don. The human hepatocellular carcinoma cell (HepG2) was used as a representative *in vitro* cancer cell line model, due to its known well-characterised property of cell line and its wide usage in many toxicity studies for screening hepatotoxic compounds[16]. To the best of our knowledge, there was no study reported on the transcriptome profiling of cancer cells treated with plant extract-mediated synthesised AgNPs. As such, this study is the first study that focuses on the transcriptome profiling of cancer cells treated with AgNPs synthesised using plant extract. This study can be a significant step in identifying potential genes that are regulated by the treatment of *C. roseus*-AgNPs on HepG2 cells, which will lead to the establishment of the underlying molecular network of the mechanistic actions of the AgNPs.

MATERIALS AND METHODS

Preparation of cell line

The hepatocellular carcinoma cell line HepG2 used in this study was purchased from American Type Culture Collection (ATCC, Cat. HB-8065™, Rockville, MD, United States). Complete RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 1% penicillin-streptomycin (v/v) and 1% L-glutamine (v/v) was used to culture and maintain the cells. All the reagents were purchased from Nacalai Tesque (Kyoto, Japan). Meanwhile, a normal liver cell line (THLE-3) (ATCC) was cultured in Bronchial Epithelial Cell Growth Basal Medium (Lonza, Basel, Switzerland) supplemented with frozen additives without gentamycin/Amphotericin and Epinephrine, 5 ng/mL EGF, 70 ng/mL Phosphoethanolamine and 10% fetal bovine serum. The incubator used for the cell culture work was set at 37 °C with 5% CO_2 (Shellab, Cornelius, OR, United States). Upon reaching 80% confluency, the cells were subcultured and transferred into new cell culture flasks. The cells were seeded at a concentration of 1×10^5 cells/mL.

Preparation of C. roseus G. Don aqueous extract

The *C. roseus* aqueous extract was prepared according to our previous study[17]. A voucher specimen of *C. roseus* plant was deposited at the Herbarium of Universiti Sains Malaysia with reference number 10933. The leaves were washed using free-flowing clean water and left dried in an oven at 40 °C. The leaves were first ground before mixing with double distilled water with a ratio of 50 g: 1 L in a conical flask. Following overnight incubation in a water bath at 40 °C, the mixture was centrifuged at 2000 rpm for 15 min. The filtered supernatant was freeze-dried and ready to be used for the preparation of *C. roseus*-AgNPs.

Preparation of C. roseus G. Don-AgNPs

The *C. roseus*-AgNPs used in this study have been successfully synthesised, optimised, and characterised in our previous study[5]. The optimised *C. roseus*-AgNPs consist of 10% of *C. roseus* aqueous extract and 5 mmol/L of silver nitrate ($AgNO_3$) solution. The mixture was allowed to react in a dark environment at room temperature for 24 h until the colour changes from light yellowish to dark brownish. The mixture was then collected and centrifuged for 15 min at 10000 rpm. The supernatant was discarded while the pellet was collected and freeze-dried.

Cell viability

The proliferative activity of HepG2 and THLE-3 cells was assessed using Cell Titer 96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, United States) which consists of (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) dye solution or also known as MTT and solubilisation solution. The method was performed according to the manufacturer's protocol. HepG2

cells and THLE-3 cells were seeded in a 96-well plate (Eppendorf, Hamburg, Germany) at a concentration of 1 × 10^5 cells/mL. Cells were treated with *C. roseus*-AgNPs (Merck, Billerica, MA, United States) in serial dilution manner which was 1.96 µg/mL, 3.91 µg/mL, 7.82 µg/mL, 15.63 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL. The cells were incubated for 24, 48, and 72 h at 37 °C, 5% incubator. Untreated cells were used as a control. Each sample size was prepared in triplicate. Following the indicated incubation time, each well was added with 20 µL of MTT reagent and further incubated for 4 h in a humidified 5% CO_2 incubator at 37 °C. After 4 h of incubation, 100 µL of stop solution was added to each well and incubated for 1 h to solubilise the formazan. The absorbance at 570 nm was recorded using a microplate reader (Bio Tek, Winooski, VT, United States). The half-maximal inhibitory concentration (IC_{50}) values were calculated based on the following formula:

% Cell Viability = [Mean OD_{sample} - OD_{blank}]/[Mean $OD_{control}$ - OD_{blank}] × 100

OD = Optical Density

Treatment of HepG2 cells with C. roseus-AgNPs and total RNA extraction

The HepG2 cells were seeded approximately at 1 × 10^5 cells/mL. The seeded cells were treated with *C. roseus*-AgNPs at a concentration of 4.95 µg/mL, which is the IC_{50} value used in our previous study[5] and incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Untreated HepG2 cells were used as a control. After 72 h of exposure, the cells were washed with PBS and immediately lysed and homogenised in TRIzol™ Reagent (Thermo Fisher, Waltham, MA, United States). Total RNA extraction was carried out using the manufacturer's protocol. The resulting pellet was solubilised in RNAse-free water and was kept at -80 °C until further processing. The purity and concentration of RNA (260/280 ratio) were determined using Nanodrop 2000 spectrophotometer (NanoDrop Products, Wilmington, DE, United States). The integrity of the total isolated RNA was assessed by Agilent 2100 Bioanalyser (Agilent RNA 6000 Nano Kit, Santa Clara, CA, United States).

Beijing Genomics Institute sequencing

All RNA samples were sent to Beijing Genomics Institute (BGI, Shenzen, China) for sequencing. The total RNA extracted was pre-processed for transcriptome sequencing. The poly-A-containing mRNA molecules were captured and purified using a technique probe. The purified RNA molecules were reverse-transcribed into the first-strand cDNA, subsequently followed by the second-strand cDNA synthesis using Polymerase I and treatment with RNase H. The resulting product was purified and enriched with PCR amplification. The PCR amplicon was quantified by Invitrogen Qubit 2.0 Fluorometer (Thermo Fisher). The amplicon libraries were pooled together to make a single-strand DNA circle (ssDNA circle). DNA nanoballs (DNBs) were generated from the ssDNA circle by rolling circle amplification and loaded into a flow cell in which DNB binding sites are patterned nano-arrays. Sequencing was carried out using a paired-end 100 bp sequencing strategy on the BGIseq500 platform.

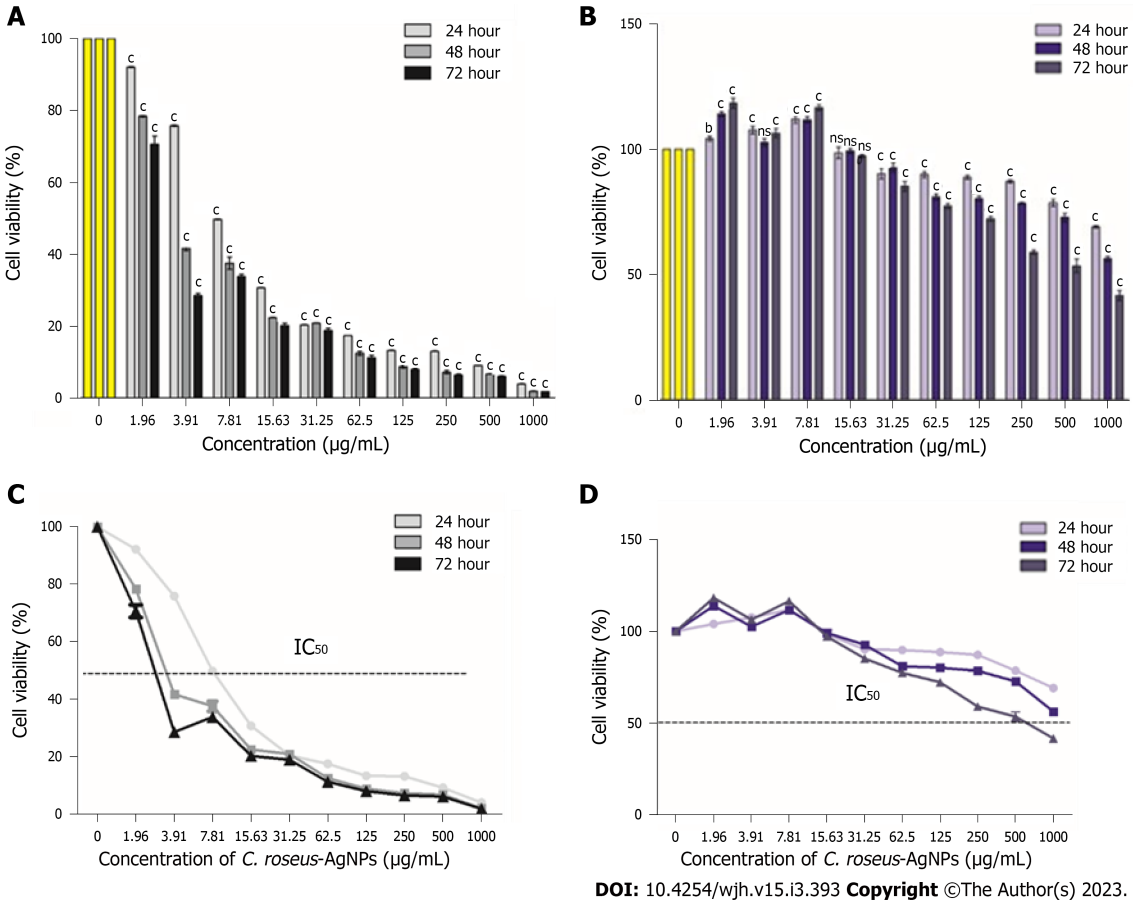
Bioinformatics analysis

High-quality genome sequencing data was developed by removing the adapter, poor quality and low complexity reads. The cleaned sequences were mapped onto the reference genome (hg19), subsequently followed by the identification of the novel genes, SNP (single nucleotide polymorphism), InDels (insertions and deletions) and the detection of gene splicing. Differential Gene Expressions were obtained by applying a paired, two-tailed t-test to the calculated expression data of the treated and untreated samples. Gene Ontology (GO) analysis was used to analyse the enrichment of gene sets associated with biological processes, molecular functions, and cellular components. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was carried out to permit the pathway annotation to the differentially expression genes (DEGs). A *P* value less than 0.1 is considered a statistically significant difference.

RESULTS

Cytotoxicity of C. roseus-AgNPs on HepG2 Cells

In this study, the cytotoxic effects of *C. roseus*-AgNPs were assessed on HepG2 cells and normal liver epithelial cells, THLE-3 cells. Figure 1A shows the cytotoxicity effects of HepG2 cells after treatment with *C. roseus*-AgNPs. In comparison to the untreated cells, *C. roseus*-AgNPs significantly ($P < 0.001$) inhibited the proliferation of HepG2 cells at all concentrations and incubation periods in time- and dose-dependent manner, indicating the cytotoxic effect of *C. roseus*-AgNPs towards HepG2, with 7.79 %, 21.59%, and 30.15% of cells were inhibited at the lowest concentration of *C. roseus*-AgNPs at 24, 48 and 72 h, respectively. HepG2 cells showed a consistent percentage decrement of cell viability upon the treatment, and only 1.78 % average of the cells survived between 24 to 72 h of incubation at the highest concentration of *C. roseus*-AgNPs. The percentage of *C. roseus*-AgNPs cytotoxicity compared to the untreated cells was used to determine the IC_{50} values as illustrated in Figure 1B where the IC_{50} were 7.81 ± 0.02 µg/mL, 3.87 ± 0.02 µg/mL, and 3.20 ± 0.04 µg/mL at 24, 48, and 72 h of incubation, respectively.



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Figure 1 Cytotoxicity evaluation of *Catharanthus roseus*-silver nanoparticles on HepG2 and THLE-3 cells. A: The cytotoxicity of HepG2 cell lines treated with different concentrations of *Catharanthus roseus*-silver nanoparticles (*C. roseus*-AgNPs); B: The IC_{50} of *C. roseus*-AgNPs on HepG2 cells; C: The cytotoxicity of THLE3 cell lines treated with different concentrations of *C. roseus*-AgNPs; D: The IC_{50} of *C. roseus*-AgNPs on THLE-3 cells. All experiments were done in triplicate, and the data represent means ± standard deviations. The comparison between each concentration with untreated cells was done using two-way ANOVA with Dunnet post-test to detect any significant differences ($^bP < 0.01$; $^cP < 0.001$; ns not significant). *C. roseus*-AgNPs: *Catharanthus roseus*-silver nanoparticles.

Figure 1C shows the effect of THLE-3 treated with *C. roseus*-AgNPs. The results demonstrate an intriguing finding, where the *C. roseus*-AgNPs demonstrated a substantial ($P < 0.001$) increment in THLE3 proliferation at concentrations of 1.96 µg/mL and 7.81 µg/mL for all incubation times. On the contrary, during all incubation times, there was no significant difference at concentrations of 15.63 µg/mL. However, at concentrations 31.25 µg/mL to 1000 µg/mL, *C. roseus*-AgNPs significantly ($P < 0.001$) inhibited the proliferation of THLE3 cells as compared to untreated THLE3 cells. After 72 h, approximately 55.78% of cells survived at the highest concentration of *C. roseus*-AgNPs. There were no IC_{50} values at concentrations for 24 and 48 h, but at 72 h, the IC_{50} was recorded at 615 ± 0.05µg/mL µg/mL, as depicted in Figure 1D. Based on the results, we observed that *C. roseus*-AgNPs was found to inhibit the growth of the HepG2 cell line with a mean IC_{50} value of 4.95 ± 0.03 µg/mL. Contrarily, *C. roseus*-AgNPs showed very weak inhibition activity toward THLE3 cells with IC_{50} value of 615 ± 0.05 µg/mL.

Quantitative and qualitative measurement of total RNA

Total isolated RNA was quantified using Bioanalyser. As depicted in Supplementary Figure 1, the representative electropherogram indicated two intact bands that are visible in each sample. These two bands represent 28s and 18s ribosomal RNA, respectively. RNA integrity number (RIN) was then determined, which is the value of the RNA integrity. The value that falls within a range between 8 to 10 showed an acceptable value of RIN[18]. The RIN and ribosomal ratio values acquired for both untreated and *C. roseus*-AgNPs treated HepG2 cells were 9.6 and 9.4, respectively. Both RIN values were within the acceptable range.

mRNA Transcriptome sequencing

Sequencing data filtering: Two samples were sequenced using the DNBseq platform and the result was about 6.98 Gb bases per sample. The distribution of the base quality was shown in Supplementary Figure 2. As observed in both Supplementary Figure 2A and B, the percentage of clean reads was 93.69% and 94.03%, respectively.

Genome mapping: After read filtering, the clean reads were mapped to the reference genome using *HISAT2*[19]. On average, 95.88 % of reads were found to be mapped to the population of human genomes and the consistency of the mapping result for each sample suggests that the samples were comparable. The mapping details are shown in Table 1.

Gene expression analysis: To get a complete reference for the gene mapping and expression, novel coding transcripts were merged with the reference transcripts, and clean reads were mapped to them using Bowtie2[20]. The gene expression level for each sample was calculated with RSEM[21]. The result of this analysis is summarised in Table 2.

The sufficiency of sequencing data for bioinformatics analysis was approached using sequencing data saturation analysis. As the number of sequenced reads increased, the number of identified genes also increased. On the other hand, when the number of sequenced reads reached a certain amount, the determining gene growth curve flattens, indicating the identified gene reached saturation. Supplementary Figure 3 displays the saturation analysis for each sample.

Reads coverage and distribution of each detected transcript are shown in Supplementary Figure 4 and Supplementary Figure 5, respectively. This approach allows access to the excellent quality of the samples and sequencing data sufficiency by showing the completely covered transcripts and evenly distributed reads throughout the transcript. These results suggest that both untreated and treated HepG2 had excellent sample quality and sufficient sequencing. Correlation between samples was assessed by Pearson correlation coefficient calculations for all gene expressions between the samples, as shown in Figure 2.

The identification of differentially expressed genes: DEGs were determined by using DEseq2 and passion Dis algorithms. The distribution of DEGs is summarised using the volcano plot as shown in Figure 3. The treatment of HepG2 cells with *C. roseus*-AgNPs revealed 296 DEGs, with 182 genes were upregulated while 114 genes were downregulated (Figure 3A).

Gene ontology analysis of DEGs: The identified DEGs were subjected to Gene ontology analysis. GO unveiled three ontologies which are related to molecular biological function, cellular components, and biological processes. The classification result is depicted in Figure 4.

Pathway analysis of DEGs: KEGG pathway classification and functional enrichment were generated based on DEGs. Pathway enrichment result is shown in Table 3 and the network enrichment is depicted in Figure 5.

DISCUSSION

C. roseus-AgNPs exhibited anti-cancer properties with negligible effect on normal cells

Our group has previously demonstrated the anti-cancer properties of *C. roseus*-AgNPs on cancer cells [22]. The anti-cancer properties of the *C. roseus*-AgNPs were estimated by IC_{50}, which represents the concentration of *C. roseus*-AgNPs required to inhibit 50% of the total cells[23]. According to the IC_{50} value (800 ± 1.55 μg/mL) observed at 72 h, the THLE3 cells substantially ($P < 0.001$) inhibited only at very high concentrations of *C. roseus*-AgNPs. On the other hand, the IC_{50} of the *C. roseus*-AgNPs on the HepG2 cells was 4.38 ± 1.59 μg/mL. This study revealed that *C. roseus*-AgNPs showed a significant ($P < 0.001$) cytotoxicity towards HepG2 cells as compared to THLE3 cells. *C. roseus*-AgNPs can inhibit the progressive development of HepG2 while causing very insignificant toxicity to normal cells at low concentrations. Several studies have also shown that biosynthesised AgNPs show no toxicity against normal cells while demonstrating cytotoxic effects against cancer cells[24-29]. For example, a study by Halkai *et al*[24] showed that fungal-derived AgNPs exerted minimal cytotoxicity against human gingival fibroblast cell line. Additionally, Sriram *et al*[25] also reported similar observations in their experiments, where AgNPs acted as an anti-proliferative agent by effectively inhibiting the development of Dalton's lymphoma ascites cell lines without causing toxicity on normal cell lines. The findings from our study agreed with the previous reports, corroborating the potentiality of *C. roseus*-AgNPs as an anti-cancer agent.

mRNA transcriptome analysis identified 296 protein-coding genes

An in-depth understanding of the anti-cancer properties of the *C. roseus*-AgNPs entails the identification of the genes that act in concert in orchestrating the effect. As transcriptome analysis can provide an overarching view of the gene expression profile under a certain condition or state, it was adopted in our effort to comprehend the underlying mechanisms of the anti-cancer activity of *C. roseus*-AgNPs against HepG2 cells. In the present study, the untreated HepG2 cells and *C. roseus*-AgNPs treated HepG2 cells were subjected to mRNA transcriptome analysis using the BGI DNBseq Platform. As revealed by the mRNA transcriptome analysis, it was found that the treatment of HepG2 cells with *C. roseus*-AgNPs has resulted in the regulation of 296 protein-coding genes, of which 182 genes were upregulated while 114

Table 1 Summary of genome mapping

Sample	Total clean reads	Total mapping ratio	Uniquely mapping ratio
Untreated HepG2	70025052	95.94%	76.60%
C. roseus-AgNPs treated HepG2	72598578	95.82%	75.94%

C. roseus-AgNPs: *Catharanthus roseus*-silver nanoparticles.

Table 2 Summary of gene mapping ratio

Sample	Total clean reads	Total mapping ratio	Uniquely mapping ratio
Untreated HepG2	70025052	66.79%	63.92%
C. roseus-AgNPs treated HepG2	72598578	62.94%	60.15%

C. roseus-AgNPs: *Catharanthus roseus*-silver nanoparticles.

Table 3 Pathway functional enrichment results

Pathway ID	Pathway	Genes	Hits	Adj *P* value
K05200	Pathway in cancer	*FAS, GADD45A, BAX, PMAIP, BID, JUN, CXCL8, HMOX1, STAT1, FOS, CEBPA, VEGFA, FGF5, EGF, RHOA, FADD, FH, SMAD, MTOR, NFκBIA, CDKN1A, WNT4*[a], *WNT7A*[a], *FGFR3*[a], *BMP4*[a], *CDK4*[a], *CDK2*[a], *MDM*[a]	29/530	3.28E-12
K04115	p53 signaling pathway	*FAS, GADD45A, SERPINE, THBS1, CDK4, BAX, CDK2, PERP, SESN1, SESN2, PMAIP1, BID, IGFBP3*[a], *MDM2*[a]	14/72	1.28E-13
K04210	Apoptosis	*FAS, GADD45A, BAX, PMAIP1, BID, JUN, FOS, FADD, NFKBIA, BCL2A1, ATF4, MCL1, TNFSF10*[a], *RIPK1*[a]	14/136	9.61E-10
K04144	Endocytosis	*ARF6, RHOA, EHD2, FGFR3, HSPA6, HSPA1L, VPS28, EEA1, VPS25, TSG101, STAM, SMAD, EHD4, LDLR, TFRC*[a], *MDM2*[a]	16/244	4.08E-08
K04010	MAPK signaling pathway	*FAS, GADD45A, JUN, FOS, VEGFA, FGF5, CDKN1A, EGF, ATF4, HSPA6, HSPA1L, CDKN1A, HSPB1, DUSP1, NR4A1, EFNA4*[a], *FGFR3*[a], *ERBB3*[a], *SKP2*[a]	16/295	5.58E-07
K04668	TNF signalling pathway	*FAS, JUN, FOS, FADD, NFKBIA, ATF4, RIPK1*[a], *CCL2, NOD2, CCL20*[a]	10/110	9.07E-07
K04350	TGF beta signaling pathway	*THBS1, RHOA, SMAD2, SMAD4, BMP4, SMAD7, BAMBI, BMP6*	8/90	1.66E-05
K02010	Cell cycle	*GADD45A, SOX15, CDK4*[a], *CDK2*[a], *MDM2, SMAD2, SMAD4, CDKN1A, PCNA*[a], *MCM3*[a], *SKP2*[a]	9/124	2.09E-05
K04978	Mineral absorption	*HMOX1, MT1F, MT1X, MT1H, MT1B, FTH1*	6/51	3.58E-05

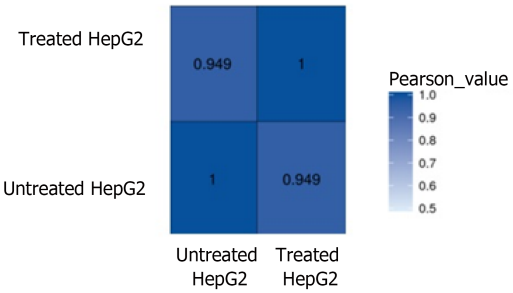
[a]Downregulated genes.

genes were downregulated, as shown in Figure 3.

GO analysis exhibited that the highest fraction of the regulated genes were involved in "cellular and signalling response" followed by "biological regulation", "regulation of biological process", "metabolic process" and "response to stimulus" (Figure 4). The underlying pathways regulated by the genes are the p53 signalling pathway, pathway in cancer, apoptosis pathway, endocytic pathway, MAPK signalling pathway, TNF signallingg pathway, TGF signallingg pathway, cell cycle pathway and mineral absorption pathway.

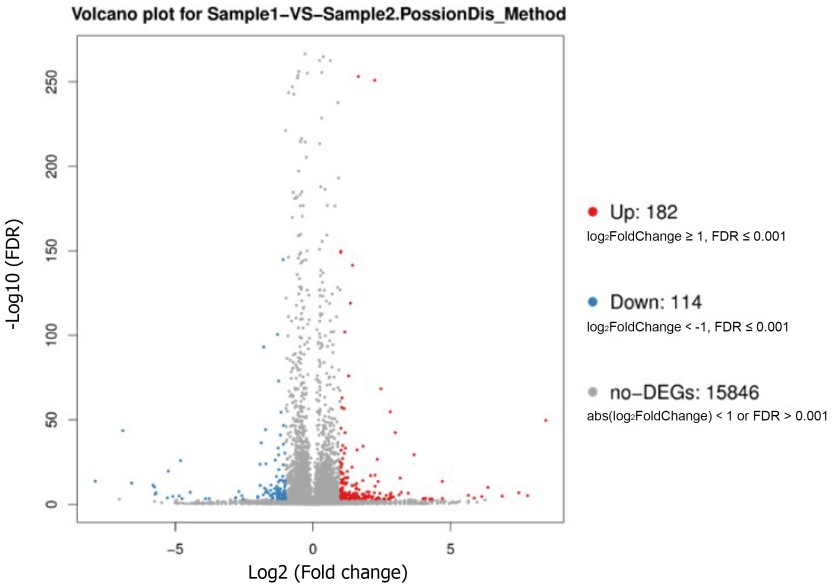
C. roseus-AgNPs induced the expression of stress-associated genes such as MT, HSP and HMOX-1

C. roseus-AgNPs treatment of the HepG2 cells was found to upregulate several members of the gene isoforms that encode metallothionein (MT), such as *MT1F, MT1X, MT1H, and MT1B*. MTs are intracellular proteins that contain approximately 30% thiol-containing cysteine residues, which can bind several cytotoxic agents, including platinum compounds, alkylating agents, and metal ions such as zinc and copper[30]. MTs also regulate various pathophysiological processes such as apoptosis, and angiogenesis and could also act as radical scavengers by protecting the cells from free radicals[31]. As such, an increased level of MT is an indicator that the cells were undergoing 'stress' and the cells are



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Figure 2 The correlation analysis between samples. The colour represents the correlation coefficient.



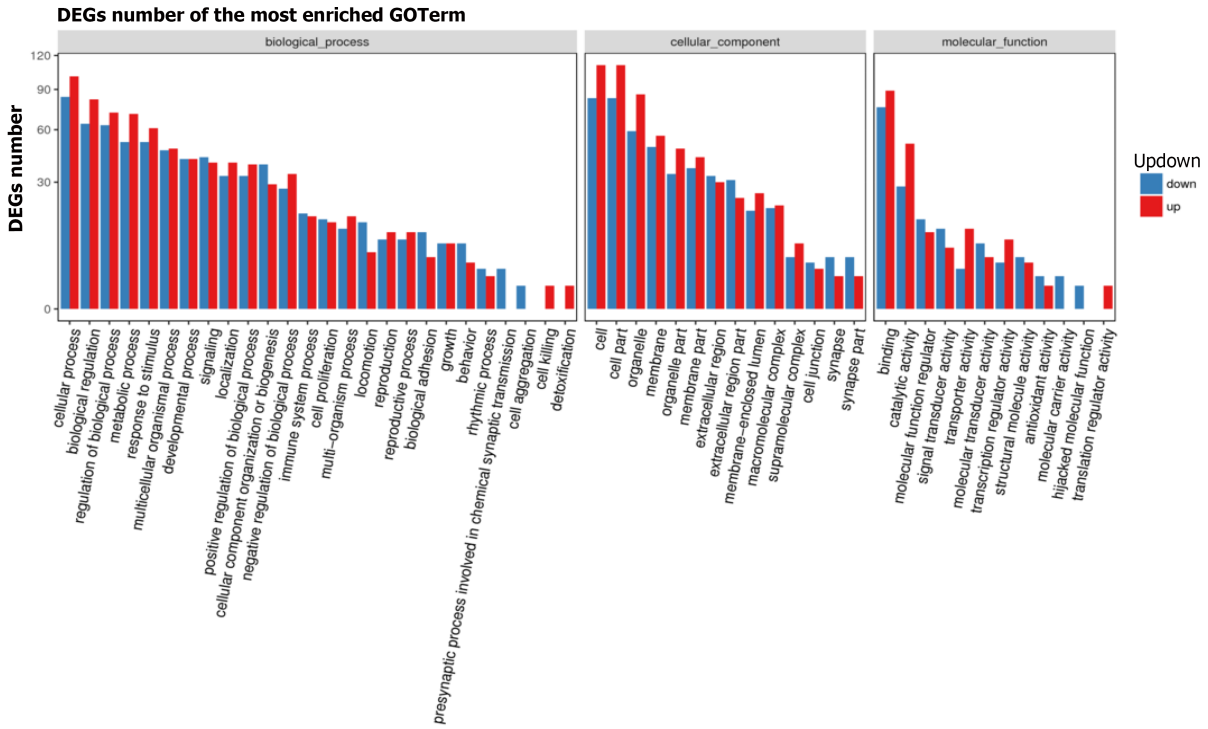
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Figure 3 Volcano plot of differentially expression genes. Red points represent upregulated differentially expression genes (DEGs). Blue points represent down-regulated DEGs. Grey points represent non-DEGs. DEGs: Differentially expression genes.

striving to mitigate the cytotoxic effect of the anticancer drug, in this case, *C. roseus*-AgNPs[32]. This finding is also in agreement with the findings by Woo *et al*[33], who reported that *Javanese medaka*, a type of seawater organism showed MT upregulation upon exposure to AgNPs. On the other hand, heat-shock genes such as *HSPA1L*, *HSPB1*, and *HSPA6* were also found to be upregulated in HepG2 cells exposed to *C. roseus*-AgNPs. *HSPs* are upregulated by stress signals such as high temperature, decreased availability of oxygen, infectious agents, and inflammatory mediators[34]. The increased expression level of HSPs is needed to counteract the stress, which is induced by *C. roseus*-AgNPs in this study. Furthermore, the up-regulation of oxidative stress-related genes *HMOX-1* was also documented in our experiment. *HMOX-1* is a reactive oxygen species (ROS) sensor that has antioxidant and anti-inflammatory properties[35]. During stress conditions, HMOX-1 catalyse the degradation of the Heme group into biliverdin, carbon monoxide, and iron[36]. Similar increased expression of *HMOX-1* was also observed by Gurunathan *et al*[37], in mouse embryonic fibroblast cells upon treatment with AgNPs. Collectively, the upregulation of stress-response genes such as *MTs, HSPs*, and *HMOX-1* in this study indicates that *C. roseus*-AgNPs exposure invokes the cell's defensive response in negating effects of cellular stresses caused by *C. roseus*-AgNPs. The increased expression of stress-response genes indirectly reflects the cytotoxic effect of *C. roseus*-AgNPs. We have also observed significant production of NO and ROS in our previous study upon treatment of HepG2 cells with *C. roseus*-AgNPs[38]. These findings are substantial and in agreement with the previous findings, whereby upregulation of *MTs, HSPs,* and *HMOX-1* was observed in cells exposed to AgNPs[39-41].

C. roseus-AgNPs increased expression of tumour suppressor genes and apoptotic genes

The most intriguing finding in our study is that *C. roseus*-AgNps treatment on HepG2 cells induces the expression of growth arrest and DNA damage-inducible alpha (*GADD45A*) gene, which is a type of



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Figure 4 Gene Ontology classification of upregulated and downregulated differentially expression genes.

tumour suppressor gene that regulates processes such as DNA repair, cell cycle control, senescence, and genotoxic stress[42]. The expression of the *GADD45A* gene in cell cycle inhibition is also regulated by p53. p53 protein is involved in maintaining genetic integrity and regulating the cellular response towards genotoxic stress by inducing cell cycle arrest or apoptosis to prevent tumorigenesis[43]. *p53* is negatively regulated by *MDM2*. Interestingly, our experimental findings demonstrated that *MDM2* was downregulated in *C. roseus*-AgNPs treated HepG2 cells, suggesting that its inhibitory effect against p53 was ameliorated, causing the upregulation of the *p53* gene. As such, the expression of the *p53* gene elevates, causing the suppression of the proliferation of cancer cells. Sahu *et al*[44] reported similar observations in their study where under normal conditions, *p53* was constitutively expressed, but inactivated by its negative regulator, *MDM2*. However, during cellular stresses, *MDM2* was downregulated which in turn caused the upregulation of *p53* gene. The upregulation of the *p53* gene is indicative of the anticancer effect of *C. roseus*-AgNPs in amplifying the tumour suppressor activity of the cancer cells. Besides the upregulation of the tumour-suppressor genes, apoptotic-related genes *BAX* and *FAS* were also found to be upregulated, suggesting the anticancer efficacy of the *C. roseus*-AgNPs in promoting apoptosis in cancer cells.

C. roseus-AgNPs activated signal transduction pathways such as MAPK signalling pathway

The MAPK pathway is a series of protein kinase cascade essential in regulating numerous physiological functions including inflammation, cell stress response, cell differentiation, cell division, cell proliferation, metabolism, motility, and apoptosis[45]. Treatment with *C. roseus*-AgNPs activated MAPK signalling pathway in HepG2 cells. In this study, several genes that are involved in MAPK pathways were found to be regulated such as *FAS, GADD45A, P53, JUN*, and *FOS*. As indicated previously, *GADD45A*, a tumour suppressor gene which could also be involved in the MAPK signalling pathway was found to be upregulated upon treatment of the HepG2 cells with *C. roseus*-AgNPs. Increased expression of *GADD45A* conduces to baicalein-induced apoptosis and activation of MAPK signalling pathway[46]. In this study, activation of MAPK signalling pathway also upregulates the *p53* gene as mentioned previously, as MAP kinase phosphorylates and activates the p53 protein in response to stressful stimuli induced by *C. roseus*-AgNPs[47]. Taken together, activation of MAPK pathway prepares the cell for counteracting actions such as inflammation, cell stress response, and apoptosis upon treatment with *C. roseus*-AgNPs, which indirectly implies the anticancer properties harboured by these nanoparticles.

C. roseus-AgNPs activated TNF signalling pathway

TNF alpha is a pro-inflammatory cytokine that acts by binding to TNF-R1 and TNF-R2 receptors, resulting in the recruitment of signal transducers that activate the effector, leading to the activation of caspases and two transcription factors, NF-κB, as well as MAPKs such as ERK, p38, and JNK, which will



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Figure 5 Network enrichment result. The darker the colour indicates the highest enrichment pathways. The larger the area, the higher the degree of enrichment.

induce apoptosis and necrosis[48]. In this study, the treatment of *C. roseus*-AgNPs caused the upregulation of several genes related to TNF signalling pathway such as FADD, NF-κbia), ATF4, CCL2, NOD2. FADD protein interacts directly with TRADD, which are signal transducers that activate NF-κB and trigger apoptosis[49]. The overexpression of the *FADD* genes in our study suggests that treatment with *C. roseus*-AgNPs eventually promotes apoptosis. Similar overexpression was also reported in the previous study, whereby AgNPs treated MDA-MB-436 cells showed an increase in the level of *FADD* gene[50]. In this study, the upregulation of *ATF4* was also found. The overexpression of *ATF4* was reported by Iwasaki *et al*[51], which happens in response to metabolic stresses caused by SFAs and ER stressors. *RIPK1* gene is involved in the system that controls cell survival, signalling nodes in cell death and inflammation and cytokine production. The downregulation of the RIPK1 gene in this study upon treatment with *C. roseus*-AgNPs can induce apoptosis *via* the cleavage activity of the caspase 3 associated pathway[52]. Qiu *et al*[53] reported similar observations in their experiments. CCL20 is known to enhance cancer cell progression[54]. The downregulation of the *CCL20* gene in this study suggests that *C. roseus*-AgNPs are able to induce inflammation through TRAIL as reported by a previous study[55].

C. roseus-AgNPs elicited the activation of TGF-β signalling pathway

TGF-β signalling pathway plays a crucial role in controlling various fundamental aspects of cellular activities such as cellular growth, development, differentiation, and apoptosis[56]. As a secreted polypeptide, TGF-β functions *via* receptor serine/threonine kinases and intracellular SMAD effectors [57]. TGF-β acts as a tumour suppressor at the early stage of cancer while it also acts as a pro-metastatic factor in the later stages of cancer[58]. Exposure of HepG2 cells to *C. roseus*-AgNPs activates TGF-β signalling pathway. The effect of *C. roseus*-AgNPs is analogous to a previous study, whereby ellagic acid was found to exert anti-proliferation effects by activating TGF-β/Smad3 signalling pathway[59]. Transcriptome analysis also showed that isoforms of SMAD, which are part of TGF-β pathway were also upregulated. Moreover, BMPs such as *BMP4* and *BAMP6*, which are extracellular signalling molecules that belong to the TGF-β pathway, were also upregulated. The tumour suppressor effect mediated by TGF-β pathway was imparted upon treatment with *C. roseus*-AgNPs, which corroborates its anticancer property.

The uptake of C. roseus-AgNPs occurred via endocytosis

Endocytosis involves the formation of small membrane vesicles (60-120 nm) that transports various

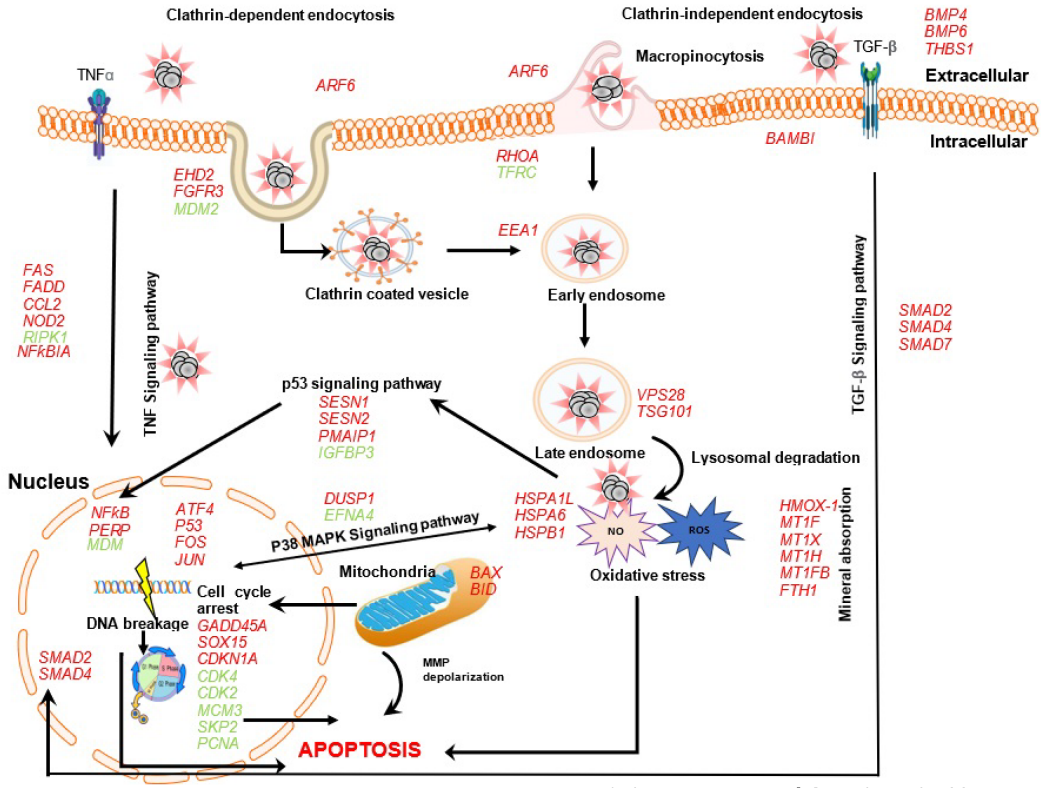
molecules or cargo from the plasma membrane to the cytoplasm. Though there are several types of endocytosis, previous studies have shown that clathrin-dependent endocytosis and macropinocytosis are the major routes of transportation of AgNPs into the cells[60]. The observations also agree with the results reported by Treuel *et al*[61] that endocytosis has been demonstrated to be a key mechanism in driving the cellular uptake of AgNPs, with NPs entering cells *via* early endosomes, late endosomes, and lysosomes. In this study, a few genes such as *ARF6, EHD2, FGFR3, RhoA, EEA1, VPS28, VPS25, and TSG101,* were upregulated, suggesting that the uptake of *C. roseus*-AgNPs can occur *via* the clathrin-dependent or clathrin-independent endocytosis pathway. *ARF6* gene, also known as ADP-ribosylation factor 6, is a small GTPase that regulates endocytic membrane trafficking and actin remodelling[62]. The upregulation of *ARF6* gene in this study is consistent with the findings of Tanabe *et al*[62], which suggest that *ARF6* gene regulates the membrane trafficking between the plasma membrane and endosome *via* clathrin-dependent or clathrin-independent endocytosis[63]. A previous study by Morén *et al*[63] showed that the overexpression of *EHD2* gene inhibited the formation of caveolae. Interestingly, our study demonstrated an upregulation of *EHD2* gene, which encodes a member of the EH domain-containing protein family. EHD2 protein has an N-terminal domain that interacts with the actin cytoskeleton and a C-terminal EH domain that binds to an EH domain-binding protein[64]. This interaction appears to link clathrin-dependent endocytosis and actin, implying that this gene is involved in the endocytic pathway, particularly clathrin-dependent endocytosis[65]. These findings suggest that clathrin-dependent endocytosis was one of the major uptake mechanisms of *C. roseus*-AgNPs while ruling out the involvement of possible involvement of caveolin-dependant endocytosis.

Another interesting finding in this study is the potential involvement of macropinocytosis, attributable to the upregulation of *RhoA* gene macropinocytosis. This gene encodes a member of the Rho family of small GTPases, which regulates macropinocytosis *via* active and inactive GTP-binding while simultaneously playing an important role in the remodelling of the actin skeleton during macropinocytosis[66,67]. According to Patel *et al*[67], after the macropinocytic cups closed to form macropinosomes, the expression of another Rho subtype, *RhoA*, increased significantly. This corroborates our findings in this study on the *RhoA* gene upregulation, which suggests that *C. roseus*-AgNPs uptake also could occur *via* macropinocytosis. The overexpression of *EEA1* in this study indicated that the formation of early endosomes occurs during the uptake of *C. roseus*-AgNPs. This finding is consistent with prior work, which demonstrated the high frequency of the *EEA1* gene in early endosomes that are the primary sorting station in the endocytic pathway[68]. On the other hand, *TSG101* and *VPS28* genes are involved in late endosomal trafficking[69]; the upregulation of *TSG101* and *VPS28* genes in this study suggests that late endosome was formed during the *C. roseus*-AgNPs uptake. This finding was in line with a previous study, where the expression of TSG101 and VPS28 was found to be increased[70]. The upregulation of the stress-responsive genes as mentioned previously in our study is also indicative of the successful uptake of *C. roseus*-AgNPs into the HepG2 cells, as the generation of free radicals that induced stress in the cells can be caused by the leaching of Ag^+ from AgNPs into the cytosol, because of high acidic lysosome rupture. *TFRC* gene encodes a cell surface receptor necessary for cellular uptake by the process of receptor-mediated endocytosis[71]. The downregulation of the *TFRC* gene in this study suggests that the expression of the gene was induced to reduce endocytosis *via* negative feedback regulation as a response to cellular homeostasis. Our finding also is in tandem with the findings of Wang *et al*[72], who have also noticed a drop in the expression of the *TFRC* gene, which could be due to negative feedback for defensive actions.

The uptake of C. roseus-AgNPs arrested cell cycle

Cancer progression is associated with aberrancy in the cell cycle, such as the anomalous expression of CDKs[73]. CDKs are usually highly expressed, causing the uncontrolled proliferation of cancer cells [74]. Upon the treatment of the HepG2 cells with *C. roseus*-AgNPs, *CDK4, and CDK2* were found to be downregulated, implying the antagonistic effect of the nanoparticles against the cell cycle protein. A previous study reported that the blockage of Go/G1 was accompanied by the downregulation of the cell cycle regulators *CDK4* and *CDK2*[75]. In this study, the downregulation of *CDK4* and *CDK2* suggested that *C. roseus*-AgNPs was arrested at Go/G1. Another important observation is the upregulation of *GADD45A*, which caused a decrease in the *SKP2* expression. Overexpression of *SKP2* is associated with the cell cycle progression and as the *SKP2* expression was found to be reduced in the present study, it is surmised that the cell proliferation is being forestalled. Moreover, the reduced expression of *SKP2* is also associated with the increased expression of *CDKN1A*, which is an inhibitor of cell cycle progression by inhibiting the activity of cyclin-dependent kinase expression[76]. The upregulation of *CDKN1A* corroborates the anticancer activities of *C. roseus*-AgNPs that can induce cell cycle arrest. *MCM3* is a member of minichromosome maintenance family that is associated with tumour invasiveness[77]. The treatment of the *C. roseus*-AgNPs caused the downregulation of *MCM3,* in HepG2 cells, ratifying the anticancer activities of *C. roseus*-AgNPs in alleviating tumour aggressiveness.

The overall proposed mechanism as depicted in Figure 6, consists of clathrin-dependent and clathrin-independent endocytosis. The signalling pathways indicate the involvement of the up and down-regulated genes in various cellular organelles. The understanding of cellular and molecular mechanisms would provide a strong justification of the rationale of *C. roseus* G. Don-AgNPs as anticancer compounds for liver cancer therapy.



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Figure 6 Model of cytotoxicity mechanism in HepG2 cell treated with *C. roseus*-AgNPs that involves the upregulated genes (in red) and downregulated genes (in green).

CONCLUSION

In this study, the treatment of HepG2 cells with *C. roseus*-AgNPs has resulted in the increase of the expression of tumour suppressor genes, apoptotic genes, and activation of signal transduction pathway such as mitogen-activated protein kinase (MAPK) signalling pathway, endocytosis signalling pathway, TNF signalling pathway, TGF-Beta signalling pathway as well as cell cycle arrest. Collectively, the findings from our study have demonstrated the anti-cancer properties of *C. roseus*-AgNPs, with insignificant effects on normal cells. The therapeutic property of the *C. roseus* G. Don-AgNPs should be further explored in the future as part of the endeavours to surrogate or complement the current conventional chemotherapeutic-based intervention.

ARTICLE HIGHLIGHTS

Research background

Conventional chemotherapy and radiotherapies based on x-ray and gamma-ray radiations are the most widespread techniques in the world for the treatment of malignant diseases due to their ability to penetrate tissues and thus allow them to reach deep sites. The only limitation of these treatments is the lack of selectivity between the tumour and the healthy surrounding tissues. Interestingly, previous studies have shown that silver nanoparticles (AgNPs) have the ability to selectively induce cytotoxic effects on cancer cells, as compared to normal cells. Therefore, the present study aims to evaluate the cytotoxic effects of AgNPs synthesised by *C. roseus* aqueous extract against liver carcinoma cells HepG2 and normal liver cells THLE-3, by assessing the proliferative activity followed by the mRNA transcriptome profiling analysis.

Research motivation

Due to the limitations of the conventional treatment like non-specificity and less effectiveness, novel strategies are in demand to solve these issues. Amongst all, the use of plant-synthesised silver nanoparticles has gained attention as they are known for non-toxic properties, are cost-effective, are easily assessable and environmentally friendly. The unique properties of nano-sized nanoparticles have been reported can penetrate cancer cells effectively. In this study, the anticancer activity was evaluated

at both cellular and molecular levels to gain insight into its mechanisms.

Research objectives

To evaluate the proliferative activity of the human hepatocellular carcinoma cells HepG2 in response to the *Catharanthus roseus*-silver nanoparticles (*C. roseus*-AgNPs), in comparison to the normal liver cells THLE-3 cells.

Research methods

To evaluate the proliferative activity, the hepatocellular carcinoma cells HepG2 and normal human liver cells THLE3 were treated with standardised *Catharanthus roseus*-silver nanoparticles (*C. roseus*AgNPs) in a double dilution manner and analysed using MTT assay. To elucidate the gene expression study, the RNA samples were extracted and sequenced using BGIseq500 platform. This is followed by data filtering, mapping, gene expression analysis, DEGs analysis, GO analysis, and pathway analysis.

Research results

The proliferative activity revealed selective effects, indicating that the *Catharanthus roseus*-silver nanoparticles were cytotoxic on hepatocellular carcinoma cells HepG2 cells but not on the normal liver cells THLE3 cells. The transcriptome analysis has resulted in the regulation of 296 protein-coding genes, of which 182 genes were upregulated while 114 genes were downregulated. The most intriguing finding is the expression of tumour suppressor gene GADD45A, responsible for the regulation of DNA repair, cell cycle control and genotoxic stress. The expression of this gene is regulated by p53. The upregulated GADD45A was supported by the downregulated MDM2, which is the negative regulator for p53. Our findings revealed the activation of several signalling pathways including the mitogen-activated protein kinase signalling pathway, TNF signalling pathway and TGF-β signalling pathway. These pathways are the main regulator in fundamental intracellular activities such as apoptosis, cell cycle and cellular growth. The upregulation of *ARF6, EHD2, FGFR3, RhoA, EEA1, VPS28, VPS25* and *TSG101* indicated that the *C. roseus*-AgNPs were taken up by HepG2 cells *via* both clathrin-dependent and clathrin-independent.

Research conclusions

The selective proliferative activity between cancerous and normal liver cells indicates a promising potential of *Catharanthus roseus*-silver nanoparticles (*C. roseus*-AgNPs) as an effective anticancer agent. The understanding of the molecular signalling pathways induced by the genes associated with oxidative stress, apoptosis and cell cycle arrest provides the novelty towards the development and establishment of *C. roseus*-AgNPs as an anticancer drug for hepatocellular carcinoma. Moreover, we propose that the uptake was *via* both clathrin-dependent and clathrin-independent endocytosis. These findings would explain the cytotoxicity mechanisms of the *C. roseus*-AgNPs at cellular and molecular level towards hepatocellular carcinoma cells HepG2.

Research perspectives

While the endocytic pathways emphasise the action of the selectively permeable plasma membrane on the nanomaterials, cytotoxicity of silver nanoparticles (AgNPs) generally involves the cells' downstream activity, including reactive oxygen species (ROS)- dependent pathway, cell cycle arrest and genotoxicity. Moreover, the small-sized AgNPs can easily penetrate the cells and bind to macromolecules including proteins and DNA, either directly or indirectly although the exact mechanism for this interaction has not been clarified. The physicochemical characteristics that make AgNPs so useful can be the main reason they might be dangerous to cells, and at a higher level to human health. Therefore, to avoid these problems, the AgNPs must be engineered from either biocompatible, nontoxic, biodegradable material or materials have with minimal toxic effects.

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FOOTNOTES

Author contributions: Ahmad NH contributed to the methodology; Ahmad NH contributed to the validation; Azhar NA contributed to the conceptualisation; Azhar NA and Abu Bakar SA contributed to the formal analysis; Azhar NA contributed to the investigation; Ahmad NH contributed to the resources; Azhar NA, Abu Bakar SA, Citartan M and

Ahmad NH contributed to the data curation; Azhar NA contributed to original draft preparation; Ahmad NH and Citartan M contributed to the review and editing; Ahmad NH and Citartan M contributed to the supervision; Ahmad NH contributed to project administration; Ahmad NH contributed to funding acquisition; all authors have read and agreed to the published version of the manuscript.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Adherence to guideline-directed hepatocellular carcinoma screening: A single-center US experience

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Abstract

BACKGROUND

The American Association for the Study of Liver Disease recommends screening patients with cirrhosis for hepatocellular carcinoma (HCC) using imaging with or without alpha-fetoprotein every six months. Unfortunately, screening rates remain inadequate.

AIM

To assess root causes of screening failure in a subspecialty hepatology clinic.

METHODS

The authors identified patients with cirrhosis seen in a subspecialty hepatology clinic and determined whether they underwent appropriate screening, defined as two cross-sectional images between five and seven months apart. The authors characterized the primary driver of screening failure. Finally, other hepatologists were surveyed to determine provider perceptions of screening failure causes.

RESULTS

1034 patients were identified with an average age of 61 years and a mean MELD of 8.1 ± 3.8. Hepatitis C virus was the most common cirrhosis etiology. 489 (47%) underwent appropriate screening. No demographic or clinical differences were detected between those who underwent appropriate screening and those who did

not. The most common etiologies of screening failure, in descending order, were: radiology unable to schedule timely imaging, provider did not order imaging, patient canceled follow up appointment, appointments scheduled too far apart, lost to follow up, no-show to radiology appointment, and provider canceled appointment. Hepatologists surveyed believed the most common cause of screening failure was no-show to radiology.

CONCLUSION
Rates of screening were poor even in a subspecialty hepatology clinic. Screening failure was mostly due to systemic factors such as radiology availability and time between hepatology appointments rather than individual error.

Key Words: Hepatocellular carcinoma; Cirrhosis; Health maintenance; Quality improvement; Screening; Hepatology

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Core Tip: This study reinforces existing knowledge that screening rates for Hepatocellular carcinoma are woefully inadequate, even in a subspecialty hepatology clinic. Unlike previous studies, ours identifies specific failure points, showing that screening failures are driven more by systemic issues than by physician or patient error.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents the sixth leading cause of cancer and the third leading cause of cancer death worldwide[1]. The most common and important risk factor for HCC is cirrhosis [2]. Estimates of the annual incidence of HCC among patients with cirrhosis range from 1 to 8%[3,4]. The lifetime incidence in patients with cirrhosis may be as high as 32% and is increasing in the United States[3,5-7].

The American Association for the Study of Liver Disease (AASLD) recommends screening for HCC with abdominal ultrasound, computed tomography, or magnetic resonance imaging, with or without alpha-fetoprotein, every six months[8-10]. Adherence to AASLD guidelines correlates with improved survival, as demonstrated in a French cohort study of 1671 patients at 35 centers. Patients who adhered to semi-annual screening protocols had increased lead-time adjusted survival[11]. A theoretical model by Sarasin *et al*[12] predicted an increase in life expectancy among patients with Child-Turcot-Pugh A cirrhosis with HCC screening if the expected incidence of HCC is at least 1.5% per year. Unfortunately, predictive algorithms to stratify patients by HCC risk have failed external clinical validation[13]. Much research now focuses on blood-based biomarkers for simple and accessible point of care screening, but these strategies are not yet ready for clinical practice[14].

Unfortunately, adherence to screening guidelines remains poor[15-17]. A 2011 retrospective cohort study of 13002 patients with cirrhosis across 128 Veterans Affairs medical centers showed that only 12% had received appropriate screening[18]. A 2012 systematic review by Singal *et al*[15] found the surveillance rate among all patients with cirrhosis to be only 18.4%, although it was higher (51.7% *vs* 16.9%) among patients followed in subspecialty gastroenterology clinics. A subsequent retrospective cohort study performed by the same group found that only 2% of patients received consistent surveillance; 33% had inconsistent surveillance, and 65% had no surveillance over 3 years[19]. A qualitative study within the Veterans Health Administration similarly found that following with a subspecialist, whether gastroenterology or infectious disease, significantly increased HCC screening rates[20]. Poor knowledge and vigilance of screening protocols among primary care providers has been well-documented[21,22]. Other factors included distance to a screening site and lead time between screening order and screening date[20]. Socioeconomic factors also contribute to screening utilization [23,24]. Primary care-based clinical reminders have also been shown to improve screening rates[25]. Singal *et al*[26] showed that a mailed outreach program increases HCC screening rates.

Many previous studies examined patients diagnosed with HCC to identify factors related to lack of screening[18,27,28]. Our group sought to collect data on all patients at a subspecialty hepatology clinic to retrospectively identify risk factors for screening failure among all patients with cirrhosis, not just those with HCC. We hypothesized that there may be additional factors not previously identified that contribute to screening failure.

The purpose of this study was two-fold: (1) To determine the rate of appropriate HCC screening in patients with cirrhosis in a subspecialty practice in which screening guidelines are well known; and (2) to identify barriers at an institutional and provider level as well as the patient-related factors. The data will be used to improve adherence to guideline-directed screening protocols *via* future quality improvement initiatives.

MATERIALS AND METHODS

The electronic medical record was queried for billing codes from the 9th revision of the International Classification of Diseases (ICD-9) or ICD-10 to identify patients. Demographic, disease etiology, and laboratory data were collected. Inclusion criteria included patients with cirrhosis who were seen at least twice in the subspecialty hepatology clinic between August 2015 and August 2017. The charts were then manually reviewed to confirm that each patient was appropriate for screening based on AASLD guidelines. Exclusion criteria included prior liver transplantation and prior HCC.

Next, the authors determined whether the patients had been appropriately screened, defined as having undergone two imaging studies (abdominal ultrasonography, contrasted computed tomography, or magnetic resonance imaging) within 150 to 210 days of each other during the study period. Because the AASLD guidelines suggested an optional role for -fetoprotein, the authors did not look for -fetoprotein measurement. The charts of these patients were reviewed to determine the primary cause of screening failure. The reason for failure was categorized based on the screening barriers listed below. For patients with multifactorial screening failure, the first failed step in the screening process was counted as the primary reason for failure. For example, if a patient canceled a hepatology appointment and subsequently did not receive orders for imaging, the reason for screening failure was attributed to the clinic cancellation. The hierarchy of steps, in order, were: loss to follow-up, patient clinic appointment cancellation, physician clinic appointment cancellation, appointments more than 7 mo apart, failure to order imaging, failure to schedule imaging, or failure to present to radiology.

Finally, eight hepatologists in the clinic who were not involved in this study were anonymously surveyed on their perceptions of risk factors for screening failure.

Statistical significance was defined using $\alpha < 0.05$. Continuous variables were abnormally distributed according to Shapiro-Wilk testing. Therefore, comparisons were made using the Mann-Whitney *U* test. Categorical variables were compared using chi-square or Fisher's exact testing. The study protocol was reviewed and approved by our institutional IRB prior to any data collection and study procedures.

RESULTS

The authors identified 1276 patients who met the inclusion criteria. 242 were removed due to meeting exclusion criteria. Therefore, a total of 1034 patients were analyzed. The study population had an average age of 61 years, was 55% male, and was 83% White. Hepatitis C virus was the most common cirrhosis etiology, accounting for 51% of participants. The mean MELD score was 8.1 (SD 3.8). No statistically significant differences were detected in baseline characteristics between patients who underwent appropriate screening and those who did not (Table 1).

489 (47%) patients underwent appropriate screening during the study period. 410 (40%) underwent two imaging studies that were outside the time range criterion. Six percent of patients had only one imaging study, and 7% had none (Figure 1). The most common cause of HCC screening failure was delays in scheduling of imaging studies (Figure 2). Patient-centered factors, including appointment cancellations, no-shows, and loss to follow up accounted for 36% of screening failures. System failures were classified as delays in radiology and hepatology scheduling as well as physician cancellation of follow-up appointments. These accounted for 40% of screening failures. Lack of physician order accounted for 21%.

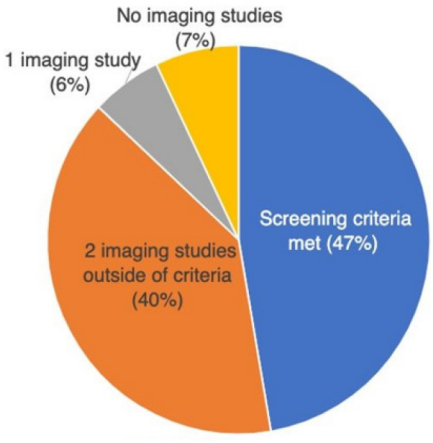
All of those who received their care exclusively within the public university medical system were referred to the radiology department within the institution. 35 patients who followed with community-based gastroenterologists and came to the institution for periodic subspecialty consultation elected to undergo HCC screening with local private radiologists.

All patients diagnosed with HCC experienced delays in screening. One was diagnosed at stage IVb and passed away due to HCC. One was lost to follow-up following discovery of a 3.1 cm nodule on an magnetic resonance imaging protocoled for liver masses. Two underwent Y-90 transarterial radioembolization and partial surgical hepatectomy. One of these patients ultimately elected to transition to hospice and passed away due to worsening hepatic decompensation; the other is still alive.

Table 1 Baseline patient characteristics, *n* (%)

Baseline patient characteristic	Met screening criteria (*n* = 463)	Failed screening criteria (*n* = 545)	*P* value
Age	61.4 ± 10.7	60.2 ± 10.5	0.06
Gender			0.37
Male	261 (56)	292 (54)	
Female	202 (44)	253 (46)	
Race			0.85
White	385 (83)	452 (83)	
African-American	48 (10)	55 (10)	
Other	20 (4)	30 (6)	
Unknown	5 (1)	5 (1)	
County of residence			0.61
Same county as institution	115 (25)	143 (26)	
Different county than institution	348 (75)	402 (74)	
Etiology			0.64
NASH	144 (31)	157 (29)	
AIH	21 (5)	25 (5)	
PBC	34 (7)	37 (7)	
PSC	19 (4)	13 (2)	
HCV	229 (49)	283 (52)	
HBV	29 (6)	37 (7)	
AALD	44 (10)	63 (12)	
MELD	8.2 ± 3.8	8.1 ± 3.8	0.65

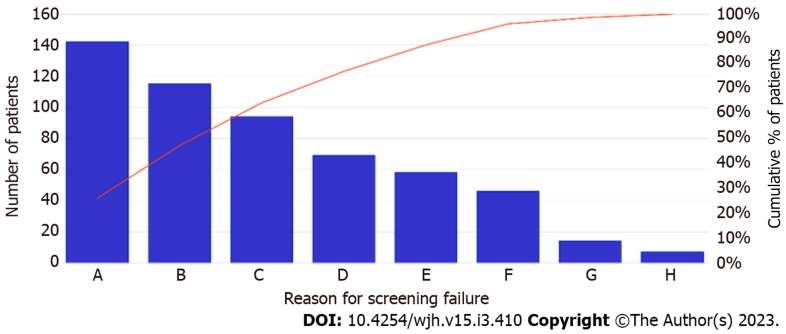
NASH: Non-alcoholic steatohepatitis; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AALD: Alcohol-associated liver disease.



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Figure 1 Rates of appropriate hepatocellular carcinoma screening in a sub-specialty hepatology clinic. HCC: Hepatocellular carcinoma.

In a poll, other hepatologists at the same institution believed the most common causes of screening failure, in order, to be: failure to present to radiology, patient clinic appointment cancellation, loss to follow up, and failure to order imaging. Human error and deferral to primary care provider (PCP) were the most cited reasons for failure to order screening.



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Figure 2 Pareto chart of root cause of screening failures. A: Radiology unable to schedule timely imaging; B: Physician/Provider did not order imaging; C: Patient canceled hepatology follow up appointment; D: Hepatology follow up appointments scheduled too far apart; E: Patient lost to follow up; F: Patient no show to radiology appointment; G-H: Physician/Provider canceled hepatology follow up appointment.

DISCUSSION

Despite guidelines that were well-known to the providers in the subspecialty hepatology practice, fewer than half of the patients in our cohort underwent appropriate screening during the study period. The findings are consistent with previous studies and add to the growing evidence that HCC screening rates are grossly insufficient.

However, our study illuminates some nuances in the reasons for screening failure. Most screening failures in our cohort were institutional rather than patient-driven or secondary to physician oversight. We were able to investigate what happened after the order was placed for screening to evaluate the system factors that contribute. Radiology scheduling failure, whether from inability to contact the patient or unavailability of timely imaging appointments, was the primary reason for lack of adherence. The multiple failure points both highlight the complexity of care coordination for cirrhosis patients in a subspecialty clinic and offer targets for intervention and improvement.

Failure to order screening was the second leading risk factor among subspecialty hepatologists in this cohort. Other investigators have demonstrated poor knowledge of screening protocols among primary care providers (PCPs), which can explain lack of adherence to guidelines. However, we do not believe a knowledge deficit was a major contributing factor in a subspecialty clinic. Many hepatologists cited deferral to PCP as a reason for not ordering screening, even though knowledge among PCPs remains poor. The authors also speculate that a busy, often overbooked clinic with competing priorities makes even the most diligent hepatologists forget to order screening. It is difficult to order abdominal imaging while counseling a patient that they will die from cirrhosis unless they overcome innumerable psychosocial barriers to abstain from alcohol for long enough to become a liver transplant candidate.

This study has several important limitations. Firstly, the window for "appropriate screening" in this study was 5 to 7 mo, which is narrower than the 4–8-mo window suggested by the AASLD, resulting in a positive bias toward ineffective screening. Secondly, patients who had two imaging studies 6 mo apart were considered "appropriately screened," regardless of whether a third imaging study was completed on time. This data simplification may have resulted in an overestimation of the screening rate. Thirdly, the attribution of screening failure to a single step fails to capture the multifactorial nature of screening failure. For example, a patient for whom radiology did not schedule an imaging study because the physician did not order one because they missed their clinic appointment would be classified as "no show," even though the provider could have ordered the screening even without the patient there. Finally, the logistical complexity of the screening process leaves room for interpretation variation between multiple investigators, even with rigorous standardization.

The debate over the proper length of screening is likely to continue, with many authors pointing out that longer intervals have not been studied. Some experts, including the National Cancer Institute, have opined that hepatologists ought to abandon screening protocols entirely due to a lack of survival benefit [29-32]. Furthermore, the World Gastroenterology Organization suggests that screening in low and middle-resource settings is appropriate only if the patient would have access to HCC treatments[33]. However, we contend that every effort be made to adhere to current practice guidelines when resources are available. Our findings demonstrate the need for future measures to address system and provider level improvements. We have implemented an automatic reminder in the electronic medical record for physicians and other healthcare professionals and targeted reminders *via* main or electronic media for patients. In addition, our findings highlight the need for serum biomarkers for HCC screening, which would eliminate the logistical delays with radiology[34].

CONCLUSION

In conclusion, the rate of appropriate HCC screening, though above the estimated national average, was inadequate in this patient population. The reasons for failure were multifactorial, but the primary driver was delays in radiology scheduling. These data immediately identify targets for future quality improvement initiatives.

ARTICLE HIGHLIGHTS

Research background

The American Association for the Study of Liver Disease recommends that patients with cirrhosis be screened for hepatocellular carcinoma (HCC) every six months. Other researchers have shown that adherence to these guidelines is poor, but little is known about the causes of this failure.

Research motivation

The authors noted that many patients in their own subspecialty hepatology practice did not undergo appropriate screening. They studied factors contributing to screening failure in order to develop a possible quality improvement initiative.

Research objectives

The authors sought to identify root causes of HCC screening failure among patients with cirrhosis in their subspecialty heaptology clinic.

Research methods

The authors identified patients with cirrhosis in their subspecialty hepatology clinic and determined whether they underwent appropriate screening. The authors reviewed the medical records of patients who did not undergo appropriate screening to identify the root causes of screening failure.

Research results

Among 1034 patients, only 489 underwent appropriate screening. The most common causes of screening failure, in descending order, were: radiology unable to schedule timely imaging, provider did not order imaging, patient canceled follow up appointment, appointments scheduled too far apart, lost to follow up, no-show to radiology appointment, and provider canceled appointment.

Research conclusions

Even in a subspecialty hepatology clinic in which providers strive to follow guideline-based HCC screening, rates of screening were still poor. Most of the barriers to appropriate screening were due to systemic factors such as radiology availability, rather than to individual error.

Research perspectives

HCC screening is vital to the comprehensive care of patients with cirrhosis, yet systemic and institutional barriers often prevent patients from receiving adequate care. The root causes identified in this article immediately suggest areas for possible quality improvement and provide guidance to those at other institutions.

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

To scan or not to scan: Use of transient elastography in an integrated health system

Libby Stein, Rasham Mittal, Hubert Song, Joanie Chung, Amandeep Sahota

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Abstract

BACKGROUND

Non-invasive tests, such as Fibrosis-4 index and transient elastography (commonly FibroScan), are utilized in clinical pathways to risk stratify and diagnose non-alcoholic fatty liver disease (NAFLD). In 2018, a clinical decision support tool (CDST) was implemented to guide primary care providers (PCPs) on use of FibroScan for NAFLD.

AIM

To analyze how this CDST impacted health care utilization and patient outcomes.

METHODS

We performed a retrospective review of adults who had FibroScan for NAFLD indication from January 2015 to December 2017 (pre-CDST) or January 2018 to December 2020 (post-CDST). Outcomes included FibroScan result, laboratory tests, imaging studies, specialty referral, patient morbidity and mortality.

RESULTS

We identified 958 patients who had FibroScan, 115 before and 843 after the CDST was implemented. The percentage of FibroScans ordered by PCPs increased from 33% to 67.1%. The percentage of patients diagnosed with early F1 fibrosis, on a scale from F0 to F4, increased from 7.8% to 14.2%. Those diagnosed with advanced F4 fibrosis decreased from 28.7% to 16.5%. There were fewer laboratory tests, imaging studies and biopsy after the CDST was implemented. Though there were more specialty referrals placed after the CDST was implemented, multi-

variate analysis revealed that healthcare utilization aligned with fibrosis score, whereby patients with more advanced disease had more referrals. Very few patients were hospitalized or died.

CONCLUSION
This CDST empowered PCPs to diagnose and manage patients with NAFLD with appropriate allocation of care towards patients with more advanced disease.

Key Words: Non-alcoholic fatty liver disease; Transient elastography; FibroScan; Clinical decision support tool; Health care utilization; Primary care

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Core Tip: This was a retrospective study of nearly 1000 patients with non-alcoholic fatty liver disease who underwent FibroScan. The purpose of this study was to compare patients before and after a clinical decision support tool was implemented. This tool was designed to guide primary care providers on the management of non-alcoholic fatty liver disease. After the tool was released, we saw higher rates of early-stage fibrosis diagnosed by FibroScan. We saw appropriate allocation of care, whereby patients with advanced fibrosis had more labs, imaging studies and specialty referrals. These results suggest non-alcoholic fatty liver disease can feasibly be diagnosed and managed in the primary care setting.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease worldwide, affecting over 25% of the population[1]. In the United States alone, this translates to over 80 million individuals[2]. In its early stage, NAFLD. is reversible. However, disease progression results in irreversible fibrosis and cirrhosis and portends significant risk of hepatocellular carcinoma.

Historically, liver biopsy was the gold standard for diagnosis of NAFLD[3]. However, advancements in non-invasive testing are beginning to change the standard, with safer, cost-effective[4,5], accurate[6] and readily accessible modalities[7,8] that can be utilized in the primary care setting[9]. In the United States, the most common modality is transient elastography, often delivered by the FibroScan device (Echosens, Paris, France).

The evolution of non-invasive tests, like FibroScan, has enabled clinical pathways by which primary care physicians (PCPs) can identify patients with liver disease prior to utilization of specialty services [10,11]. Recently, professional societies[12,13] have started to embrace these diagnostic tools and clinical pathways in their recommendations. However, very few retrospective[14] and prospective[15,16] studies have assessed the effectiveness of these pathways in clinical practice. To date, no singular study has assessed management, appropriateness of care and patient outcomes for NAFLD patients who have undergone FibroScan.

In 2018, a clinical decision support tool (CDST) for NAFLD was implemented in Kaiser Permanente Los Angeles Medical Center (KPLAMC), a tertiary care center in Southern California. The goals of this CDST were to: (1) Educate and guide PCPs in identifying patients with NAFLD; (2) Risk-stratify patients *via* non-invasive tests; and (3) Triage patients based on risk, whereby lower risk patients were educated about lifestyle modification and higher risk patients were offered specialty referral for advanced care. We sought to determine the impact of this CDST on health care utilization, practice patterns and patient outcomes.

MATERIALS AND METHODS

Clinical pathway

This study was centered around a CDST, part of a user-facing app, called Aura, on the electronic health record (EHR). Aura-based CDSTs populate patient clinical data to allow clinicians to calculate scores and receive recommendations. This CDST was based on the Fib-4 index, a validated calculator to predict liver fibrosis and cirrhosis[17]. If the score was below 1.3, the recommendation included lifestyle

counseling and repeating the score in 3 years. If the score met a threshold of 1.3, a FibroScan was recommended. If the score was above 3.25, FibroScan and specialty referral to gastroenterology and hepatology was recommended (Figure 1).

Study population

The primary population included persons ≥ 18 years who underwent FibroScan for NAFLD indication at KPLAMC from January 1, 2015 to December 31, 2020. KPLAMC is the tertiary referral center for Kaiser Permanente Southern California (KPSC), the largest integrated health system in the state of California. KPLAMC cares for over 275000 adult members, representing about 16% of the population [18].

Study design and data source

A retrospective study. The cohort was identified using an internal database of patients for whom FibroScan was performed. The population was stratified by time of FibroScan, either before (pre-CDST) or after (post-CDST) introduction of the CDST to clinical workflow. Patients were excluded from analysis if pregnant within 1 year of FibroScan. Patients with incomplete data were also excluded (Figure 2).

Data were gathered and extracted from this cohort *via* the KPSC Health Connect Database using International Classification of Diseases and Current Procedural Terminology codes (Supplementary Tables 1 and 2). Certain variables were confirmed by manual chart review. The KPSC Institutional Review Board approved the study.

Outcomes

The primary outcome was health care utilization - who underwent FibroScan and what was the result of the scan. Variables included age, sex, body mass index (BMI), race, insurance type and medical comorbidities such as concomitant chronic liver diseases and risk factors for metabolic syndrome. FibroScan results included fibrosis score, steatosis score, probe type used and category of physician who ordered the scan, either primary or specialty care.

The secondary outcomes included clinical management, hospitalization rate and mortality within one year of FibroScan. Clinical management was subdivided into three categories - laboratory tests, imaging studies, biopsy and specialty referral. Laboratory tests included liver function test, international normalized ratio, creatinine and complete blood count. Imaging studies included computerized tomography (CT)-4 phase liver, magnetic resonance imaging (MRI) liver, right upper quadrant ultrasound and repeat FibroScan. Specialty referral included gastroenterology, hepatology and health education, for services like diet and weight loss. Primary hospital admission diagnoses included hepatic encephalopathy, variceal bleeding, spontaneous bacterial peritonitis and liver cancer (Supplementary Table 2). Deceased patients who died within the first year after FibroScan were captured and cause of death was identified.

Statistical analysis

Statistical significance was calculated by chi-square and Kruskal-Wallis for categorical and continuous variables, respectively. All *P*-values were determined to be significant if they were below the 0.05 threshold.

Subgroup analysis included a multivariable logistic regression to quantify the relationship between clinical management - laboratory tests, imaging studies and specialty referrals - and fibrosis score. The multicollinearity and variance inflation factor were checked and determined to be negligible. For multivariate logistic regression, the p-value was calculated by the Wald Test, with multicollinearity between variables checked with high correlation of 0.8, tolerance below 0.1 and variance inflation factor of above 10. All analyses were done using SAS 9.4 and SAS Enterprise Guide 7.15 (SAS Institute, Cary, NC, United States).

RESULTS

Patient characteristics

We identified 958 patients who underwent FibroScan from January 1, 2015 to December 31, 2020. Of these, 115 patients had FibroScan from January 1, 2015 to December 31, 2017 (pre-CDST) and 843 patients had FibroScan from January 1, 2018 to December 31, 2020 (post-CDST). Patient demographics and clinical characteristics are represented in Tables 1 and 2.

In the pre-CDST cohort, mean age was 58.3 ± 13.78 years with over half (53.9%) being female. Mean BMI was 31.6 ± 6.13. The majority racial group was Hispanic (47.8%), followed by non-Hispanic White (25.2%) and Asian (19.1%). Most patients had commercial health insurance (64.3%) while many others had Medicare (24.3%). Patients carried comorbid diagnoses of diabetes mellitus (45.2%), hyperlipidemia (52.2%) and obstructive sleep apnea (14.7%). Very few patients had comorbid liver diseases. The post-CDST cohort had statistically similar data to the pre-CDST cohort with one exception. Mean BMI in the

Table 1 Patient demographics

Characteristic	Pre-clinical decision support tool (*n* = 115)	Post-clinical decision support tool (*n* = 843)	*P* value
Age, year	58.3 ± 13.78	57.1 ± 14.02	0.3777
Female (%)	53.9	53.3	0.8956
Body mass index	31.6 ± 6.13	33.1 ± 7.10	0.0358
Race (%)			0.4486
African American	4.3	3.3	
Asian	19.1	17.4	
Hispanic	47.8	56.8	
Non-Hispanic White	25.2	19.9	
Other, unknown	3.5	2.5	
Insurance plan type (%)			0.1312
Commercial, private pay	64.3	64.7	
Dual	3.5	5.9	
Medicaid	6.1	4.5	
Medicare	24.3	24.7	
Other, unknown	1.7	0.2	
Medical Comorbidities (%)			
Chronic hepatitis B	2.6	1.8	0.5415
Chronic hepatitis C	4.3	1.3	0.0172
Diabetes mellitus	45.2	42.4	0.5572
Hepatocellular carcinoma	0	0	
Hyperlipidemia	52.2	58.6	0.1891
Liver transplant	0	0.4	0.5212
Obstructive sleep apnea	14.7	13.3	0.6662
Polycystic ovarian syndrome	1.7	0.5	0.1076
Primary biliary cholangitis	0.9	0.4	0.4242
Primary sclerosing cholangitis	0	0	

Data are expressed as mean ± SD or *n* (%).

post-CDST cohort was 33.1 ± 7.1 (P = 0.0358).

FibroScan data

In the pre-CDST cohort, 33% of FibroScans were ordered by PCPs. In the post-CDST cohort, 67.1% of FibroScans were ordered by PCPs. In both cohorts, a little over half (55.7%-56%) of probes used during FibroScan were XL.

Regarding FibroScan results, 9 patients, representing 7.8% of the pre-CDST cohort, had low grade F1 fibrosis. In the post-CDST cohort, this increased to 120 patients with F1 fibrosis, representing 14.2% (P = 0.0142). Additionally, 33 patients in the pre-CDST cohort had advanced F4 fibrosis, representing 28.7%. This decreased to 16.5%, a total of 139 patients, in the post-CDST cohort with F4 fibrosis (P = 0.0142). The percentage of patients with advanced steatosis S3 increased from 43.5% in the pre-CDST cohort to 67.3% in the post-CDST cohort ($P \leq 0.0001$).

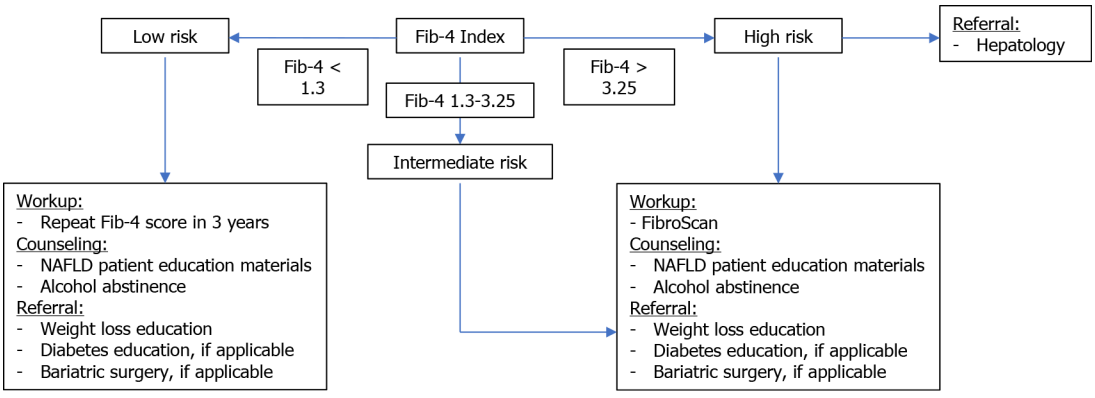
Clinical management

Laboratory tests: In the pre-CDST cohort, an average of 7.2 tests were performed *per* patient in the first year after FibroScan. This significantly decreased to 5.3 Laboratory tests in the post-CDST cohort ($P < 0.0001$). When subdivided by type of test, this significant decrease remained true (Figure 3A).

Table 2 FibroScan data

Parameter	Pre-clinical decision support tool (*n* = 115)	Post-clinical decision support tool (*n* = 843)	*P* value
Physician ordering FibroScan (%)			< 0.0001
Primary care	33	67.1	
Specialty care	67	32.9	
Exam probe used (%)			0.9453
Medium	44.3	44	
Extra large (XL)	55.7	56	
FibroScan result (%)			
Fibrosis score			0.0142
F0	32.2	38.1	
F1	7.8	14.2	
F2	17.4	17.9	
F3	13.9	13.3	
F4	28.7	16.5	
Steatosis score			< 0.0001
S0	43.5	8.1	
S1	4.3	10	
S2	8.7	14.7	
S3	43.5	67.3	

Data are expressed as *n* (%).

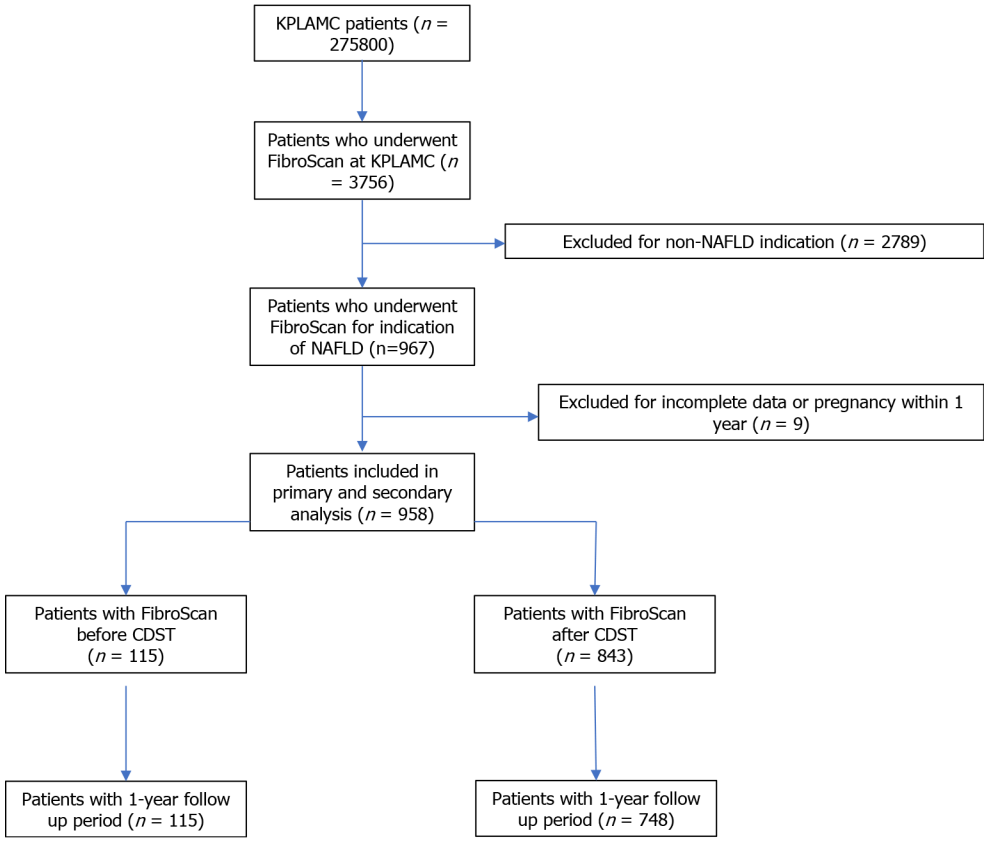


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Figure 1 Clinical decision support tool workflow. Fib-4: Fibrosis-4; NAFLD: Non-alcoholic fatty liver disease.

Imaging studies: The percentage of patients who had an MRI 4-phase liver decreased from 13.9% in the pre-CDST cohort to 12.8% in the post-CDST cohort (P = 0.7486). The percentage of patients who had an MRI liver decreased from 16.5% in the pre-CDST cohort to 10.6% in the post-CDST cohort (P = 0.0607). The percentage of patients who had a right upper quadrant ultrasound decreased significantly from 30.4% in the pre-CDST cohort to 20.7% in the post-CDST cohort (P = 0.0193). The percentage of patients who had a repeat FibroScan increased from 1.7% in the pre-CDST cohort to 2.7% in the post-CDST cohort (P = 0.5538, Figure 3B).

Biopsy: In the pre-CDST cohort, 8.7% of patients had liver biopsy within the first year. This decreased significantly to 2.7% in the post-CDST cohort (P = 0.001). The average number of months to biopsy was similar in both cohorts, 3.9 mo in the pre-CDST cohort *vs* 3.5 mo in the post-CDST cohort (P = 0.9822, Figure 3B).



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Figure 2 Cohort flow chart. KPLAMC: Kaiser Permanente Los Angeles Medical Center; NAFLD: Non-alcoholic fatty liver disease; CDST: Clinical decision support tool.

Of those who were referred for liver biopsy, 82.8% of patients had fibrosis scores of F3 or F4 from FibroScan. Of the biopsies done, 76.9% resulted in fibrosis scores that agreed with the patient's FibroScan result. The remaining 23.1% were discordant to the FibroScan result. In all the discordant biopsy results, Fibroscan overestimated the fibrosis score from the biopsy pathology.

Specialty referral: The percentage of patients for whom gastroenterology referral was placed increased from 5.2% in the pre-CDST cohort to 7.5% in the post-CDST cohort (P = 0.3803). The percentage of patients for whom hepatology referral was placed increased significantly from 2.6% in the pre-CDST cohort to 12.8% in the post-CDST cohort (P = 0.0014). The percentage of patients for whom health education referrals were placed increased significantly from 21.7% in the pre-CDST cohort to 35.2% in the post-CDST cohort (P = 0.0045, Figure 3B).

Patient outcomes

Morbidity: In the pre-CDST cohort, no patients were hospitalized for complications of liver disease in the first year. In the pre-CDST cohort at any time in the study time frame, 4 patients were hospitalized for hepatic encephalopathy, 1 patient was hospitalized for variceal bleeding and 1 patient was hospitalized for spontaneous bacterial peritonitis.

In the post-CDST cohort, 1 patient was hospitalized for hepatic encephalopathy and 2 patients were hospitalized for liver cancer in the first year. In the post-CDST cohort at any time in the study time frame, 4 patients were hospitalized for hepatic encephalopathy and 5 patients were hospitalized for liver cancer (Table 3).

Mortality: In the pre-CDST cohort, 1 patient died in the first year. In the pre-CDST cohort at any time, 9 patients died. In the post-CDST cohort, 7 patients died in the first year. In the post-CDST cohort at any time, 17 patients died. No patients died of complications of liver disease. Cause of death was primarily cardiovascular or complications of coronavirus disease 19 (COVID-19) (Table 3).

Multivariable analysis: The likelihood of healthcare utilization across all categories - laboratory tests, imaging studies and specialty referrals - increased with advancing fibrosis, most prominent in F4 fibrosis (Table 4). The reference group for this analysis was F0 fibrosis patients, unless otherwise specified.

Table 3 Patient morbidity and mortality

Variable	Pre-clinical decision support tool	Post-clinical decision support tool
Patients hospitalized in first year for:		
Hepatic encephalopathy	0	1
Variceal bleeding	0	0
Spontaneous bacterial peritonitis	0	0
Liver cancer	0	2
Patients hospitalized at anytime for:		
Hepatic encephalopathy	4	4
Variceal bleeding	1	0
Spontaneous bacterial peritonitis	1	0
Liver cancer	0	5
Patients deceased in first year	1	7
Patients deceased at any time	9	17

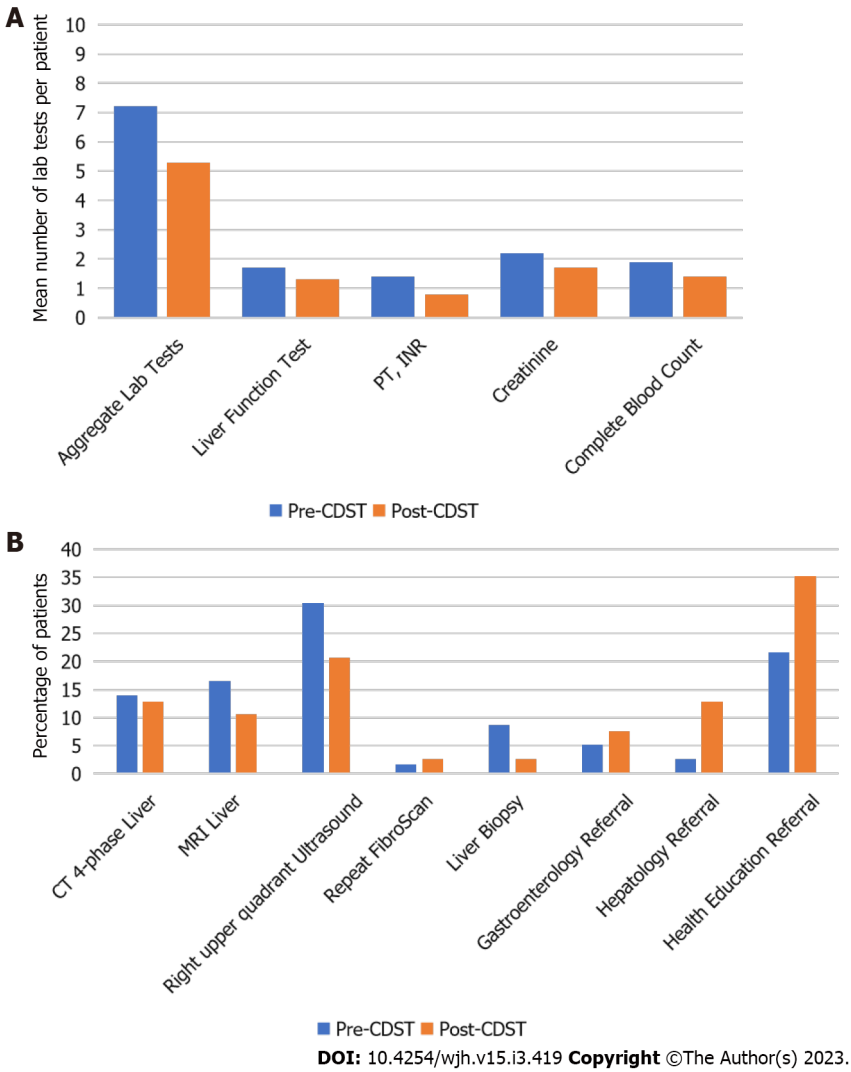
Data are expressed as raw numbers.

Table 4 Multivariable analysis

Variable	Fibrosis score	Odds ratio	95% confidence interval	*P* value
Lab tests	F1 *vs* F0	0.955	0.621-1.469	0.8354
	F2 *vs* F0	1.055	0.711-1.566	0.7886
	F3 *vs* F0	1.507	0.946-2.4	0.0845
	F4 *vs* F0	2.477	1.522-3.953	0.0001
Imaging study	F1 *vs* F0	0.825	0.506-1.343	0.4386
	F2 *vs* F0	1.287	0.855-1.937	0.2259
	F3 *vs* F0	4.703	3.064-7.218	< 0.0001
	F4 *vs* F0	7.188	4.793-10.78	< 0.0001
Gastroenterology referral	F1 *vs* F0	2.362	0.909-6.141	0.0778
	F2 *vs* F0	1.47	0.549-3.939	0.4431
	F3 *vs* F0	6.195	2.786-13.775	< 0.0001
	F4 *vs* F0	4.122	1.85-9.14	0.0005
Hepatology referral	F1 *vs* F2	0.181	0.04-0.813	0.0258
	F3 *vs* F2	4.438	2.253-8.739	< 0.0001
	F4 *vs* F2	4.55	2.385-8.681	< 0.0001
Health education referral	F1 *vs* F0	1.415	0.882-2.272	0.1501
	F2 *vs* F0	1.463	0.957-2.236	0.0786
	F3 *vs* F0	2.054	1.305-3.233	0.0019
	F4 *vs* F0	3.589	2.391-5.387	< 0.0001

Those with F3 fibrosis were 1.507 times as likely to have a laboratory test than those with F0 fibrosis ($P = 0.0845$). Those with F4 fibrosis were 2.477 times as likely to have a laboratory test than those with F0 fibrosis ($P = 0.0001$).

Those with F3 fibrosis were 4.703 times as likely to have an imaging study than those with F0 fibrosis ($P < 0.0001$). Those with F4 fibrosis were 7.188 times as likely to have an imaging study than those with F0 fibrosis ($P < 0.0001$).



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Figure 3 Clinical management in first year following FibroScan. A: Laboratory tests; B: Imaging studies and specialty referral. CDST: Clinical decision support tool; CT: Computerized tomography; MRI: Magnetic resonance imaging.

Those with F3 fibrosis were 6.195 times as likely to have a gastroenterology referral than those with F0 fibrosis ($P < 0.0001$). Those with F4 fibrosis were 4.122 times as likely to have a gastroenterology referral than those with F0 fibrosis ($P = 0.0005$).

Due to low numbers, comparisons for hepatology referrals were made with F2 fibrosis patients rather than F0 or F1 fibrosis patients. Those with F3 fibrosis were 4.438 times as likely to have a hepatology referral than those with F2 fibrosis ($P < 0.0001$). Those with F4 fibrosis were 4.55 times as likely to have a hepatology referral than those with F2 fibrosis ($P < 0.0001$).

Those with F3 fibrosis were 2.054 times as likely to have a health education referral than those with F0 fibrosis ($P = 0.0019$). Those with F4 fibrosis were 3.589 times as likely to have a health education referral than those with F0 fibrosis ($P < 0.0001$).

DISCUSSION

This study examined the demographics, clinical management, morbidity and mortality of a cohort of patients with NAFLD who underwent FibroScan, a non-invasive test to diagnose liver fibrosis. This study was centered around a CDST designed to guide PCPs in the care of patients with NAFLD. We compared patients before and after the CDST was implemented to determine its impact on health care utilization, practice patterns and patient outcomes.

The CDST pathway, combining Fib-4 and FibroScan, was chosen in particular because of robust clinical data supporting its use in the NAFLD population. When compared head-to-head with other scoring systems, Fib-4 has a high negative predictive value[19], making it an ideal rule out test in detecting advanced fibrosis and cirrhosis[20,21]. Furthermore, FibroScan has a high positive predictive value for the measurement of liver stiffness[22], to rule in advanced fibrosis and cirrhosis, and thus risk-

stratify patients. When Fib-4 and FibroScan are used in tandem, it is predicted that 87% of unnecessary further assessments may be avoided[23].

Our data revealed three important findings. First is regarding FibroScan orders. Prior to the CDST, about two-thirds of all FibroScans were ordered by specialty providers. Additionally, the overall number of scans ordered by any provider during that time was low. This indicates either poor understanding of the test's presence or low level of confidence in the test itself. After the CDST, not only did the overall number of scans increase 7-fold, but also, the majority of scans - about two-thirds - were ordered by PCPs. This drastic shift shows that the CDST achieved its goal of educating PCPS on the utility of FibroScan and fostered a new confidence in the test, leading to higher rates of utilization.

As such, the average BMI of patients in the post-CDST cohort was statistically significantly higher than those in the pre-CDST cohort. We attribute this difference to provider education regarding risk factors for NAFLD. When the CDST was implemented, PCPs were alerted of its presence and provided educational materials in the form of EHR alerts, informational emails and formal lectures. Since obesity is a known risk factor for NAFLD, it is likely that PCPs thought to screen patients with higher BMIs.

The second important finding is regarding fibrosis score. In the pre-CDST cohort, fibrosis scores had a bi-modal distribution. About half of the patients either had no fibrosis (F0) or had advanced fibrosis (F4). Conversely, the post-CDST cohort contained almost half the number of patients with advanced fibrosis (F4) and also twice the number of patients with early fibrosis (F1). This change shows that the CDST captured patients earlier in the disease process. As we know, while early fibrosis is reversible, advanced fibrosis and cirrhosis is not. Early recognition and diagnosis are crucial.

The third important finding is regarding care utilization. In aggregate, the utilization rates of laboratory tests, imaging studies and biopsy decreased with the introduction of the CDST. In particular, there was no significant difference in gastroenterology referral for patients with early fibrosis (F0-F1). Furthermore, patients with advanced fibrosis (F3-F4) had more tests and studies done and more referrals placed. This not only represents appropriate allocation and utilization of care, but also may serve to quell providers' worries that identification of NAFLD patients may lead to unnecessary testing, in particular endoscopies for variceal surveillance[24].

Regarding strengths and weaknesses, this study cohort is robust and diverse and can reasonably be extrapolated to the national population. To date, no singular study of a clinical pathway has assessed management, appropriateness of care and patient outcomes in the NAFLD population. Unfortunately, the study period included the COVID-19 pandemic, which is known to have resulted in decreased rates of care utilization and delivery[25].

CONCLUSION

This study is of particular importance. PCPs see more than 300 cases of NAFLD for every 1000 patient encounters[26]. The average annual cost of care *per* NAFLD patient with private health insurance in the United States is $7804 for a new diagnosis and $3789 for long-term management[27]. Not only is NAFLD independently associated with 17% higher annual attributable healthcare costs, but also more advanced disease, F3 and above, is associated with a 40% increase in median annual healthcare cost when compared to F2 and below[28]. The lion share of this increase in cost can be attributed to liver biopsy, imaging and hospitalizations[27].

Not only is the prevalence of NAFLD and NASH projected to increase by up to 56% in the next 10 years[29], but also high primary care workload and physician burnout[30] necessitates action and education. Early and accurate diagnosis of fibrosis in NAFLD patients, particularly those with advanced disease, is necessary to determine the patient's prognosis and guide clinical decision making.

Workflows such as this CDST can not only help patients attain adequate, appropriate, preventative care, but also can help streamline primary care clinical practice and empower physicians beyond the liver clinic to appropriately recognize and manage high risk NAFLD. Future directions for this work include longitudinal study of this population and clinical workflow in multiple centers on a national and international scale.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) is a growing problem, affecting over 25% of the global population. Non-invasive tests are being used more and more to risk stratify and diagnose patients with NAFLD. However, there is a paucity of data for how these tests are being used for clinical decision making in real-world practice.

Research motivation

We examined a clinical decision support tool (CDST) designed to guide primary care providers (PCPs)

in the care of patients with NAFLD.

Research objectives

To evaluate health care utilization, practice patterns and patient outcomes of patients who underwent Fib-roScan for NAFLD indication.

Research methods

A retrospective review of 958 adult patients who underwent FibroScan. Patients were compared before and after introduction of the CDST. Univariate and multivariate logistic regression models were performed in statistical analyses.

Research results

Introduction of the CDST allowed for more patients with early fibrosis and fewer patients with advanced fibrosis to be identified. Overall, fewer labs, imaging studies and biopsies were ordered after the CDST. Providers appropriately ordered more specialty referrals for patients with more advanced fibrosis.

Research conclusions

This CDST empowered PCPs to diagnose and manage patients with NAFLD with appropriate allocation of care towards patients with more advanced disease.

Research perspectives

Non-alcoholic fatty liver disease can feasibly be diagnosed and managed in the primary care setting. Future research is required to streamline and refine care of this patient population.

FOOTNOTES

Author contributions: Stein L, Mittal R and Sahota A designed the study; Song H and Chung J gathered the data and performed the statistical analysis; Stein L wrote the manuscript; all authors read, edited and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Kaiser Permanente Southern California Institutional Review Board (Approval No. #12674).

Informed consent statement: Because of the nature of a retrospective study, signed informed consent form is not needed. However, Kaiser Permanente Los Angeles Medical Center has given permission to conduct this study.

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

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Retrospective Study

Coexistent alcohol-related cirrhosis and chronic pancreatitis have a comparable phenotype to either disease alone: A comparative retrospective analysis

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Abstract

BACKGROUND

Alcohol use disorder is a prevalent disease in the United States. It is a well-demonstrated cause of recurrent and long-standing liver and pancreatic injury which can lead to alcohol-related liver cirrhosis (ALC) and chronic pancreatitis (ACP). ALC and ACP are associated with significant healthcare utilization, cost burden, and mortality. The prevalence of coexistent disease (CD) ranges widely in the literature and the intersection between ALC and ACP is inconsistently characterized. As such, the clinical profile of coexistent ALC and ACP remains poorly understood. We hypothesized that patients with CD have a worse phenotype when compared to single organ disease.

AIM

To compare the clinical profile and outcomes of patients with CD from those with ALC or ACP Only.

METHODS

In this retrospective comparative analysis, we reviewed international classification of disease 9/10 codes and electronic health records of adult patients with verified ALC Only (n = 135), ACP Only (n = 87), and CD (n = 133) who received

care at UPMC Presbyterian-Shadyside Hospital. ALC was defined by histology, imaging or clinical evidence of cirrhosis or hepatic decompensation. ACP was defined by imaging findings of pancreatic calcifications, moderate-severe pancreatic duct dilatation, irregularity or atrophy. We compared demographics, pertinent clinical variables, healthcare utilization, and mortality for patients with CD with those who had single organ disease.

RESULTS

Compared to CD or ACP Only, patients with ALC Only were more likely to be older, Caucasian, have higher body mass index, and Hepatitis B or C infection. CD patients (*vs* ALC Only) were less likely to have imaging evidence of cirrhosis and portal hypertension despite possessing similar MELD-Na and Child C scores at the most recent contact. CD patients (*vs* ACP Only) were less likely to have acute or recurrent acute pancreatitis, diabetes mellitus, insulin use, oral pancreatic enzyme therapy, and need for endoscopic therapy or pancreatic surgery. The number of hospitalizations in patients with CD were similar to ACP Only but significantly higher than ALC Only. The overall mortality in patients with CD was similar to ALC Only but trended to be higher than ACP Only ($P = 0.10$).

CONCLUSION

CD does not have a worse phenotype compared with single organ disease. The dominant phenotype in CD is similar to ALC Only which should be the focus in longitudinal follow-up.

Key Words: Alcohol; Cirrhosis; Chronic pancreatitis; Overlap; Phenotype

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Core Tip: Patients with coexistent alcohol-related cirrhosis and alcohol-related chronic pancreatitis do not have a worse phenotype when compared with single organ disease patients. The dominant phenotype in patients with coexistent disease (CD) in terms of overall survival and markers of advanced liver disease was similar to patients with Alcohol-related Cirrhosis Only. Coexistent disease patients also had lower prevalence of disease-related manifestations when compared with those who had single organ disease. Patients with CD may not need to be monitored at a higher degree, but the primary focus for longitudinal follow-up should be on alcohol-related cirrhosis.

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INTRODUCTION

Alcohol use disorder (AUD) is a disease affecting over 14 million adults in the United States[1]. Long-standing alcohol use is a well-established cause of liver and pancreatic injury that can culminate in alcohol-related liver cirrhosis (ALC) and alcohol-related chronic pancreatitis (ACP)[2,3]. The complications of ALC and ACP are major causes of morbidity and mortality associated with alcohol misuse[4-6].

The liver and pancreas are developmentally related and share a number of functional similarities; they also exhibit common features of alcohol-induced injury. The quantity of alcohol misuse is the primary risk factor for developing both diseases and leads to the metabolic stress and low-grade inflammation that stimulates maladaptive fibrotic changes[7]. Susceptibility for developing ALC and/or ACP also relates to non-modifiable risk factors such as race, genetics, and environment[8-11]. ALC-related complications range from ascites and portosystemic encephalopathy to hepatorenal syndrome and hepatocellular carcinoma, and it is estimated that alcohol use accounts for 20%-36% of cirrhosis cases [12-14]. The rate of cirrhosis-related hospitalizations and annual costs have been increasing[15,16]. Comparably, the long-standing inflammatory state in chronic pancreatitis (CP) results in irreversible parenchymal destruction and dysfunction. ACP often begins with an index acute pancreatitis event that progresses to CP as dictated by the severity and number of recurrent episodes of acute pancreatitis[17]. Commonly attributed to alcohol consumption in the North American population, complications from CP include chronic pain, exocrine/endocrine insufficiency, and pancreatic adenocarcinoma[18-20] and poor quality of life[21].

Although ALC and ACP have been well-studied in isolation, patients with overlap of ALC and ACP (*i.e.*, Coexistent Disease) is inconsistently characterized in the literature. Some studies have failed to demonstrate any association between ALC and ACP[22,23] while others suggest interconnectivity between alcohol-related liver and pancreas disease. For instance, alcohol-related liver disease can lead to pancreatic exocrine insufficiency and accumulation of fatty acid ethyl esters which contributes to further progression of alcohol-related liver[24] and pancreas disease[25], while ACP can cause and exacerbate portal hypertension which worsens the complications of liver disease[26]. Furthermore, emerging data from the United States in recent years suggests that coexistent disease (CD) represent only a small fraction of patients with AUD. Although estimates of prevalence of CD in the literature range widely from 0%-75%, a meta-analysis performed by our group revealed a pooled prevalence of ACP in ALC and ALC in ACP to be 16.2% and 21.5% respectively[27].

To date, published studies have yet to define the clinical profile of patients with CD and its differences from single-organ disease. We hypothesized that patients with CD will have a more advanced phenotype and worse outcomes when compared with patients who have single organ (ALC Only or ACP Only) disease. To test this hypothesis, we performed a detailed comparative analysis of well-characterized patients with ACP Only, ALC Only, and CD who received care in a large healthcare system cohort.

MATERIALS AND METHODS

Study population

The study was approved by the University of Pittsburgh's Institutional Review Board. The patient pool consisted of those who were aged ≥ 18 years, had one or more inpatient, emergency room, and outpatient encounters at any UPMC facility from January 1, 2006 to December 31, 2017 with international classifications of diseases (ICD) versions 9 and/or 10 codes for AUD, alcohol-related liver disease or pancreatitis (Supplementary material), had 12 or more months of contact with the UPMC system, and received care at UPMC Presbyterian-Shadyside campus at some time during their care at UPMC[28]. Among these patients, we randomly identified a subset who received a diagnosis of ALC Only (n = 202), ACP Only (n = 200) and both ALC and ACP (n = 200). Unlike ALC for which etiology-specific codes are routinely used in clinical practice, ICD-9 classification for pancreatitis did not include etiology-specific codes, which became available with the ICD-10 coding system. In our dataset, as only a small portion of patients received an ICD-10 diagnosis of ACP, we identified patients as ACP by the diagnosis of AUD at any time in addition to CP, as was described previously[28].

Analysis and review of the Electronic Health Records of the 602 randomly identified patients was performed by 2 authors (ML, YS) under the supervision of the senior author using pre-defined criteria to verify the diagnosis of cirrhosis and CP. Cirrhosis was defined by histologic findings, imaging evidence of cirrhosis or portal hypertension, or clinical signs of hepatic decompensation. CP was defined by imaging findings of pancreatic calcifications, moderate-severe pancreatic ductal dilation, pancreatic ductal stricture or gland atrophy. To ensure that patients with ALC Only did not have any clinical pancreatic disease, we excluded patients with a verified diagnosis of ALC who had prior acute or recurrent acute pancreatitis. Similarly, among patients with verified ACP Only, we excluded those who had prior alcohol-related hepatitis. Patients with a verified diagnosis of ALC Only, ACP Only and both ALC and ACP (CD) formed the study population.

Data collection

For each patient with a verified diagnosis, we reviewed the Electronic Health Records to retrieve detailed information on demographics, alcohol and tobacco use, pertinent clinical information for ALC and ACP, healthcare utilization and overall survival until March 3, 2021. Information relevant to liver disease included details of verification criteria fulfilled, clinical features of portal hypertension, hepatic decompensation, history of alcohol-related hepatitis, Child-Pugh and MELD scores, need for liver transplantation, and treatments received. For CP, in addition to the verification criteria fulfilled, information was collected on clinical features of CP, laboratory tests, dual-energy X-ray absorptiometry (DEXA) scan results, and treatments for CP or its complications.

Analytic approach and statistical analyses

We report demographic and disease-specific information for each of the three groups. Continuous variables are presented as mean ± standard deviation or median (interquartile range), and categorical variables were reported as n (%). Statistical comparisons were made using t-test and Kruskal-Wallis test for continuous variables and chi-square tests for categorical variables. Survival from time of first diagnosis is reported using the Kaplan-Meier method. Cox proportional-hazards models are used to report the hazard ratio (HR) and 95% confidence intervals (CI) for patients with ALC Only *vs* ACP Only and CD *vs* ACP Only while adjusting for age at diagnosis, sex, and race. All statistical analyses were performed using R, version 4.1.3 by biomedical statisticians (RF, AA).

RESULTS

Study population and demographics

The final study population consisted of 355 patients with verified diagnosis - 135 with ALC Only, 87 with ACP Only, and 133 with CD. Select characteristics of these patients are presented in Table 1. When compared with CD, patients with ALC Only were older at the time of study entry, had higher body mass index, were more likely to be Caucasian and more likely to have Hepatitis B and C infections. While roughly one-thirds of patients with CD or ALC Only were female, only 23% of ACP patients were female. The median duration of contact was greater than 10 years and was comparable between groups. The median number of non-elective hospital admissions for CD and ACP Only were comparable and significantly greater than patients with ALC Only. During follow-up, the number of patients who died in the CD, ALC Only, and ACP Only group was 80 (60%), 82 (61%), and 36 (41%), respectively. Survival analysis using Cox-regression after controlling for age, sex and race (Figure 1) demonstrated that the survival between ALC Only and ACP Only was similar (HR 1.22, 95%CI 0.82-1.82, $P = 0.32$), while there is a trend towards lower survival in patients with CD when compared to ACP Only (HR 1.40, 95%CI 0.94-2.09, $P = 0.10$).

Comparisons between CD vs ALC Only

Select disease-specific characteristics of patients with CD and ALC Only are shown in Table 2. Patients with ALC Only underwent liver biopsy more often than those with Coexistent disease (33.3% *vs* 16.5%, $P = 0.002$). Patients with ALC Only were more likely to have radiographic evidence of cirrhosis (93% *vs* 76%, $P \leq 0.001$) and portal hypertension (74% *vs* 59%, $P = 0.006$) on imaging. Although MELD and Child-Pugh scores at most recent contact were similar among patients with CD and ALC Only, some specific clinical features differed between the two groups. Specifically, while patients with CD were more likely to have a history of spontaneous bacterial peritonitis, those with ALC Only were more likely to have esophageal varices, need for variceal banding, treatment with beta blockers, and hepatocellular carcinoma. Other features of decompensated liver disease (*e.g.*, ascites) or treatments (*e.g.*, TIPS) were similar between the two groups.

Comparisons between CD and ACP Only

Morphologic appearance of the pancreas was generally similar among patients with CD and ACP Only (Table 3). In regards to the clinical manifestations, patients with ACP Only were more likely to have a history of acute or recurrent acute pancreatitis, receive pancreatic enzyme replacement therapy, ERCP, and pancreatic surgery than patients with CD. Patients with ACP Only were also more likely to have endocrine dysfunction, as characterized by a higher prevalence of diabetes, need for insulin therapy, and poor glycemic control as reflected by a higher hemoglobin A1c level at the time of last contact. Other clinical features or therapies were similar between the two groups.

DISCUSSION

As the largest study of its kind, this work endeavors to further characterize patients at the intersection of ALC and ACP. Our retrospective analysis of patients with a verified diagnosis of ALC Only, ACP Only or CD reveals that during a similar period of observation, although patients with CD had differences in some disease-related manifestations, they did not have worse phenotype than counterparts with single organ disease. Furthermore, our findings suggest that patients with CD potentially need not be monitored at a higher degree, but the primary focus should be on the management of ALC.

Patients included in this study represent the most severe phenotypes of alcohol-related liver or pancreas disease who received care at a tertiary care center during the course of their illness. Among them, we observed that the dominant phenotype in patients with CD to be similar to that of ALC, specifically the two most important indicators of outcomes (*i.e.* overall survival and MELD-Na and Child C scores in patients with CD were similar to patients with ALC Only). This suggests that patients with alcohol-related pancreatic disease who are identified to have alcohol-related liver disease need to be assessed and monitored for early identification of cirrhosis or cirrhosis-related complications so they can be managed in a timely manner.

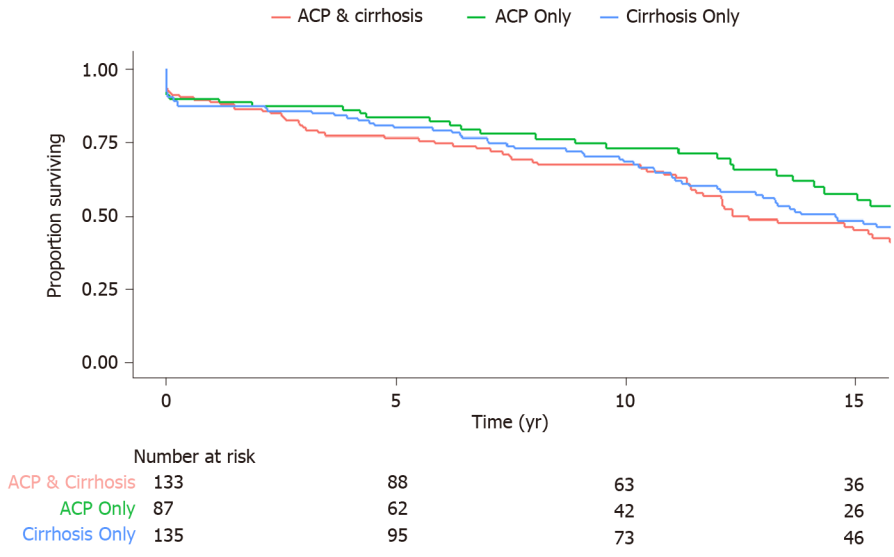
Patients with CD shared similar demographic attributes with those of single organ disease such as the sex distribution of ALC Only patients as well as age, racial distribution and body mass index (BMI) of ACP Only patients. Of note, although our prior study showed that the prevalence of alcohol-related pancreatic disease in those with alcohol-related liver disease was 2-4 folds higher in blacks compared to other races[28], the racial difference was not present in this study. This may be related to the inclusion of patients with the most severe phenotypes in this study as noted above, which may not be representative of the full spectrum of alcohol-related liver and pancreas disease.

When comparing patients with CD with those who had single organ disease, we observed some demographic differences. For instance, patients with CD were younger than those with ALC Only but similar to patients with ACP Only. Although our retrospective study was not designed to evaluate this

Table 1 Select demographics and characteristics in the study population, *n* (%)

	CD (*n* = 133)	ALC only (*n* = 135)	ACP only (*n* = 87)	*P* value (CD *vs* ALC only)	*P* valve (CD *vs* ACP only)
Age (at study entry), yr – mean ± SD	51.7 ± 12.0	54.6 ± 9.8	51.0 ± 12.3	**0.029**	0.684
Female	49 (38)	42 (31)	20 (23)	0.322	**0.03**
Race				**0.015**	0.52
Caucasian	97 (73)	113 (84)	61 (70)		
Black	30 (23)	22 (16)	24 (28)		
Other	6 (5)	0 (0)	2 (2)		
Body mass index[a] – mean ± SD	24.2 ± 7.0	27.8 ± 6.5	23.3 ± 5.4	**< 0.001**	0.281
Tobacco use	117 (88)	109 (81)	81 (93)	0.104	0.127
Smoking (one or more packs per day	31 (23)	23 (17)	24 (28)	0.29	0.392
Alcohol use (duration), yr – mean ± SD	26.7 ± 16.0	29.4 ± 13.8	23 ± 18.4	0.595	0.762
Hepatitis B Infection	7 (5)	17 (13)	2 (2)	**0.036**	0.278
Hepatitis C Infection	31 (23)	58 (43)	14 (16)	**0.001**	0.194
Non-Elective Hospital Admissions[b] – median (IQR)	4 (1 - 12)	3 (0 - 7)	4 (1 - 8)	**0.007**	0.57
Duration of observation, yr – mean ± SD	10.8 ± 7.9	12.4 ± 7.6	11.8 ± 7.6	0.107	0.36

[a]At most recent contact.
[b]Compared using Kruskal-Wallis test. ACP: Alcohol-related chronic pancreatitis; ALC: alcohol-related liver cirrhosis; CD: Coexistent disease; IQR: Interquartile range.



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Figure 1 Survival analysis for the three clinical groups from time of first diagnosis to the last contact. ACP: Alcohol-related chronic pancreatitis.

systematically, a potential explanation is an earlier identification of CP based on clinical symptoms and/or imaging studies in patients with alcohol-related liver disease. Similarly, patients with CD had BMI similar to ACP but lower than patients with ALC likely related to malabsorption. The alternative explanation in a subset of patients with ALC may be fluid retention related to portal hypertension.

Other than spontaneous bacterial peritonitis, patients with CD in general had a lower burden of disease-related manifestations when compared with patients who had ALC Only and ACP Only. The reason for this is unclear but a possible explanation may be the recognition of disease overlap at an earlier stage, *e.g.* alcohol-related liver disease in patients with ACP or alcohol-related pancreatitis in patients with ALC. In terms of healthcare utilization, the burden of non-elective admissions in patients

Table 2 Select disease-specific characteristics in patients with coexistent disease *vs* alcohol-related liver cirrhosis only, *n* (%)

Characteristics present	CD (*n* = 133)	ALC only (*n* = 135)	*P* value
Verification criteria fulfilled			
Liver biopsy performed	22 (17)	45 (33)	**0.002**
Cirrhosis on biopsy	13 (59)	22 (49)	0.349
Cirrhosis on imaging[b]	101 (76)	126 (93)	**< 0.001**
Portal hypertension features on imaging	78 (59)	100 (74)	**0.006**
Alcohol-Related hepatitis	48 (36)	34 (25)	0.052
MELD score[a]	19.3 ± 8.98	18.7 ± 8.89	0.614
Child-Pugh score[a]			0.69
A	41 (31)	48 (36)	
B	49 (39)	48 (36)	
C	43 (32)	39 (29)	
Complications of portal hypertension			
Esophageal varices on EGD	46 (35)	68 (50)	**< 0.001**
Esophageal variceal hemorrhage	11 (8)	17 (13)	0.247
Ascites	96 (72)	83 (62)	0.063
Spontaneous bacterial peritonitis	22 (17)	9 (7)	**0.011**
Portosystemic encephalopathy	62 (47)	68 (50)	0.539
Hepatocellular carcinoma	6 (5)	23 (17)	**0.001**
End-stage renal disease requiring CRRT/HD	15 (11)	13 (10)	0.644
Treatment of portal hypertension/complications			
Esophageal variceal banding	11 (8)	22 (16)	**0.046**
TIPS	3 (2)	8 (6)	0.13
Beta-blocker use[a]	18 (14)	38 (28)	**0.004**
Diuretic use[a]	57 (43)	65 (48)	0.415
Large volume paracentesis	29 (22)	39 (29)	0.183
Antibiotics for SBP prophylaxis[a]	9 (7)	11 (8)	0.68
Lactulose and/or rifaximin use[a]	50 (38)	55 (41)	0.632
Transplant evaluation	14 (11)	19 (14)	0.402
Liver transplantation	3 (2)	10 (7)	**0.05**

[a]At most recent contact.

[b]Ultrasound, computed tomography scan magnetic resonance imaging, elastography. ALC: Alcohol-related liver cirrhosis; CD: Coexistent disease; CRRT: Continuous renal replacement therapy; HD: Homeodomain; EGD: Esophagogastroduodenoscopy; SBP: Spontaneous bacterial peritonitis.

with CD mirrored those of ACP Only patients.

Strengths of our study include the largest sample size to evaluate the phenotype of patients with CD, rigorous review of medical records to verify diagnosis and data collection by review of medical records and a long observation period which ensures capture of clinical events. Our study also has limitations. Being a retrospective study from a single-center tertiary academic medical center may have resulted in our study population to be of higher complexity and limit generalizability of our findings. Our study population includes patients with concomitant Hepatitis B and C infections. While the prevalence of these infections rates represent the traits of our underlying clinical population, hepatitis B and C infections may attribute to or confound the severity of hepatic disease. Although our review of records within the UPMC system was complimented by availability of medical records from other institutions whenever possible through Care Everywhere, there is a possibility of underestimation of clinical events. Finally, clinical events and demographics have the potential to be misclassified in the dataset due to missing or incomplete information.

Table 3 Select disease-specific characteristics in patients with coexistent disease *vs* alcohol-related chronic pancreatitis only, *n* (%)

Characteristics present	CD (*n* = 133)	Only ACP (*n* = 87)	*P* value
Verification criteria fulfilled on imaging			
Pancreatic calcifications	88 (66)	64 (74)	0.246
Moderate-severe ductal dilatation	38 (29)	30 (35)	0.354
Moderate-severe ductal structure	18 (14)	14 (16)	0.599
Any gland atrophy	77 (58)	46 (53)	0.463
Moderate-severe gland atrophy	10 (13)	7 (15)	0.88
Gland atrophy not reported	67 (87)	37 (80)	0.25
Diagnosis based on EUS alone	6 (5)	5 (6)	0.681
Chronic pancreatitis features			
Acute pancreatitis	101 (76)	74 (85)	**0.009**
Age at first pancreatitis, yr – mean ± SD	48.1 ± 15.2	41.5 ± 10.6	0.112
Recurrent acute pancreatitis	61 (46)	53 (61)	**0.023**
Chronic abdominal pain[a]	56 (42)	44 (51)	0.189
Pancreatic pseudocyst	29 (22)	22 (25)	0.549
Diabetes mellitus	54 (41)	50 (58)	**0.011**
Exocrine pancreatic insufficiency (Fecal elastase < 100 and/or steatorrhea)	24 (18)	14 (16)	0.708
Pancreatic adenocarcinoma	2 (2)	4 (5)	0.163
Treatment of chronic pancreatitis/complications			
Oral anti-diabetic therapy[a]	11 (20)	13 (26)	0.113
Insulin therapy[a]	46 (85)	37 (74)	0.209
Pancreatic enzymatic replacement therapy[a]	35 (26)	39 (45)	**0.004**
Chronic opiate therapy[a]	59 (44)	33 (38)	0.381
Treatment by chronic pain specialist	20 (15)	20 (23)	0.124
Celiac plexus block	0 (0)	2 (2)	0.077
ERCP	41 (31)	43 (49)	**0.004**
Pseudocyst drainage (endoscopic/surgical)	18 (14)	13 (15)	0.743
Pancreatic surgery	13 (10)	19 (22)	**0.012**
Pertinent test results			
Hemoglobin A1C[a] – mean ± SD	6.4 ± 2.3	7.3 ± 2.1	**0.01**
Vitamin D deficiency	48 (36)	25 (29)	0.104
DEXA scan performed	28 (21)	19 (22)	0.855
Osteopenia on DEXA scan	12 (43)	10 (53)	0.51
Osteopenia on DEXA scan	8 (29)	5 (26)	0.865

[a]At most recent contact. ACP: Alcohol-related chronic pancreatitis; CD: Coexistent disease; ERCP: Endoscopic retrograde cholangiopancreatography; DEXA: Dual-energy-xray-absorptiometry.

CONCLUSION

Contrary to our working hypothesis, patients with Coexistent ALC and ACP did not have a worse phenotype when compared with single organ disease patients. The dominant phenotype in patients with CD in terms of overall survival and markers of advanced liver disease was similar to patients with ALC Only. CD patients also had lower prevalence of disease-related manifestations when compared with those who had single organ disease. Our findings suggest that patients with CD may not need to be monitored at a higher degree, but the primary focus for longitudinal follow-up should be on ALC.

ARTICLE HIGHLIGHTS

Research background

Heavy alcohol use is a known cause of liver and pancreatic injury that can lead to alcohol-related liver cirrhosis (ALC) and alcohol-related chronic pancreatitis (ACP). These diseases are associated with significant morbidity, mortality, and healthcare utilization and spending.

Research motivation

While both ALC and ACP are well-characterized, there is a subset of patient with both ALC and ACP (coexistent disease) that is poorly understood.

Research objectives

We aim to characterize the clinical profile of patients with coexistent disease (CD) and its differences from those with ALC Only or ACP Only.

Research methods

The study population consisted of adult patient encounters at UPMC facilities from 2006 to 2017 with more than 12 mo of contact. We identified subsets of patients with ACP Only, ALC Only, and CD based on international classifications of diseases codes and reviewed the Electronic Health Record to verify diagnoses and abstract clinical information. Statistical comparisons were made using t-test and Kruskal-Wallis test for continuous variables and chi-square tests for categorical variables. Survival from time of first diagnosis is reported using the Kaplan-Meier method. Cox proportional-hazards models are used to report the hazard ratio and 95% confidence intervals while adjusting for age at diagnosis, sex, and race.

Research results

The median duration of contact was greater than 10 years and was comparable between groups. The median number of non-elective hospital admissions for CD and ACP Only were comparable and significantly greater than patients with ALC Only. The number of patients who died in follow-up in CD, ALC Only, and ACP Only groups was 80 (60%), 82 (61%), and 36 (41%). Using Cox regression, survival was similar between ALC Only *vs* ACP Only and CD *vs* ACP Only. Despite comparable MELD-Na and Child-Pugh scores between CD and ALC Only patients, those with ALC Only were more likely to have esophageal varices, need for variceal banding, treatment with beta blockers, and hepatocellular carcinoma. Patients with ACP Only were more likely to have acute pancreatitis, need for endoscopic or surgical intervention, and endocrine dysfunction.

Research conclusions

Patients with CD did not have a worse phenotype compared to patients with ACP Only or ALC Only.

Research perspectives

As the largest study of its kind, this work hopes to characterize patients at the intersection of ALC and ACP. Given our findings, we observed that the dominant phenotype in CD is similar to that of ALC Only, suggesting that patients with alcohol-related pancreatic disease who are newly identified to have alcohol-related liver disease should be closely monitored for liver cirrhosis and its complications.

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REVIEW

COVID-19 and liver injury: Pathophysiology, risk factors, outcome and management in special populations

Romina Roshanshad, Amirhossein Roshanshad, Reza Fereidooni, Mahnaz Hosseini-Bensenjan

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Abstract

The coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 is an ongoing health concern. In addition to affecting the respiratory system, COVID-19 can potentially damage other systems in the body, leading to extra-pulmonary manifestations. Hepatic manifestations are among the common consequences of COVID-19. Although the precise mechanism of liver injury is still questionable, several mechanisms have been hypothesized, including direct viral effect, cytokine storm, hypoxic-ischemic injury, hypoxia-reperfusion injury, ferroptosis, and hepatotoxic medications. Risk factors of COVID-19-induced liver injury include severe COVID-19 infection, male gender, advanced age, obesity, and underlying diseases. The presentations of liver involvement comprise abnormalities in liver enzymes and radiologic findings, which can be utilized to predict the prognosis. Increased gamma-glutamyltransferase, aspartate aminotransferase, and alanine aminotransferase levels with hypoalbuminemia can indicate severe liver injury and anticipate the need for intensive care units' hospitalization. In imaging, a lower liver-to-spleen ratio and liver computed tomography attenuation may indicate a more severe illness. Furthermore, chronic liver disease patients are at a higher risk for severe disease and death from COVID-19. Nonalcoholic fatty liver disease had the highest risk of advanced COVID-19 disease and death, followed by metabolic-associated fatty liver disease and cirrhosis. In addition to COVID-19-induced liver injury, the pandemic has also altered the epidemiology and pattern of some hepatic diseases, such as alcoholic liver disease and hepatitis B. Therefore, it warrants special

vigilance and awareness by healthcare professionals to screen and treat COVID-19-associated liver injury accordingly.

Key Words: SARS-CoV-2; COVID-19; Liver injury; Chronic liver disease; Management; Liver transplant

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Core Tip: Severe acute respiratory syndrome coronavirus-2 can involve the liver and cause damage through different mechanisms. Liver injury can be diagnosed based on alterations in the liver function tests, which can also predict the disease severity and fatality. In patients without underlying liver disease, liver injury is typically mild and can be treated with supportive care. However, it requires additional awareness and appropriate therapy in patients with chronic liver diseases, including autoimmune hepatitis, viral hepatitis, liver cirrhosis, liver transplantation, and nonalcoholic fatty liver disease, which we have discussed in detail.

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INTRODUCTION

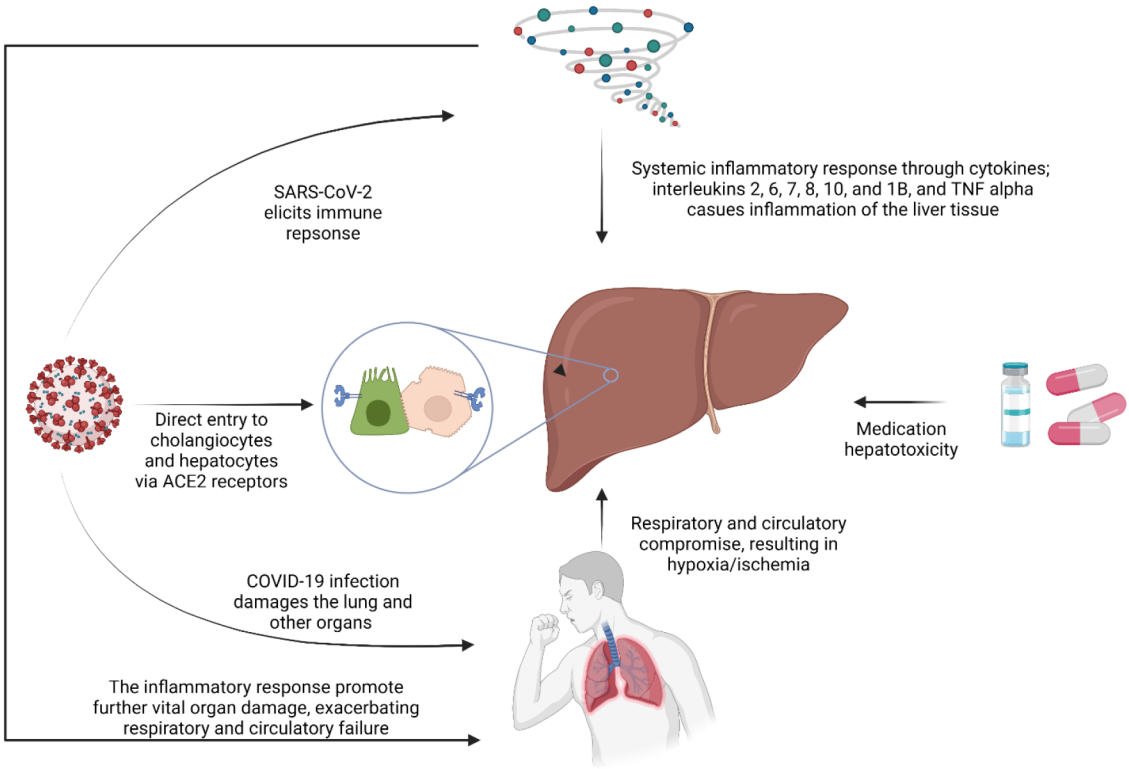
The coronavirus disease 2019 (COVID-19) pandemic has tremendously influenced global public health since its emergence in December 2019. Despite the effectiveness of worldwide vaccinations, the disease is still a substantial threat[1]. As of 6 January 2023, the World Health Organization (WHO) recorded over 657 million confirmed cases of COVID-19 and over 6.6 million deaths[2].

COVID-19 was first recognized as a respiratory disease with variable manifestations, from asymptomatic and mild symptoms to acute respiratory distress syndrome and death. However, further investigations determined that COVID-19 can cause various extra-pulmonary manifestations and result in multi-organ dysfunction[3]. Various theories have been suggested to explain how COVID-19 causes gastrointestinal (GI) system involvement. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) penetrates the host cells *via* angiotensin converting enzyme 2 (ACE2) receptors, which exist in different tissues, including the GI system. This may result in GI manifestations such as nausea, vomiting, diarrhea, anorexia, and hepatic manifestations[4]. After the lungs, the liver is the second most frequent organ impacted by COVID-19[5]. Liver involvement usually presents as mild to moderate elevations of liver enzymes. Although, in certain circumstances, including patients with severe COVID-19 infection and underlying comorbidities (*i.e.*, diabetes and hypertension) significant elevations of liver enzymes and liver dysfunction are more probable to happen[6,7]. COVID-19-induced liver injury may develop in patients regardless of the presence of underlying liver diseases; however, the likelihood of poorer outcomes is higher in patients with underlying chronic liver disease (CLD)[5,8]. Chronic liver diseases, including viral hepatitis, autoimmune hepatitis, liver cirrhosis, and fatty liver disease constitute a significant worldwide health burden. About 3% of COVID-19 patients have CLD, and this group has a higher risk of developing severe disease and death[9]. Moreover, these individuals have impaired immune systems due to their use of immunosuppressive drugs. Therefore, it is essential to consider stringent monitoring and develop appropriate treatments for these patients[10].

In this review, we aimed to comprehensively investigate different related aspects of liver injury in COVID-19. We discuss the pathophysiology, epidemiology, clinical manifestations, management, and outcomes of COVID-19 patients with liver injury. We also covered important topics like the interaction between COVID-19 and various types of liver disease, as well as unique considerations for particular populations with CLD and liver transplant (LT) recipients.

MECHANISMS AND PATHOPHYSIOLOGY OF LIVER INJURY IN COVID-19 PATIENTS

Although the exact mechanism of liver injury in COVID-19 is yet to be understood, several pathways have been proposed. The direct viral effect, drug hepatotoxicity, systematic inflammatory response (cytokine storm), decompensation of pre-existing liver disease, and hypoxic liver injury are among the suggested hypotheses[11]. Figure 1 demonstrates the proposed mechanisms of liver injury in COVID-19.



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Figure 1 Proposed mechanisms of liver injury in coronavirus disease 2019 patients. SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; ACE2: Angiotensin converting enzyme 2; COVID-19: Coronavirus disease 2019; TNF alpha: Tumor necrosis factor alpha.

Direct viral effect

Although almost none of the current histopathological reports has shown a typical hepatitis picture[12], some evidence argues in favor of liver tropism and the direct effect of the virus on the liver. Firstly, the SARS-CoV-2 receptor, ACE2, is found on the surface of the hepatic Kupffer cells, hepatocytes, and cholangiocytes which helps the virus enter these cells and, as a result, makes the liver a potential target organ for the virus[13-15]. Secondly, Wang *et al*[16] discovered the SARS-CoV-2 spike structures in the cytoplasm of hepatocytes in two COVID-19 cases. The spatial presence of SARS-CoV-2 RNA and spike protein was also proved by previous studies in hepatic cells, which implies replication of the virus and direct infection of hepatic parenchyma[13,17]. Wanner *et al*[13] discovered a lower viral load in the liver than in the lungs of one patient, suggesting this may be the reason we do not typically observe cytopathic changes (*i.e.*, hepatitis pattern) in the liver of COVID-19 patients.

Systemic inflammatory response (cytokine storm)

In response to COVID-19 and in order to hinder viral replication and evoke the adaptive immune response, the innate immune system is activated[18]. Severe cases of COVID-19 are associated with an exaggerated immune response indicated by high levels of C-reactive protein (CRP), pro-inflammatory T helper 17 cells, and cytokines[18,19]. Cytokines in severe cases of COVID-19 include interleukins (IL)-2, 6, 7, 8, 10, and 1B and Tumor necrosis factor alpha (TNF-α)[18-20]. Pathological studies have demonstrated non-specific inflammatory changes in the hepatocytes, including steatosis, lymphocyte infiltration, and Kupffer cell hyperplasia[19]. This immune system dysregulation can result in multiple organ involvement, including the liver. The cytokine storm not only affects the liver by triggering the inflammatory response and recruiting macrophages; but also plays a role in further indirect damage by promoting thrombotic events, circulatory changes, and failure of other organs[20].

Hypoxic-ischemic injury, hypoxia-reperfusion injury, and liver congestion

While shock as the result of the COVID-19 infection itself is not a common finding[21], it is a common entity in intensive care units (ICU)[22]. COVID-19 patients are susceptible to all types of shock (*i.e.*, cardiogenic, septic, hypovolemic, and obstructive)[21,23,24]. While shock reduces liver perfusion, respiratory failure can also produce hypoxic harm to the liver, even in the absence of ischemia[22,25]. On the other hand, COVID-19 is known to induce microangiopathy and thromboembolism that further compromises the liver blood supply[26]. In the context of COVID-19, liver damage and coagulopathy are connected. Rise in transaminases have been shown to correlate with abnormal coagulopathy markers, such as prothrombin time, international normalized ratio, fibrinogen, D-dimer, fibrin/

fibrinogen degradation products and platelet count[27]. Different organ involvements in COVID-19, including cardiac, pulmonary, and vascular involvement, paired with the high metabolic activity of the liver, makes it a highly susceptible organ to hypoxic-ischemic damage[28]. In the autopsy of COVID-19 patients, ischemic-type hepatic necrosis and lipid droplet accumulation (steatosis) have been found in histopathologic evaluations, both findings in favor of hypoxic-ischemic liver damage[12,29]. However, the hallmark of hypoxic hepatitis is a significant increase in liver enzymes, and the increase reported in the COVID-19 scenario is notably less[30]. Hence some have hypothesized that hypoxia-reperfusion is another explanatory mechanism[23]. Impairment of the venous drainage of the liver can contribute to blood stasis and hepatic congestion. Hepatic congestion is caused by the stasis of blood within the liver parenchyma due to the compromise of hepatic venous drainage[31]. Congestion of the hepatic sinusoids was estimated at 34.7% in a histopathologic study[32]. Cardiac events resulting in right-sided heart failure, decompensation of heart failure, and pulmonary thromboembolism can contribute to liver congestion in COVID-19 patients[23]. In a review by Kukla *et al*[33], hepatic dysfunction was associated with mechanical ventilation, especially with high rates of positive end-expiratory pressure (PEEP). They hypothesized that high PEEP (18-20 cm H_2O) causes high right atrial pressure resulting in liver congestion in a mechanism similar to right-sided heart failure. In a hemodynamic study of COVID-19 patients, mechanical ventilation and PEEP were associated with left ventricular underfilling[25].

Ferroptosis

Ferroptosis is a novel form of cell death, which is characterized by iron accumulation and lipid peroxidation[34]. The oxidative stress caused by iron overload is an important trigger for liver injury as well as other vital organ damage[35]. COVID-19 is believed to impact iron metabolism[36]. In the condition of cytokine storm, IL-6 stimulates the production of hepcidin in the liver, which is an inhibitor of iron export and leads to iron sequestration inside cells[37]. It is hypothesized that SARS-CoV-2 triggers the recognition of blood iron-binding molecules by receptors in many organs, including the liver, thereby causing an influx of iron ions. The accumulated iron, along with agents like lipid and hydrogen peroxide, triggers the Fenton reaction, producing massive lipid reactive oxygen species that cause cell membrane damage[38]. In conclusion, ferroptosis is a potentially important mechanism of organ damage in COVID-19; however, the extent of its role and whether treatments that inhibit ferroptosis can be helpful in preventing organ damage in COVID-19 remains to be determined[37,39].

Medications

Medication hepatotoxicity has strong evidence as the cause of liver injury in the context of COVID-19 [40]. Owing to the novel nature of the COVID-19 infection and the lack of evidence-based guidelines, various medications have been used for treatment, many of which have potential hepatotoxic properties. These include antivirals (including remdesivir, lopinavir/ritonavir, Favipiravir), hydroxychloroquine, antibiotics (*e.g.*, azithromycin), corticosteroids, tocilizumab, and antipyretics, specifically acetaminophen[23,40,41]. Remdesivir has been associated with transient increases in liver enzymes in Gilead company trials in less than 5% of participants and was indicated to possibly be cytotoxic to human hepatocytes at clinically relevant exposures[42,43]. Consistently, liver injury in remdesivir-treated COVID-19 cases was mostly mild, according to published literature[44-46]. Wang *et al*[34] reported that 7% of a remdesivir-treated group developed grade 1-2 aspartate aminotransferase (AST) elevation compared to 12% in a placebo-controlled group, and 1% developed grade 1-2 alanine aminotransferase (ALT) elevation. Case reports have revealed that associate Favipiravir, another antiviral agent used to treat COVID-19, with liver injury[47,48]. However, a systematic review on the safety of Favipiravir in COVID-19 failed to prove the association between the drug and liver injury[49]. The use of lopinavir/ritonavir has been linked to liver toxicity in multiple studies of COVID-19 patients [40]. Serum aminotransferases increase in a large number of individuals using antiretroviral regimens containing ritonavir. Moderate to severe elevations in the serum aminotransferase levels are detected in up to 15% of patients treated with full dosages of ritonavir[50]. In the setting of COVID-19, Cai *et al*[51] also found lopinavir/ritonavir to be associated with increased odds of liver injury. Yip *et al*[52] showed that the use of lopinavir/ritonavir ± ribavirin + interferon beta was independently associated with elevated liver enzymes. The use of systemic corticosteroids is highly encouraged in COVID-19, especially in patients requiring oxygen supplement therapy[53]. A cohort study in Hong Kong reported the independent association of corticosteroids with elevated AST/ALT[52]. However, extensive data on the hepatotoxicity of corticosteroids is non-existent, and it's usually confined to case reports[53]. Some have even proposed them to treat drug-induced liver injury[54]. One potential way of corticosteroid-induced liver toxicity may be its role in inducing non-alcoholic steatohepatitis[53]. Tocilizumab, a humanized recombinant monoclonal antibody, is used to combat systematic inflammatory response in COVID-19 because of its anti-IL-6 properties[55]. Hepatotoxicity with transient, mild to moderate transaminase increase is its well-known side effect, but severe drug-induced liver injury is a rare event [55,56]. Hydroxychloroquine and chloroquine are the standard medications to treat malaria and had been explored as treatment options for COVID-19 due to their *in vitro* anti-SARS-CoV-2 properties[57]. Liver injury associated with hydroxychloroquine is uncommon and limited to case reports[58-60]. Azithromycin, a macrolide antibiotic, was also advocated for the treatment of COVID-19 owing to its cytokine-suppressing features, potentially preserving the epithelial cells and inhibiting lung fibrosis

[61]. Azithromycin is known to cause liver injury, with both hepatocellular and cholestatic patterns[62,63]. Ivermectin, an anti-parasite drug, was reported to be associated with one case of severe hepatitis; however, there is insufficient data to comment on the effects of ivermectin on the liver function of COVID-19 patients, and more research is needed to clarify this[40,64]. So far, there is no report in the literature about acetaminophen-associated hepatotoxicity in COVID-19 patients; this is likely because acetaminophen-induced hepatotoxicity is highly dose-dependent, and its safe dose is well-established [65]. Additionally, it should be highlighted that, despite dosages used in patients normally belonging within safe ranges, combination therapy with various hepatotoxic drugs may have a synergistic effect.

COVID-19 AND LIVER MANIFESTATIONS

COVID-19 is usually regarded as a respiratory disease; however, subsequent research revealed extra-pulmonary manifestations of COVID-19, including cardiovascular, gastrointestinal, renal, ocular, neurological, psychiatric, dermatological, and endocrinology symptoms[66,67]. GI manifestations of COVID-19 are among the most common extra-pulmonary manifestations, and they can emerge even before the appearance of respiratory symptoms. Also, they can be the sole manifestation in some COVID-19 patients[68]. It seems that patients with COVID-19 who experience GI symptoms have a higher risk of elevated liver enzymes and liver injury[18,69]. The prevalence of liver injury in COVID-19 patients ranges widely from 21.5% to 45.71%[70]. This wide range is because of the specific characteristics of different populations and cut-off level discrepancies[71]. Liver injury commonly manifests as abnormalities in liver enzymes in the absence of particular clinical symptoms. Hence, close monitoring of liver enzymes in COVID-19 patients is essential for timely diagnosis of liver injury. In a meta-analysis by Yadav *et al*[70], the prevalence of liver enzymes abnormalities among COVID-19 patients ranged from 37.2% to 76.3%. In another meta-analysis, an elevation in the liver enzymes was reported in about 25% of COVID-19 patients, and the prevalence of increased AST and ALT was 23.2% and 21.2%, respectively. Gamma-glutamyltransferase (GGT) was elevated in 15%, total bilirubin in 9.7%, and alkaline phosphatase (ALP) in 4% of the patients[69].

RISK FACTORS FOR LIVER INJURY AND PREDICTORS OF DISEASE SEVERITY

Certain risk factors can predispose patients to develop liver injury. Some laboratory findings, such as lymphopenia, elevated AST/ALT ratio, and higher erythrocyte sedimentation rate and CRP levels are linked to the development of liver injury in COVID-19 patients. It is hypothesized that a systemic inflammatory response and cytokine storm are the cause of liver damage[70,72]. In addition, male gender, obesity, and advanced age are the risk factors for liver injury in COVID-19 patients[51]. The higher odds of COVID-19 induced liver injury among males may be due to the higher prevalence of existing risk factors, such as nonalcoholic fatty liver disease (NAFLD) and alcohol intake[73,74]. Previous studies have linked abnormalities in liver enzymes and liver injury to disease severity and mortality[75]. The abnormalities in liver function tests (LFT) can be used to predict the outcome of the disease. In a study conducted by Cai *et al*[51], the patients who had hepatocellular and mixed patterns of liver involvement were at higher risk of developing severe disease. A hepatocellular pattern was determined as an increase in aminotransferases (ALT/AST) levels higher than three times the upper limit of normal (ULN), while a mixed pattern was determined as an increase in aminotransferases greater than three times the ULN and ALP/GGT greater than twice the ULN.

Weber *et al*[76] demonstrated the correspondence between abnormal baseline liver enzymes and disease progression in patients without underlying liver disease. Increased GGT, AST, and ALT levels with hypoalbuminemia upon admission predicted the severity of the illness and the requirement for ICU hospitalization. Other studies have confirmed the link between low albumin levels and the severity of COVID-19. Hypoalbuminemia in severely ill COVID-19 patients is likely due to a combination of impaired hepatic albumin production and dietary deficiencies[18,77]. Phipps *et al*[75] revealed that higher levels of peak ALT and markers of inflammation, such as ferritin and IL-6, were strongly linked to progression to severe liver injury and a poorer outcome.

RADIOLOGIC FINDINGS OF THE LIVER IN COVID-19 PATIENTS

Some radiologic manifestations of liver damage have been reported in COVID-19 patients. On abdominal computed tomography (CT) scans of COVID-19 patients, hepatic hypodensity and fat stranding around the gallbladder were observed[78]. Some findings, such as a decrease in liver-to-spleen attenuation ratio and liver CT attenuation, may be relevant to the severity of the disease[79,80]. Chen *et al*[72] demonstrated that hepatic steatosis on CT scan was observed in 11.3% of COVID-19 cases, which enhanced the risk of hepatic dysfunction. In addition, they mentioned that while a decrease in

hepatic attenuation on a baseline CT scan is rampant, it is usually a temporary condition that improves on subsequent CT scans. The most prevalent findings of abdominal ultrasound of COVID-19 patients in ICU were hepatomegaly and biliary abnormalities, including common bile duct dilatation, gallbladder distention, and wall thickening[81]. Also, it was shown that in patients with mildly increased liver enzymes, hepatic ultrasonography was often not significant. However, in patients with a substantial rise in liver enzymes, hepatic ultrasound may detect vascular abnormalities or cholestatic changes, which is tied to higher fatality rates[82].

COVID-19 VACCINATION AND LIVER INJURY

With widespread vaccination against COVID-19, many reports of immune-mediated liver injury (ILI) associated with vaccination have emerged[83-87]. Most cases develop a hepatocellular pattern of injury [86]. The proposed hypothesis is that due to the similarity between S protein and liver specific proteins, the immune response elicited against the S protein, which is encoded by the vaccines, may cause autoimmune-like hepatitis[88]. Histopathological evaluation of the patients with ILI demonstrated portal lymphoplasmacytic infiltration and interface hepatitis[89]. The prognosis of immune-mediated vaccine-induced liver injury seems to be excellent with corticosteroid treatment, with only 4.3% death among patients who received steroids[89].

Roy *et al*[89] reviewed ILI following COVID-19 vaccinations. Most of the patients with ILI were female, with a mean age of 55.3 years. Moderna mRNA–1273 was the most common culprit for ILI following COVID-19 vaccinations, followed by Pfizer-BioNTech BNT162b2 mRNA and AstraZeneca ChAdOx1 nCoV-19 vaccine. Inactivated vaccines can also contribute to ILI; however, the reports are much more scarce than mRNA vaccines[90]. The most common presentation of ILI was jaundice, which was present in 78.3% of the patients, and bilirubin, ALT, and ALP levels were elevated. There is a dispute over the diagnosis of ILI following vaccination. Physicians should consider the clinical symptoms, liver function tests, histopathological findings, and chronological association between vaccine injection and presentations of the symptoms.

OUTCOME, MANAGEMENT, AND CONSIDERATIONS IN SPECIAL POPULATIONS

Liver involvement is a common finding in patients with COVID-19; however, it is frequently mild, transitory, and resolves without management[75,91]. Nevertheless, patients with underlying liver diseases, including hepatitis, cirrhosis, or LT recipients, are at higher risk of severe diseases. It is estimated that the probability of developing severe disease and death due to COVID-19 was 2.44 times greater in patients with CLD compared to those without underlying liver diseases [Odds ratio (OR) for severity = 2.44, 95%CI: 1.89-3.16; OR for mortality = 2.35, 95%CI: 1.84-3.00][92]. Among different underlying liver diseases, NAFLD was associated with the highest odds of severe disease and death due to COVID-19, followed by metabolic-associated fatty liver disease (MAFLD) and cirrhosis[92]. Severity and mortality rates after COVID-19 were not impacted by viral hepatitis significantly. Table 1 demonstrates the strength of the association between several major underlying liver diseases and the severity and mortality of COVID-19.

Autoimmune hepatitis

The most common presentations of COVID-19 in patients with autoimmune hepatitis (AIH) were respiratory (74%) and GI (26%) manifestations[93]. However, AIH patients are more likely to present with GI manifestations than the general population (26% *vs* 14%). The mortality rate and cause of death of the AIH group were not significantly different from the non-AIH CLD group. Age (OR *per* 10 years: 2.01, 95%CI: 1.07-3.81), Child-Pugh B (OR: 42.48, 95%CI: 4.41-409.53), and Child-Pugh C (OR: 69.30, 95%CI: 2.83-1694.50) cirrhosis were the determinants of mortality within AIH patients[93].

AIH is an immune-mediated liver disease, which is mainly responsive to immunosuppressive therapy[94]. Single corticosteroid therapy or in combination with azathioprine are the standard immunosuppressive treatments for AIH[95]. Other drugs used to treat AIH include mycophenolate mofetil (MMF), calcineurin inhibitors, and TNF-α blockers[95]. The immunosuppression status following the use of these drugs enhances the risk of bacterial and viral infections in these patients[95] and might theoretically lead to a more severe COVID-19. Therefore, it is crucial to closely monitor the immunosuppressed AIH patients affected by COVID-19. In patients with simultaneous AIH and COVID-19, immunosuppressive therapy should be given after evaluating the risks and benefits. It is suggested that immunosuppressive therapy can be lifesaving in severe AIH patients[96]. Thus, it is not wise to discontinue the treatment as it might predispose the patients to a higher risk of relapse. In conclusion, empirical reduction in the doses of immunosuppressive drugs in these patients during the course of COVID-19 can be potentially harmful[97]; however, the immunosuppression therapy can be delayed until the COVID-19 polymerase chain reaction (PCR) test becomes negative in milder forms of

Table 1 The association of different underlying liver diseases with clinical outcomes of coronavirus disease 2019

Underlying liver disease/condition	Findings
Autoimmune hepatitis	AIH *vs* other CLDs: No significant differences in hospital admission (76% *vs* 85%; *P* = 0.06), ICU admission (29% *vs* 23%; *P* = 0.240), and mortality (23% *vs* 20%; *P* = 0.643) rates; AIH *vs* non-CLDs: Higher hospitalization rate, but similar rate for other outcomes; Severity of AIH associates with COVID-19 mortality, as follows: Age OR *per* 10 years: 2.01 (95%CI: 1.07-3.81); Child-Pugh B cirrhosis OR: 42.48 (95%CI: 4.41-409.53); Child-Pugh C cirrhosis OR: 69.30 (95%CI: 2.83-1694.50)[93]
Viral hepatitis	The risk of severe COVID-19 is higher (RR: 1.68, 95%CI: 1.26-2.22)[191]; however, another meta-analysis showed no association between viral hepatitis and poorer outcomes (pooled OR = 1.29, 95%CI: 0.36-4.63)[92]
Cirrhosis	Cirrhotic patients experienced more severe disease (pooled OR = 3.09, 95%CI, 1.95–4.89)[92]; Severity of cirrhosis associates with COVID-19 severity, as follows: Child-Pugh A cirrhosis OR: 1.90 (95%CI: 1.03-3.52); Child-Pugh B cirrhosis OR: 4.14 (95%CI: 2.4-7.65); Child-Pugh C cirrhosis OR: 9.32 (95%CI: 4.80-18.08)[111]
Liver transplant	No significant difference in mortality rates between LT and non-LT participants (OR: 0.8, 95%CI: 0.6-1.08); The time between the transplantation and COVID-19 did not affect the mortality rate (OR: 1.5, 95%CI: 0.63-3.56); Severe COVID-19 infection was observed in 23% of the participants with LT[129]
NAFLD	NAFLD was associated with more severe COVID-19 (AOR: 2.60, 95%CI: 2.24-3.02), more ICU admission (AOR: 1.66, 95%CI: 1.26-2.20), but not higher mortality rates (AOR: 1.01, 95%CI: 0.65-1.58)[148]
MAFLD	MAFLD increased the risk of severe COVID-19 (OR: 1.80, 95%CI: 1.53-2.13). No association was found between the presence of MAFLD and the occurrence of COVID-19 death[192]
Pregnancy	29.7% of pregnant patients with COVID-19 had liver injury; Liver injury can predispose pregnant females to experience more severe COVID-19, however, their neonates do not have worsen prognosis[157]

COVID-19: Coronavirus disease 2019; AIH: Autoimmune hepatitis; AOR: Adjusted odds ratio; CLD: Chronic liver disease; ICU: Intensive care unit; LT: Liver transplant; MAFLD: Metabolic associated fatty liver disease; MMF: Mycophenolate mofetil; NAFLD: Non-alcoholic fatty liver disease; OR: Odds ratio; RR: Relative risk.

AIH[96].

In addition to the impacts of COVID-19 on the management of AIH during the pandemic, SARS-CoV-2 has been proposed as a possible trigger for AIH. In a study by Kabaçam *et al*[96], COVID-19 patients were diagnosed with AIH after presenting with high serum aminotransferase and IgG levels. It should be noted that there is an AIH-like liver injury that might occur following COVID-19 vaccination. The laboratory findings are similar to AIH in these cases, and there is a good response to corticosteroids in these patients[98,99].

Viral hepatitis B

The prevalence of hepatitis B virus in patients with COVID-19 ranges from 0.1% in a study in the United States to 12.2% in China[100,101]. Interestingly, these prevalence rates were not significantly higher than the general population. Furthermore, there is no well-established association between HBV infection and severe COVID-19 disease[101-104]. Surprisingly, some studies reported a milder course of COVID-19 in patients with HBV[101,103,105,106]. One hypothesis is that patients with HBV might experience a state of "immune exhaustion", which can be responsible for a lower chance of developing cytokine storm[107].

The treatment of COVID-19 with corticosteroids and other immunosuppressive drugs, including tocilizumab, may lead to the reactivation of HBV infection in these patients. Therefore, screening for HBV in COVID-19 patients with elevated liver enzymes in endemic HBV populations is highly recommended[104]. Several treatments were recommended for COVID-19 infection in HBV patients. A suggested method is drug repositioning, which means that the antiviral HBV drugs could also be used to treat COVID-19 infection[108,109]. In COVID-19 HBsAg-positive patients who are not receiving anti-HBV medication, continuous corticosteroid or immunosuppressive drugs consumption necessitates prophylaxis with tenofovir disoproxil fumarate, tenofovir alafenamide, and entecavir to reduce the likelihood of HBV reactivation and liver failure[104].

Liver cirrhosis

COVID-19 is one of the main contributors to the exacerbation of preexisting liver cirrhosis[110]. It is found that liver cirrhosis decompensation occurs in nearly half of the cirrhotic patients with COVID-19, one-fifth of whom might not develop any pulmonary manifestations[111]. Therefore, physicians should be advised to consider COVID-19 in patients with cirrhosis exacerbation, even in the absence of respiratory symptoms. Because of cirrhosis-associated immune dysfunction, patients with decompensated liver cirrhosis are more susceptible to COVID-19[112,113]. The mortality rate in COVID-19 patients with decompensated liver cirrhosis was shown to be about 50%[114]. The main predictor of mortality in cirrhotic patients with COVID-19 is the severity of underlying cirrhosis[111]. Child-Pugh C cirrhosis has the highest odds for mortality among patients with CLD (OR = 9.32, 95%CI: 4.80-18.08),

while the odds ratio for Child-Pugh A was 1.90 (95%CI: 1.03-3.52). Other predictors of mortality were higher age and concurrent alcoholic liver disease. One study reported that mortality, ventilation support, hospitalization, and ICU admission rates were significantly higher in patients with higher model for end-stage liver disease (MELD) scores, especially a score above ten[115]. However, whether COVID-19 can worsen the outcomes in hospitalized patients with cirrhosis remains controversial. While some reports demonstrate that COVID-19 can elevate mortality rates in cirrhotic patients[110,116,117], other studies found that COVID-19 did not significantly increase mortality in these patients[118,119]. Monitoring liver function tests, early detection of liver complications caused by a coronavirus, and broad-spectrum antibiotic therapy in case of secondary bacterial infection are highly recommended for successful management[112].

The humoral response to COVID-19 vaccines is 94% in patients with CLD or cirrhosis. The mRNA vaccines are more efficacious than inactivated vaccines in these patients[120].

LT

The success rate of transplantation is associated closely with the postoperative immunosuppression treatment to prevent organ rejection[121]. Since the emergence of the pandemic, there has been a debate on the use and dosing of immunosuppressive drugs for these patients, as they act like a double-edged sword[122]. While they can profoundly decrease the chance of organ rejection, severe immunosuppression can theoretically lead to uncontrolled viral replication and severe COVID-19[123,124]. MMF and calcineurin inhibitors, including tacrolimus are among the main immunosuppressive drugs used in LT patients[125,126]. MMF inhibits the proliferation of lymphocytes and suppresses cell-mediated immunity and antibody formation[127]. LT patients who were previously treated with MMF have a higher risk of experiencing severe COVID-19 infection, especially those who have received doses more than 1000 mg/day[127]. They also suggested a reduction in the dose of MMF or temporary conversion to other immunosuppressive drugs, including tacrolimus in LT patients[127]. On the other hand, a meta-analysis found that the continued administration of certain immunosuppressive drugs during COVID-19 resulted in a lower probability of severe disease[128]. Among the immunosuppressive drugs, the mammalian target of rapamycin inhibitor (mTORi) was the most effective in preventing severe disease, followed by calcineurin inhibitors, steroids, and antimetabolites.

Further studies, including a meta-analysis, indicated that the rate of severe COVID-19 and death was not higher in LT patients[129]. Most deaths due to COVID-19 were associated with the cytokine storm and inflammatory process, not the result of direct viral replication[130]. Therefore, not only is immunosuppression not harmful, but also it could prevent cytokine storm and reduce mortality. Guarino *et al*[131] demonstrated that LT patients were more likely to become symptomatic; however, they did not exhibit more severe disease and death due to COVID-19. They did not reduce the dose of immunosuppressive drugs unless in severe and critical patients. This heterogeneity among the results can be attributed to the baseline characteristics of the patients, including the presence of other comorbidities, mean age, time from the transplant to COVID-19, and immunosuppressive drug. The strengths of the associations of these factors were as follows: (1) Age > 70 [Hazard ratio (HR): 4.16, 95%CI: 1.78-9.73]; (2) Tacrolimus use (HR: 0.55, 95%CI: 0.31-0.99); (3) Diabetes (HR: 1.95, 95%CI: 1.06-3.58); (4) Chronic kidney disease (HR: 1.97, 95%CI: 1.05-3.67); and (5) Time between transplantation and COVID-19 (HR: 0.55, 95%CI: 0.31-0.99)[132,133]. Therefore, we suggest that physicians assess the risk of severe disease in LT patients by considering age, comorbidities, and the time between the transplant and COVID-19. It is not necessary to modify the immunosuppressive drugs for all the patients; they can be closely monitored for the occurrence of risk factors and manifestations of severe disease and, if present, reducing the dose or changing the drugs can be applied. Besides, tacrolimus-based or mTORi-based immunosuppressive regimens have been proven to be safer with more favorable outcomes[128,134].

In addition, the response to COVID-19 vaccination is another important challenge in immunosuppressed patients. It is found that discontinuation or dose reduction of MMF optimizes the COVID-19 vaccination response in LT patients[135,136]. Moreover, it is necessary to consider booster shots in immunosuppressed patients who do not demonstrate the appropriate response to vaccinations[137]. On the other hand, pre-exposure prophylaxis with tixagevimab/cilgavima can be beneficial in LT patients with severe immunosuppression who did not respond adequately to COVID-19 vaccination or COVID-19 vaccine contraindication[138]. The humoral response to COVID-19 vaccines is 66% for patients with LT[120]. Luo *et al*[120] also revealed that the worse humoral response to COVID-19 vaccinations in LT patients was observed in patients with a history of using MMF (OR: 3.27, 95%CI: 1.45-7.41), diabetes (OR: 2.75, 95%CI: 1.48-5.09), and the use of > 2 immunosuppressive drugs (OR: 3.13, 95%CI: 1.22-7.99).

Transplant candidates waiting for a donor are also considered high-risk due to their underlying liver disease. COVID-19 can complicate the LT process as an affected patient cannot undergo transplant surgery until declared recovered from COVID-19. It is suggested that the transplant should be performed with one negative COVID-19 reverse transcription-PCR test 24 h prior to the transplantation [139]. It was found that COVID-19 infection before liver transplantation in patients with high MELD scores was not associated with a higher mortality rate[140]. As a result, postponing the surgery to a later time than what is suggested above is not recommended.

Nonalcoholic fatty liver disease

NAFLD is a liver manifestation of metabolic syndrome and is the most common CLD all over the world, affecting 30%-40% of the world population[141,142]. The spectrum of NAFLD ranges from hepatocellular steatosis to advanced liver cirrhosis[143]. There was a great heterogeneity across different studies which assessed the severity outcomes of COVID-19 in patients with NAFLD[144-147]. Two meta-analyses suggested a more severe disease course and higher rates of ICU admission in NAFLD patients[92,148].

MAFLD is a more recent term to describe fatty liver disease in the context of metabolic syndrome [142]. It is considered a better reflection of the disease pathogenesis and can contribute to a more appropriate disease classification and management. NAFLD patients were diagnosed and included in the studies mainly based on their imaging (computed tomography or sonography) findings; however, metabolic risk abnormalities should be considered beside the imaging findings of hepatic steatosis to label a patient as MAFLD[149]. The existence of MAFLD can lead to the release of more inflammatory cytokines and worsens the inflammatory process in COVID-19 infected patients[150]. Gao *et al*[151] found that the elevated level of IL-6 was associated with poorer COVID-19 outcomes in MAFLD patients. Leptin dysregulation in metabolic syndrome and obesity can be responsible for the more prominent cytokine storm observed in these conditions[152]. Therefore, it seems that metabolic syndrome features which are incorporated in the definition of MAFLD are the determinants of the disease severity in patients with fatty liver. This also clarifies the inconsistency of the results about disease severity in NAFLD patients with COVID-19, but a more consistent result for MAFLD that we described above.

Another predictor of disease severity is the degree of NAFLD or MAFLD and liver fibrosis. Targher *et al*[146] found that MAFLD patients with intermediate to high fibrosis-4 (FIB-4) scores had poorer COVID-19 outcomes (OR for intermediate FIB-4 = 4.32, 95%CI: 1.94-9.59; OR for high FIB-4 = 5.73, 95%CI: 1.84-17.9). Another study found that the increase in various liver fibrosis scores, including FIB-4, aspartate aminotransferase to platelet ratio index, NAFLD fibrosis score, and Forns scores was associated with worse COVID-19 outcomes and, as a result, can be used as prognostic factors[153]. However, their accuracy in identifying liver fibrosis is not acceptable in young individuals and both extremes of body mass index[154,155].

Pregnancy

The presence of liver involvement can partially impact COVID-19 prognosis in pregnant patients. It is shown that the duration of admission in COVID-19 pregnant females is more prolonged in those with liver involvement[156]. Moreover, pregnant females with liver injury are at higher risk of developing severe disease[157]. Nevertheless, their neonates do not have an excessive risk for worsening prognosis.

The prevalence of COVID-19-related liver injury in pregnant women is nearly 30%, which is relatively similar to the general population[157]. The first step in managing a pregnant patient who presents with COVID-19 and liver injury is to identify the cause of liver damage and exclude obstetric diagnoses. COVID-19-related liver involvement can be mistaken for preeclampsia[158], HELLP syndrome[159], acute fatty liver of pregnancy[158], and intrahepatic cholestasis[160]. All these conditions warrant early diagnosis and require certain urgent management. Therefore, it is highly recommended to monitor liver function tests in pregnant females[157].

Myalgia is one of the most common presentations of COVID-19, which is usually managed with acetaminophen plus non-steroidal anti-inflammatory drugs (NSAID)[161]. As the use of high-dose NSAID is not recommended during pregnancy, especially during the third trimester, higher doses of acetaminophen might be prescribed to control the pain, which might exacerbate the underlying liver disease. Therefore, physicians should calculate the safe dose of acetaminophen to prevent the exacerbation of underlying liver disease[162].

Infants and children

Severe COVID-19 disease in adults is linked to dysfunctional cellular immunity and unchecked inflammatory cytokines production[163]. The unaffected immunosuppressive and cellular elements of the immune system in children can result in a less aggressive course of COVID-19. This milder course of COVID-19 can explain the lower rate of elevated AST and ALT in children (6%-22%)[164,165], compared to adults (14%-53%)[166]. The most common hepatic presentation of COVID-19 is the elevation of liver enzymes. Elevated ALT is usually seen in older male children[167]. Supportive treatment is generally considered for managing COVID-19-affected children with liver involvement.

In addition to COVID-19-related liver injury, pediatricians should be aware of the management of COVID-19 in children with pre-existing liver disease. It was revealed that the severity of COVID-19 was not higher in children with CLD[168]. Therefore, children with stable CLD can be managed virtually with a well-organized telemedicine system. Similar to adults, in children with autoimmune liver disease or LT, who are receiving immunosuppressive drugs, it is not recommended to decrease the dose of drugs in mild cases, as it can increase the chance of organ rejection[163].

Neonates can be affected by COVID-19 *via* three routes: antenatal, peripartum, and postnatal. Liver involvement in neonates is mainly characterized by elevated liver enzymes. In these cases, the first step

is to exclude other causes of abnormal liver function tests[169]. Remdesivir should be administered in neonates with COVID-19 with great caution and only in severe cases as it can worsen liver injury in COVID-19- infected neonates[170].

EPIDEMIOLOGICAL ASPECTS OF LIVER DISEASE DURING THE COVID-19 PANDEMIC

COVID-19 can cause liver injury *via* different mechanisms discussed above. In addition to these mechanisms, the COVID-19 pandemic contributed to some changes in the pattern and epidemiology of some of the hepatic diseases.

Alcoholic liver disease

The amount of alcohol consumption and prevalence of alcoholic liver disease have risen during the COVID-19 pandemic. Several reports from different countries worldwide demonstrated a significant rise in the amount of alcohol use during the pandemic, including the United States[171], China[172], and England[173]. It is postulated that this increase resulted from financial problems, job loss, and mental distress during the pandemic[174-176]. Besides, the psychological impacts of the loss of beloved ones, social isolation, lockdown, and other mitigation strategies that were implemented to control the disease have resulted in a greater level of mental distress and, as a result, aggravated alcohol use even more [177-179]. Therefore, it was expected to observe higher rates of alcoholic liver diseases and their burden. Numerous reports from across the globe provided data regarding the increased burden, hospitalization, and mortality due to excessive alcohol consumption during the pandemic. A study from Canada found that the rates of monthly hospitalization due to alcoholic hepatitis increased from 11.6 before March 2020 to 22.1 cases *per* 10000 amid the pandemic, which indicates a nearly two-fold increase in the number of cases[180]. A similar increase rate was also observed in another study from England[181]. Two reports from the United States suggested a nearly 50% increase in the case admission due to alcoholic hepatitis during the pandemic[182,183]. In concordance with the increase in case hospitalization, we also observed a 20% rise in mortality rates due to alcoholic hepatitis[184]. Females and younger individuals are more likely to experience these increasing trends in hospitalization and mortality rates due to alcoholic liver disease[183,184]. Several strategies can be implemented to mitigate the detrimental effects of the COVID-19 pandemic on patients with alcohol use disorders. Developing a well-organized telemedicine system can help better identification of high-risk persons and provide consultation, health-related suggestions, and surveillance[179]. The screening programs can also be used for earlier identification of at-risk groups for alcohol use disorder. Alcohol Use Disorders Identification Test – Consumption and Single Alcohol Screening Question are two recommended screening tools for assessing alcohol use disorder and are suggested to be used for all persons over 18 during primary care evaluation[185,186].

Hepatitis B virus

In addition to alcoholic liver disease, HBV infection is another hepatic disease which is affected by the COVID-19 pandemic. Implementation of the strategies to mitigate the spread of the pandemic, including the lockdown, resulted in more difficult access to the healthcare facilities and, thus, reduced the willingness of many patients to seek medical services[187]. As a result, the number of HBV tests was reduced, and HBV diagnosis and treatment were impacted negatively amid the pandemic[188]. In addition, there are reports which indicate that the failure of the screening programs lowers the number of HBV vaccination and aggravates the preexisting health inequalities during the pandemic[188,189]. All these can contribute to a notable rise in the number of HBV cases. Similar to what we proposed to tackle the burden associated with the alcoholic liver disease during the pandemic, telemedicine and remote screening programs can be useful to resolve the pandemic-related disruptions in HBV infection management. Also, integrating the messaging system and contact tracing into the screening programs is suggested to increase the efficacy of screening; however, their effectiveness is yet to be determined by future studies[190].

CONCLUSION

Liver injury is a relatively common manifestation of COVID-19, mostly presenting with elevated liver enzymes. Different mechanisms for COVID-19-induced liver injury, including direct viral effect, cytokine storm, hepatic congestion, hypoxia, and reperfusion injury are proposed. Most cases of liver injury are mild and only require supportive treatment; however, there are some underlying liver diseases which might require special considerations. NAFLD and MAFLD were associated with the highest OR for severe COVID-19. Also, more severe disease is expected in patients with liver cirrhosis, especially cases with more advanced liver fibrosis. Despite the immunocompromised status in LT and AIH patients, they are not at a significantly higher risk of severe disease, and there is no need to

empirically reduce or change the immunosuppressive drugs.

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REVIEW

Recent advances in recurrent hepatocellular carcinoma therapy

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Abstract

Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer, accounting for 75%-85% of cases. Although treatments are given to cure early-stage HCC, up to 50%-70% of individuals may experience a relapse of the illness in the liver after 5 years. Research on the fundamental treatment modalities for recurrent HCC is moving significantly further. The precise selection of individuals for therapy strategies with established survival advantages is crucial to ensuring better outcomes. These strategies aim to minimize substantial morbidity, support good life quality, and enhance survival for patients with recurrent HCC. For individuals with recurring HCC after curative treatment, no approved therapeutic regimen is currently available. A recent study presented novel approaches, like immunotherapy and antiviral medication, to improve the prognosis of patients with recurring HCC with the apparent lack of data to guide the clinical treatment. The data supporting several neoadjuvant and adjuvant therapies for patients with recurring HCC are outlined in this review. We also discuss the potential for future clinical and translational investigations.

Key Words: Recurrent hepatocellular carcinoma; Liver transplantation; Therapy; Immunotherapy; Neoadjuvant and adjuvant therapy

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Core Tip: Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer, and up to 50%-70% of individuals may experience a relapse of the illness in the liver after 5 years. This review will provide novel approaches to improve the prognosis of patients with recurring HCC with the apparent lack of data to guide the clinical treatment. Neoadjuvant and/or adjuvant therapy methods potentially elevate the opportunity of cure in refractory patients with recurrent HCC and contribute to a better long-term prognosis.

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INTRODUCTION

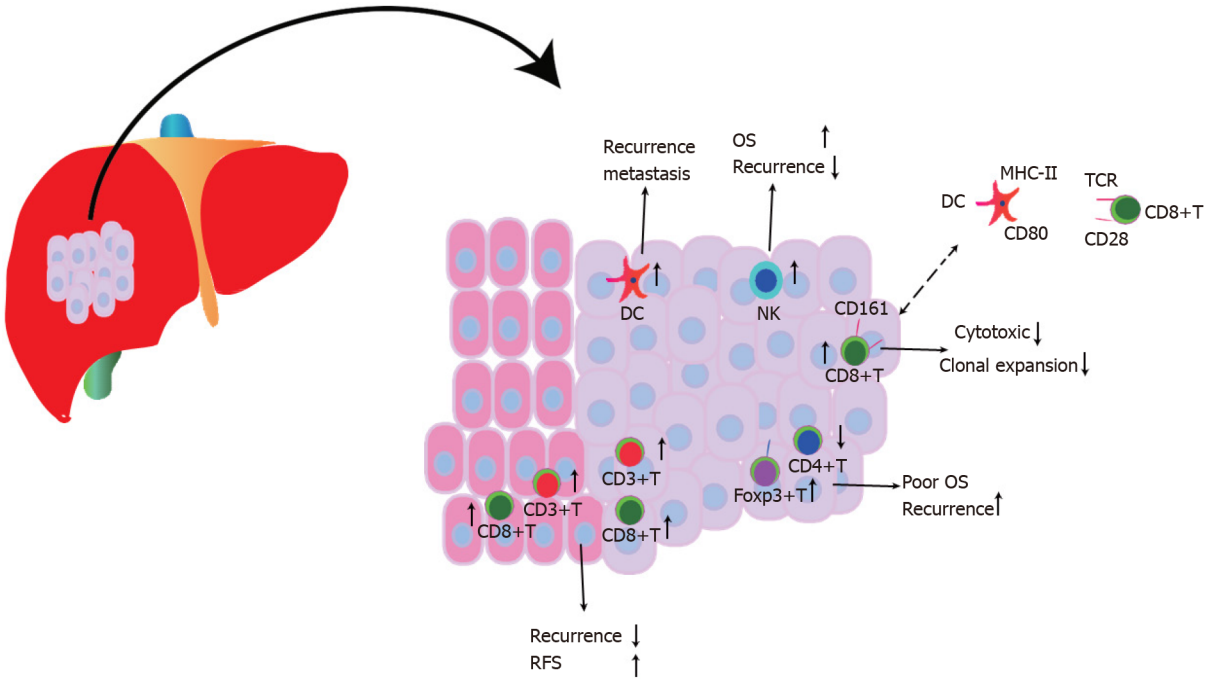
With an expected 906000 new cases and over 800000 fatalities, primary liver cancer is ranked as the sixth most commonly diagnosed malignancy and the third most prevalent cause of cancer-related deaths worldwide in 2020[1]. Hepatocellular carcinoma (HCC) accounts for 75%-85% of cases of primary liver cancer[2]. As medical care has improved, liver transplantation (LT) has emerged as the best option for individuals with HCC that is either incurable or who have progressive liver damage as a result of their HCC[3]. Although patients receive treatments for early stage HCC intending to cure the disease, up to 50%-70% of patients may experience disease relapse in the liver after 5 years[4,5]. This is not only related to the inadequacy of the surgery (*i.e.*, positive surgical margin) but is also frequently associated with the development of *de novo* tumors as the disease progresses. Additionally, 70% of patients with recurrent HCC experience an early relapse within 2 years of surgery, which is nearly incurable and has been linked to apoor prognosis[6]. The molecular mechanisms underlying the prompt relapse of HCC are still unclear.

In a small percentage of HCC patients with multifocal intra or extrahepatic relapse, liver function impairment, and tumors that cannot be removed, rehepatectomy is necessary[7]. According to reports, HCC patients with tumors that meet the Milan criteria had excellent 5-year survival rates and minimal risks of relapse after LT[8]. Until the disease worsened or tolerance was established, monotherapy was thought to be the only course of therapy. At least two treatments administered simultaneously or within 4 wk of each other were considered multimodal therapy[9]. Preclinical findings suggest that neoadjuvant and adjuvant dosages should be used in combination instead of using either of them individually[10].

Therefore, it is crucial to devise the best treatment plans and fully comprehend the mechanism of HCC relapse. As a result of these problems, numerous researchers have looked into the benefits of neoadjuvant and adjuvant therapeutic approaches to lower relapse rates and enhance prognosis. Adjuvant therapy is not typically advised following curative treatment since its benefits are unclear[11]. It is clear that some additional treatment modalities are necessary, and in this regard, either neoadjuvant or adjuvant approaches are mostly taken into consideration. These include adjuvant antiviral therapy, repeated excision, transarterial chemoembolization (TACE), transarterial radioembolization, radiofrequency ablation (RFA), LT, tyrosine kinase inhibitors, and immunotherapy. Here, we will discuss the current state of knowledge on and recent advances in the therapy of recurrent HCC in this narrative review.

MECHAMISM OF RECURRENT HCC

Up to 70% of early HCC recurrence cases were thought to manifest within the first two years following curative therapy; relapses that occur after this point are referred to as late HCC recurrences[12]. Malignant, immunological, and stromal cells are made up of heterogeneous cell types that interact spatiotemporally in complex tumor ecosystems[13]. Recent research has found similarities between the genetic variants of primary and early recurrent HCC[14]. Nevertheless, explanations for differences between the cellular ecosystems of primary and recurrent HCC are still being sought after. Early-relapse HCC displayed decreased regulatory T cells (Tregs) and higher dendritic cells (DCs) and CD8+ T cells compared to primary HCC, which were associated with a poor prognosis[15], as shown in Figure 1. Treg recruitment is a characteristic of the immunosuppressive milieu of primary HCC[16]. In contrast to the traditional depletion state in primary HCC, CD8+ T cells in relapsed HCC displayed higher CD161



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Figure 1 Mechanism of action of immune cells under investigation for recurrence of hepatocellular carcinoma. Early-relapse hepatocellular carcinoma displays decreased regulatory T cells, and higher dendritic cells and CD8+T cells. FoxP3(+) regulatory T cells encourage a gradual decline in CD4+ cytotoxic T cells, which contributes to poorer survival and high recurrence rates. OS: Overall survival; RFS: Relapse-free survival; DC: Dendritic cells; NK: Natural killer cell.

expression, a low cytotoxic state that was innate, and reduced clonal expansion. This is significant because the immune escape mechanism underlying HCC relapse was connected to the inhibition of DC antigen presentation and infiltration of innate-like CD8+ T cells[15], which caused intrahepatic dissemination of HCC. Tregs and intragraft Toll-like receptor 4/C-X-C motif chemokine 10/CXCR3 C-X-C motif chemokine receptor 3 levels were higher in patients with HCC recurrence following LT, which was further substantiated in a rat transplantation model[17].

According to a meta-analysis, HCC patients with high Foxp3+ T cell infiltration had worse 1-, 3-, and 5-year survival rates and a greater rate of recurrence than patients with low Foxp3+ T cell infiltration [18]. The frequency of CD8+ tumor-infiltrating lymphocytes in the intratumor and margin area was positively correlated with overall survival (OS) and disease-free survival (DFS) in two HCC cohorts (a combined total of 449), and a larger proportion of CD8+ tumor-infiltrating lymphocytes was associated with a lower recurrence rate[19]. As a result of their inflammatory condition, increased densities of CD8+T lymphocytes that infiltrate the liver in HCC patients contribute to tumor recurrence and carcinogenesis[20]. High CD3+ and CD8+ T cell densities were associated with minimal relapse and extended relapse-free survival in both the tumor center and margin[21]. In HCC patients, increased FoxP3(+) regulatory T cells encouraged a gradual decline in CD4+ cytotoxic T cells, which contributed to poorer survival and high recurrence rates[22]. A high Foxp3/CD8 ratio indicated a higher Edmondson-Steiner nuclear grade, relapse, and shorter OS and DFS, along with worse differentiation[23]. In HCC patients who received LT but not Foxp3+ T-lymphocytes, a correlation between CD4/CD8 ratio and tumor recurrence was established[24]. After surgical excision, high DC infiltration in HCC nodules can be used as a predictor of the recurrence and metastasis of the disease[25]. The response to sorafenib improved relapse-free survival, and OS in patients was significantly influenced by the increased density of natural killer (NK) cells[26].

In HCC patients after surgery, higher interleukin (IL-11) levels enhanced tumor expansion, and in genetic mouse model, suppression of IL-11-STAT3 signaling greatly reduced cell proliferation and post-surgical recurrences of HCC tumors[27]. Local recurrence is caused by the invasion of local tumor blood flow and peritumoral diffusion, whereas systemic dissemination is caused by the "rehoming" of circulating tumor cells that have spread from the initial nodules[28]. HCC recurrence can occur as a result of tumor cells that are circulating or at rest, evading the host's immune responses. The total somatic mutations and copy number depletion of WNK2 (WNK lysine deficient protein kinase 2) were associated with low levels of WNK2 protein expression, premature tumor relapse, and poor cumulative survival in patients with HCC following curative excision, indicating a tumor-suppressor role of WNK2 [29]. WNK2 inactivation results in the recruitment of tumor-associated macrophages, ERK1/2 signaling activation, tumor growth, and metastasis.

Following therapy, HCC shows pathological modifications; therapy induced pathological variation, particularly sarcomatous transformation, results in random and frequent recurrences after RFA[30]. It is generally accepted that recurrent HCC following curative therapy was caused by both initial incomplete treatment as well as current technological and biomarker limitations that make it difficult to detect preexisting microscopic tumors[31]. Occasionally, local therapies like TACE result in the direct diffusion of tumor cells from the RFA needle, which eventually causes a relapse of HCC[32]. However, multicentric origin HCC developed from *de novo* carcinogenic effect following curative excision, and the latter has a better OS than the former[33], consistent with the results of Kuo *et al*[34]. The incidence of intrahepatic metastasis and multicentric recurrent HCC was 59.4% and 27.5%, respectively, which were accompanied by loss of heterozygosity (63.8%) and microsatellite instability (30.0%) between primary and recurrent tumors[34]. Concerning previously unidentified circulating tumor cells or preexisting metastasis caused by the current technology that contribute to extrahepatic relapse, metastatic tumor lesions in the graft are originally formed from circulating cells or extrahepatic locales, providing a greater potential for biological advancement[35].

ADJUVANT ANTIVIRAL TREATMENT

Adjuvant antiviral therapy has been shown to decrease multicentric HCC recurrence, which in turn reduces post-treatment recurrence[33]. However, the ideal time for starting therapy with direct-acting antivirals (DAAs) for hepatitis C virus (HCV)-related HCC patients following surgical resection, and the impact of DAA on HCC recurrence remain unclear. Low risk of HCC recurrence after DAA treatment was suggested in some studies, while others have reported contrasting outcomes. Furthermore, there is conflicting evidence concerning HCV-related HCC recurrence in previously cured patients following virus elimination with DAAs. With a 5.7-mo follow-up in 20 DAA-treated HCV patients, a high rate of early HCV-related HCC recurrence was observed[36]. Even though DAA treatment was not associated with HCC or early HCC recurrence, a higher proportion of DAA-treated patients accepted potential curative therapy for recurrent HCV-related HCC compared to untreated patients (32.0% *vs* 24.6%) and developed a non-significant complete or partial response (45.3% *vs* 41.0%)[37]. A systematic review has highlighted an association of HCC recurrence with the status of previous HCC recurrences and the shorter interval between HCV-related HCC complete response and initiation of DAA treatment, and similar recurrences in patients treated with DAAs, those treated with interferon, or untreated patients [38]. HCV-related HCC patients, who had a shorter interval between HCC treatment and DAA therapy (less than 4 mo), appeared to be at greater risk, with a relapse rate of 41.2%[39]. DAA treatment following curative HCC therapy was not associated with early or advanced cancer recurrence[40]. DAA treatment is not associated with a high risk of recurrence in LT patients with HCV and HCC who achieved an original complete response to local-regional therapy, but rather involves a low risk of waitlist dropout due to cancer aggression or death[41]. In three separate prospective cohorts, no increased risk of HCC recurrence following DAA therapy was found, particularly in patients who received curative treatment, such as LT[42].

Even though the impact of DAAs on HCV-related HCC recurrence remains debatable, the results of anti-hepatitis B virus (HBV) treatment following HCC therapy showed that NAs might potentially inhibit HCC recurrence after curative hepatectomy in patients with HBV-related HCC[43]. Managing viral conditions and reactivation of viral replication plays a major role in suppressing HCC recurrence, maintaining liver function, and improving survival for HBV-related HCC post-therapy[44]. After curative therapy, NAs significantly improved recurrence-free survival and OS in HBV-related HCC patients, and entecavir was on par with other NAs, including lamivudine and adefovir, in this regard [45]. Another study discovered that after curative therapy, antiviral therapy with NA could increase survival and reduce early recurrence in patients with HBV-related HCC[46]. NA with or without anti-HBs immunoglobulins was significantly effective in inhibiting post-LT HBV recurrence[47]. In a limited sample cohort, NA did not lower the short-term recurrence rate but increased the elimination of postoperative serum HBV and remnant liver volume, which resulted in significantly improved tolerance to follow-up treatment for HCC recurrence[48]. In a large cohort of 4569 patients with HBV-related HCC who underwent curative resection, the anti-HBV therapy cohort had a significantly lower 6-year HCC recurrence rate than the control cohort [anti-HBV therapy, 45.6%; 95% confidence interval (CI): 36.5%-54.6% *vs* control, 54.6%; 95%CI: 52.5%-56.6%][49]. According to a previous study, recipients who accepted LT by removing HBV-infected initial liver at undetected serum HBV DNA levels continue to have an elevated risk for posttransplant recurrent HBV due to the absence of any particular treatment [50]. In comparison to lamivudine for HCC after curative therapy, entecavir is associated with a four-fold higher one-year OS rate and lower HCC recurrence, suggesting that entecavir may be more suitable for HBV-related HCC patients[51].

REPEAT RESECTION

Only a small proportion of patients with recurrent HCC are candidates for repeat hepatectomy due to recurrent multifocal tumors and compromised liver function[52]. Twenty-two patients with recurrent HCC following LT (2 intrahepatic HCC patients and 20 extrahepatic HCC patients) received complete hepatectomy and had a longer median survival of 35 mo than unresected patients with a median survival of 15 mo[53], suggesting a less aggressive tumor biology. According to a retrospective cohort study, 15 patients with HCC recurrence who underwent LT had a better 5-year OS rate and 5-year DFS rate than the patients with RFA treatment (35% *vs* 28%, and 16% *vs* 0%, respectively)[54]. A recent study reported that repeat laparoscopic liver resection (LR) for recurrent HCC is both feasible and suitable with promising short-term results[55]. Laparoscopic repeat LR was associated with shorter hospitalization and prolonged operation time compared to open repeat LR for recurrent HCC but they had similar perioperative results for primary HCC except for a longer operation time[56]. Patients who underwent wedge resection during laparoscopic repeat LR showed a significantly lower postoperative complication rate than open repeat LR (7.2% *vs* 21.8%)[57]. Even though patients with open LR have a higher morbidity rate than those who underwent LR for primary HCC, there are no striking differences in the clinical characteristics of repeat laparoscopic LR based on prior resection method (open or laparoscopic) or tumor location (segments 7 and 8 or other)[55].

A meta-analysis of 767 patients, 334 of who had repeat laparoscopic hepatectomy and 433 of whom had repeat open hepatectomy, discovered that repeat laparoscopic hepatectomy resulted in less intraoperative blood loss, fewer major complications, shortened hospitalization, and a higher rate of R0 resection[58]. The repeat-surgery group had better liver function, long recurrence-free survival (16.5 mo *vs* 11.4 mo), and better 5-year survival after recurrence (repeat surgery group *vs* non-surgery group: overall, 53.0% *vs* 25.7%; intrahepatic recurrence, 73.8% *vs* 37.2%; extrahepatic recurrence, 30.0% *vs* 0%; intrahepatic and extrahepatic recurrence, 34.1% *vs* 10.6%) compared to the non-surgery group[59] for recurrent HCC. Patients with recurrences within 6 mo of resection had poor survival outcomes than those who experienced recurrences later, and patients with intrahepatic-only recurrences had a better prognosis than those with either extrahepatic-only or intra and extrahepatic recurrences[60]. Additionally, repeated resection of recurrences with a remediable objective produced better outcomes than other therapy options[60]. After 18 mo of initial hepatectomy, repeat hepatectomy may be suggested as a treatment for recurrent HCC. When compared to patients with intrahepatic metastasis, repeat hepatectomy improves survival rates in HCC patients with multicentric occurrence[61]. Although RFA is associated with lower grade 3 morbidity and shorter hospital stay, repeated hepatic resection resulted in a longer median recurrence-free survival *vs* RFA (23.6 mo *vs* 15.2 mo) in patients with recurrent HCC[62]. Resection can be advised as a treatment option for patients with extrahepatic recurrent HCC in conjunction with local treatment for intrahepatic recurrent HCC due to the superior outcomes[63]. At the third (71.3% *vs* 65.7%), fifth (59.9% *vs* 45.4%), and tenth (35.4% *vs* 32.2%) year follow-up, repeat hepatectomy improved long-term OS more than RFA and showed a late survival advantage for patients with recurrent HCC despite a higher morbidity rate[64].

LT

According to reports, the results of salvage LT (SLT) for recurrent HCC following hepatectomy are comparable to the outcomes of initial transplantation, even when examined on an intention-to-treat basis[65]. LT seems to be the most effective treatment for HCC patients to remove both tumors and underlying liver diseases, but the scarcity of organ donors available globally and stringent criteria for patients who are not eligible for transplantation are the major challenges. However, recurrent HCC patients following LT have a poorer prognosis, with a median OS of 10-13 mo as opposed to 2-3 years for patients who had hepatectomy[66-68]. In 2000, Majno *et al*[69] made the first suggestion for SLT, which was used in patients with recurrent HCC or liver dysfunction following primary hepatectomy as initial treatment. Fortunately, liver transplants were an option for 80% of patients with recurrent HCC following curative hepatectomy[70]. A case of salvage living donor LT in a patient with tumor recurrence following surgical resection of combined HCC and cholangiocarcinoma has multiple tumor recurrences after 21 mo due to the more aggressive tumor biology of this type of cancer[71]. The patients receiving SLT therapy demonstrated better DFS than those receiving re-resection or RFA, which is a beneficial strategy for intrahepatic recurrent HCC, particularly for patients with multicentric occurrence that is related to better long-term outcomes than the intrahepatic metastasis pattern[72].

SLT (n = 16) revealed poorer short-term perioperative results than repeat LR (n = 16), with a higher incidence of morbidity (57.8% *vs* 5.4%), reoperations (39.1% *vs* 0%), renal dysfunction (30.1% *vs* 3%), bleeding (19.8% *vs* 2.2%), prolonged intensive care unit stay (4 d *vs* 0 d), and hospitalization (19.8 d *vs* 7.1 d) but significantly decreased recurrence (15.4% *vs* 70.3%) and 5-year cumulative incidence of recurrences (19.4% *vs* 68.4%) to improve long-term survival outcomes for recurrent HCC[73]. SLT was found in a meta-analysis to have higher blood loss, longer hospital stays and surgeries, increased DFS, and elevated risk of postoperative morbidity than repeat LR, while there was no clear difference in

postoperative mortality or OS[74] for recurrent HCC. In terms of disease-specific and recurrence-free survival of patients with intrahepatic HCC recurrence, SLT with transplantable patients is superior to repeat resection, even in patients with Child-Pugh class A liver cirrhosis[75]. Only 56% of cases can be cured using the SLT strategy. A successful SLT strategy is predicted by higher end-stage liver disease scores at the start of the strategy and the absence of pre-resection TACE[76]. Even though SLT is associated with a higher rate of surgical complications, SLT for recurrent HCC following primary hepatic resection is still an efficient and safe treatment that increases survival and reduces tumor recurrence compared to patients with HCC exceeding the Milan criteria who accepted primary orthotopic LT[77]. After hepatectomy, HCC patients with larger tumor sizes were more likely to experience relapse, even with SLT. As a result, LT should be recommended as soon as possible, ideally within a year, for patients with recurrent HCC after LR, followed by meeting the requirements for transplantation[78]. For patients with recurrent HCC after hepatectomy, SLT has a poorer OS and RFS, as well as a higher risk of recurrence and death compared to primary LT, particularly for those who meet the Milan criteria[79]. Another study discovered no difference between patients receiving primary LT and SLT for HCC recurrence following primary treatment with LR or RFA in terms of the 5-year risk of recurrence and 5-year actuarial survival[80]. SLT for relapsed HCC patients after initial LR followed by SLT showed overall and recurrence-free survival rates on par with primary LT. Despite this, there are higher rates of Child-Pugh class A, more than three transplant treatments, and reoperation for postsurgical bleeding[81]. Patient background possibly has various effects on therapy, as Hong Kong patients with recurrent HCC following LR who received SLT, but not Roman patients, showed an increased recurrence rate[82].

RFA

Clinical therapy for HCC frequently involves ablation. Following ablation, the tumor experiences residual and local recurrence due to asymmetrical heat diffusion and heat absorption *via* circulating blood or air around the tumor[83]. For HCC patients who experience recurrence but cannot undergo a suitable operation, ablation is used as a safe and efficient therapy[84]. With ablation alone, the 5-year recurrence rate of HCC patients was 70%[85]. Although a small set of 11 patients with relapsing HCC following LT embraced microwave ablation without serious side effects, this safe technique still needs to be validated in larger studies or compared with other treatment options[86]. RFA and repeat resection are better choices for late-relapsing HCC patients post-curative hepatectomy who meet the Milan criteria[87]. Although the 1-, 3-, and 5-year OS (90.7%, 69.04%, and 55.6% *vs* 87.7%, 62.9%, and 38.1%, respectively) and progression-free survival (PFS) (56.5%, 27.9%, and 14.6% *vs* 50.2%, 21.9%, and 19.2%, respectively) were comparable between the RFA and repeat resection groups for locally recurrent HCC following primary resection, the former was superior to the latter in term of complications and hospitalization[88]. In a different study, repeat resection was found to increase survival for recurrent HCC, particularly for patients who had relapsed within two years and whose primary tumor burden exceeded the Milan criteria[89]. Primary HCC (94.8%, 75.7%, 61.6%, and 47.3%, respectively) and recurrent HCC (91.9%, 71.2%, 58.7%, and 45.2%, respectively) did not differ in the 1-, 3-, 5-, or 10-year OS rate[90]. RFA offers comparable long-term survival whether treatment is for the first-time or recurrent HCC that is 5 cm or less. Although LR with long-term survival results is superior to RFA for recurrent HCC patients, RFA is a good alternative to LR in patients with small-sized recurrence or patients with a limited number of recurrent nodules, even though LR has better long-term survival outcomes for patients with recurrent HCC[91]. Multiprobe stereotactic RFA as first-line therapy for recurrent HCC following LR has such low morbidity that the OS and DFS rates at 1, 3, and 5 years were 94.0%, 70.2%, and 53.3%, and 52.6%, 19.7%, and 15.8%, respectively[92]. RFA is beneficial and effective for intrahepatic recurrent HCC with 1-, 3-, and 5-year OS rates of 68.5%, 40.3%, and 40.3%, respectively, particularly for recurrent HCC following LT in the absence of finite extrahepatic metastases[93]. Due to its advantages of being less invasive, extreme selectivity, and reproducibility, RFA is suggested as a better therapy for intrahepatic HCC recurrence given that it is associated with a lower recurrence-free survival than LT[94].

In patients with recurrent HCC (tumor size < 3 cm, tumor number ≤ 2), a phase III non-inferiority trial found that the 2-, 3-, and 4-year local PFS of proton beam radiotherapy was comparable to that for RFA. However, the most common adverse outcomes were radiation pneumonitis (32.5%) and decreased leukocyte counts (23.8%) for proton beam radiotherapy, and increased alanine aminotransferase levels (96.4%) and abdominal pain (30.4%) for RFA[95], which suggested that proton beam radiotherapy was tolerable and safe with long PFS values comparable to those of RFA.

TACE

TACE is generally considered a standard therapeutic method for patients with unresectable HCC[11]. The most widely used treatment for postoperative recurrence is TACE, especially when there is a large mass or multifocal relapsed HCC[96]. The outcomes of TACE in the neoadjuvant setting are debatable.

OS showed no difference between 71 patients treated with TACE before surgery and 21 patients who underwent surgery without TACE[97]. In a retrospective study, 1457 patients were evaluated, of whom 120 were treated with preoperative TACE, and it was found that 5-year DFS was improved in patients treated with TACE[98]. Patients with primary HCC who undergo embolization have a strikingly higher chance of survival than those with recurrent HCC. A study revealed that primary HCC patients who received TACE had a median survival of 30 mo and a 29% 3-year survival rate[99]. The results of treating patients with recurrent HCC, however, showed a low median survival time of 19 mo and an 11% survival rate. Patients with primary HCC and microvascular invasion (MVI) experience recurrence after resection, and TACE treatment is more effective than resection and RFA for recurrent HCC[100]. There was no significant difference in prognostic factors or OS between the initial and recurrent TACE groups[101]. TACE was administered to 28 patients with recurrent HCC following LT; 19 of these patients (67.9%) experienced tumor-shrinking by over 25%. However, the 1-, 3-, and 5-year survival rates were lower (47.9%, 6.0%, and 0%, respectively) due to extrahepatic metastases or intrahepatic recurrences[102]. According to a different study, patients who underwent chemoembolization without experiencing any serious side effects had a significantly longer OS time following the diagnosis of HCC recurrence post-LT than those who did not receive the treatment[103]. The 1-, 3-, and 5-year OS rates did not significantly differ between the repeat resection or RFA and the TACE groups, suggesting that TACE likely was as effective as repeat resection or RFA for preventing early intrahepatic relapse following curative resection of HCC[87]. Although there is no obvious difference between RFA and TACE treatment for isolated intrahepatic recurrent HCC following LT in terms of 2-year DFS rate (20% *vs* 14%) and 4-year OS rate (33% *vs* 25%), TACE treatment seems to be more beneficial in isolated intrahepatic recurrent HCC patients following LT when RFA therapy is not suitable[104]. In contrast to TACE-alone treatment for intrahepatic recurrent HCC after hepatectomy, apatinib, a vascular endothelial growth factor receptor 2 inhibitor, in conjunction with TACE significantly improved the median PFS, short-term objective responses, and disease control rate, and had a tendency of increasing the 1- and 2-year OS rates[105]. The patients who received TACE-RFA for recurrent HCC that was less than 5 cm following LT had a higher DFS than those who received TACE alone[106]. After a follow-up of 24 mo, the median OS for patients with the first recurrence of HCC treated with multimodality therapy was 40 mo (range 8-85 mo), far exceeding that of patients with LR/ablation (27 mo, range 4-75 mo), TACE/XRT (13 mo, range 4-68 mo), and systemic treatments (26 mo, range 3-59 mo)[9].

SORAFENIB

Sorafenib, a multitarget tyrosine kinase inhibitor (TKI) and the first approved drug for HCC patients, is most frequently used as an adjuvant therapy in resected HCC patients[107] and as a frontline systemic treatment in patients with HCC recurrence after LT. However, the current data are mainly based on observational research due to the exclusion of Randomized Protocol studies and Asia-Pacific trials of sorafenib from the registered studies for HCC[107,108]. Sorafenib has a few drawbacks, including poor oral bioavailability and drug toxicities, and its OS is only marginally improved by 2.8 mo[107,109]. The impacts of sorafenib in patients with recurrent HCC who underwent an incurable liver transplant have been estimated in several retrospective studies. Based on a retrospective cohort study of 50 patients with recurrent HCC following liver transplants who initially accepted sorafenib, an objective response rate of 16% and stable disease in 50% of this population were observed, and the median OS was 18 mo[110]. Patients with HCC recurrence following LT treated with sorafenib had a better median survival of 42 mo compared to 16.2 mo for patients not receiving sorafenib, supporting the notion that sorafenib increases survival[111].

According to this study, patients with relapsed HCC have a better chance of longer OS and a better prognosis by receiving the sorafenib treatment. For patients with recurrent HCC, sorafenib-lenvatinib continuous treatment and radical resection together with nonoperative therapy were both independent favorable factors for post-recurrence survival[112]. According to Lee *et al*[113], sorafenib for recurrent HCC is associated with a better prognosis because it involves smaller intrahepatic HCC combined with favorable liver function in LT recipients, which may explain why the median OS (16.8 mo *vs* 7.1 mo) and time to development were higher in 42 HCC patients in the LT group than in 790 non-LT patients. Sequential sorafenib treatments are similarly common in recurrent HCC patients following LT. These treatments improve OS compared to non-LT treatments and do not suppress systemic treatments with concurrent antirejection strategy[35]. Treatment with sorafenib and TACE was associated with a higher 5-year OS and PFS compared to those treated with TACE alone in patients with recurrent intermediate-stage HCC and lesions positive for MVI, but patients with MVI-negative lesions did not show a survival benefit from combined therapy[114]. Treatment with sorafenib plus TACE improves hepatic reserve, leads to a better OS, and results in longer intervals between TACE rounds in TACE-refractory patients with recurrent advanced HCC than repeated TACE treatments[115]. The RFA plus sorafenib treatment resulted in a significantly improved OS than RFA alone (1-, 3-, and 5-year OS rates of RFA-sorafenib *vs* RFA: 97.7%, 83.7%, and 54.7% *vs* 93.1%, 61.3%, and 30.9%, respectively), suggesting that adjuvant sorafenib combined with RFA was superior to RFA alone in improving survival results in patients with

recurrent HCC who meet the Milan criteria after initial LT[116]. Prussian blue (PB) nanomaterial is safe and has multiple roles as an antidote to thallium poisoning[83]. With minimal injury to surrounding healthy tissues, photothermal therapy is a highly effective and noninvasive therapeutic option[117]. By using human and mouse HCC cell lines, Zhou *et al*[118] developed HCC-targeted SP94 peptide and cyanine (Cy) 5.5-conjugated PB nanoparticles loaded with sorafenib for HCC-targeted multimodality imaging and combined photothermal therapy/sorafenib treatment. These nanoparticles accumulated in HCC tumor sites and then controlled the release of sorafenib to eradicate the tumor without any local recurrence and with a minimal amount of toxic side effects.

OTHER TKIS

The United States and Europe approved lenvatinib in the first line, cabozantinib, and ramucirumab in the second line as a potential systemic therapeutic approach for liver transplant recipients with relapsed HCC. A retrospective multicenter study discovered that regorafenib, a multitarget TKI, was safe and effective for patients with recurrent HCC following LT who were tolerable to sorafenib, with a median OS of 12.9 mo[119]. Lenvatinib, as a TKI, has been used as an optional frontline treatment strategy. Patients with recurrent HCC treated with lenvatinib who are tolerable to sorafenib have a longer median OS (19.5 mo), far exceeding those who are receiving intermittent sorafenib or regorafenib following sorafenib failure (12 mo)[112]. Cabozantinib, a TKI of vascular endothelial growth factor receptor 2 (VEGFR2), is used as an effective and safe monotherapy to proceed with third-line systemic therapies in advanced HCC[120]. Patients with recurrent HCC who received lenvatinib treatment had decreased expression of programmed death ligand 1 (PD-L1) and Treg infiltration in the tumor compared to the matched primary tumor, suggesting that lenvatinib targets fibroblast growth factor receptor 4 to increase the antitumor immune response of anti-programmed cell death-1 (PD-1) treatment, which is accompanied by decreased expression of tumor PD-L1 and Treg infiltration[121]. As a multi-kinase inhibitor, cabozantinib is expected to be an effective treatment for advanced HCC patients with sorafenib tolerance[122]. A case study identified a patient with recurrent HCC who had more than 10 years of survival after receiving an intensive multimodal therapeutic strategy that included surgery, RFA, and systemic therapy with cabozantinib as the second-line therapy in living-donor LT[123]. In patients with HCC recurrence following LT with sorafenib tolerance, regorafenib treatment resulted in a longer median OS (28.8 mo) than best supportive care (15.3 mo). This makes regorafenib a safe and effective second-line treatment option[124].

IMMUNE CHECKPOINT INHIBITORS

Although the importance of immune evasion in the progression of HCC recurrence was widely acknowledged, the lack of effective medications to reverse cancer-related immune suppression remained an untreatable condition until recently. Programmed cell death receptors on T cells and their ligands PDL-1 and PDL-2 on tumor cells are the targets of immune checkpoint inhibitors (ICPIs). Only 15–20% of patients benefit from anti-PD-L1 monoclonal antibodies (mAbs), which block interactions with PD-1 and PD-L1 and restore the roles of T cells in the tumor microenvironment[125,126]. Stimulation-induced immune surveillance has notable antitumoral outcomes in advanced and recurrent HCC, with significant response rates and even complete responses. Despite their promising prospects, ICPIs must be used with caution in transplant patients due to the complexity of HCC. In particular, HCC patients with multifocal tumors, higher AFP levels, larger tumor volume, and poorer differentiation presented a high risk of post-LT relapse when given neoadjuvant ICPIs[127]. The perioperative nivolumab *vs* ipilimumab/nivolumab combination had fine effects, according to a phase II study, with a 29% complete response rate[128]. The immune checkpoint blockade remedy resulted in only 16%-20% response rates among patients with advanced HCC[129]. Combination therapy with anti-PD-1 plus RFA for recurrent HCC achieved a superior recurrence-free survival compared to RFA monotherapy[130]. By combining anti-PD-L1 mAb with SP94-PB-sorafenib-Cy5.5 nanoparticles plus near-infrared therapy, Zhou *et al*[118] also observed the production of extraordinary results, such as suppression of distant metastases and obstruction of cancer relapse. Note that, different from primary HCC, the therapeutic strategy for recurrent HCC following LT has to be discrete due to the higher risk of allograft rejection or graft loss[131,132]. For early HCC recurrence after radical resection, TKIs combined with PD-1 therapy demonstrated a better survival benefit than TKIs alone[133]. In a patient with recurrent, refractory, metastatic HCC following LT, PD-1 inhibitor eliminated lung metastases and resulted in a partial radiological response of metastatic retroperitoneal lymph nodes after 13 cycles[134].

HBV-SPECIFIC T-CELL IMMUNOTHERAPY

Chimeric or classical T-cell receptors (TCRs)-redirected T cells target HBV antigens/epitopes expressed on HBV-infected hepatocytes or in HCC cells as an immunotherapeutic approach. According to a case study, the HBV antigen was expressed in the metastases of a patient with HBV-related HCC after LT [135]. To treat extrahepatic metastases of chemotherapy resistance, HCC autologous T cells were genetically redirected to express an HBsAg-specific T cell receptor. This resulted in decreased HBsAg levels without worsening liver inflammation or other toxicity[135]. In two patients with metastatic recurrence of HBV-related HCC after LT, immunotherapy of HBV-specific TCRs was safe and did not cause any damage to liver function over a year[136]. Notably, a patient appeared to have a reduced volume in 5 of 6 pulmonary metastases during the first year of T-cell management[136]. HBV-specific TCR T-cells transiently escape the immunosuppressive effects of tacrolimus and mycophenolate mofetil owing to the activation of CD39+ Ki67+ peripheral blood mononuclear cells, which are positively correlated to clinical outcomes in patients with HBV-related HCC relapses following LT[137].

OTHER IMMUNOTHERAPIES

Cytokine-induced killing (CIK) cell-based immunotherapy has gained popularity as a promising new adjuvant therapy approach. CIK cells are a mixture of T lymphocytes, which are *ex vivo* amplified with cytokines and comprised of CD3+/CD56+ and CD3+/CD56- T cells, as well as CD3-/CD56+ NK cells, which have potent antitumor activity with the combined ability of both T cells and NK cells and minimal cytotoxicity to normal cells, but tremendous specificity to cancer cells[138]. Multiple clinical trials revealed that CIK cell-based immunotherapy increased RFS in HCC patients who underwent surgical resection[139,140]. The production of an individual autologous CIK cell-based immunotherapeutic agent involves activating peripheral blood mononuclear cells from the relevant patients with IL-2 and anti-CD3 antibodies[141]. According to research by Lee *et al*[141], the average RFS for HCC patients who accepted the CIK cell-based agent after curative therapy was 44.0 mo, as opposed to 30.0 mo for those who did not receive adjuvant immunotherapy. The results of a meta-analysis reported that the results of DC-based immunotherapy increased antitumor immunity, enhanced survival rate, and improved survival times in HCC patients[142]. Another meta-analysis listed 22 distinct studies with 3756 HCC patients that received DC-based vaccine and/or CIK-based adoptive therapy after receiving different HCC interventional therapies. These studies showed a prolonged OS (6 mo, 1, 3, and 5 years) and reduced mortality and recurrence at 1, 2, and 3 years but not 5 years[143]. For HCC patients, a personalized neoantigen vaccine served as a safe, practical, and effective anti-recurrence treatment [144]. After a radical operation on seven postoperative HCC patients who had received all of the planned neoantigen vaccinations, five of them showed neoantigen-activated cell responses and longer RFS than the other five patients, who had only received primary vaccination and had propensity scores that matched with those of control patients[144]. After curative resection or RFA in the first stage, the personalized neoantigen-loaded DC vaccine and neoantigen-activated T-cell therapy were successfully used on ten patients with HCC without unexpected delay or grade 3 therapy-related side effects[145]. New circulating multiclonal neoantigen-specific T-cell responses, activated neoantigen-specific immunity, an upregulated immune stimulatory signature, increased immune-cell infiltration, and elevated T-cell inflammatory gene expression, were produced in 70% of patients who had improved DFS compared to non-responders, and 71.4% of patients were without relapse for 2 years after curative treatment. Neoantigen depletion (immunoediting) also increased in recurrent tumors compared to primary tumors, suggesting that immune evasion developed as a result of immunological therapy[145].

CONCLUSION

With its unique characteristics, recurrent HCC is still a difficult disease to treat. Every stage of the disease calls for a multidisciplinary approach, which is still predominantly evolving. LT and hepatectomy remain successful therapeutic strategies for patients with recurrent HCC. Additionally, neoadjuvant and/or adjuvant therapy techniques may improve the long-term prognosis and increase the chance of cure in refractory patients with recurrent HCC. Relying on the tumor biology and possible hepatic reserve, multimodality therapy should be used in patients with recurrent HCC. By simultaneously optimizing oncologic outcomes and minimal side effects, this therapy helps these patients have better OS and tolerability.

FOOTNOTES

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REVIEW

Comparison between metabolic-associated fatty liver disease and nonalcoholic fatty liver disease: From nomenclature to clinical outcomes

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Abstract

As a result of the obesity epidemic, Nonalcoholic fatty liver disease (NAFLD) and its complications have increased among millions of people. Consequently, a group of experts recommended changing the term NAFLD to an inclusive terminology more reflective of the underlying pathogenesis; metabolic-associated fatty liver disease (MAFLD). This new term of MAFLD has its own disease epidemiology and clinical outcomes prompting efforts in studying its differences from NAFLD. This article discusses the rationale behind the nomenclature change, the main differences, and its clinical implications.

Key Words: Metabolic associated fatty liver disease; Non alcoholic fatty liver disease; Fatty liver disease; Obesity; Diabetes mellitus

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Core Tip: A new nomenclature to represent the underlying pathophysiology of fatty liver disease has been created and labeled metabolic-associated fatty liver disease. This article discusses the rationale behind the nomenclature change, the main differences to nonalcoholic fatty liver disease, and its clinical implications.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a well-established terminology that was first coined by Ludwig and colleagues in 1980[1] to describe fatty liver disease arising in the absence of significant alcohol intake. Over the last four decades, there has been a rapidly growing global burden of NAFLD and its subtype nonalcoholic steatohepatitis (NASH)[2] which has a potentially progressive course that can lead to cirrhosis, hepatocellular carcinoma, liver transplantation, and potential death[3]. Currently, it is one of the most common causes of liver disease worldwide affecting nearly a quarter of the population[4,5], with increased recognition and diagnosis in younger individuals[6].

The rising body of research on NAFLD/NASH has led to a better understanding of its underlying pathophysiology and its relationship with metabolic syndrome[7]. Indeed, obesity and diabetes are the strongest risk factors associated with NAFLD/NASH. The public health and economic impacts of fatty liver disease have provoked extensive clinical trial activity targeted toward finding treatments for NASH among patients, regulators, and the biotechnology and pharmaceutical industries[8]. Despite this rapidly evolving activity, NASH resolution, and fibrosis regression rates are only 20%-30%[9].

In an effort to recognize the importance of metabolic abnormalities in an inclusive rather than an exclusive diagnosis, a group of international experts suggested a change of the name from NAFLD to metabolic-associated fatty liver disease (MAFLD)[10-12]. The criteria to diagnose MAFLD are based on evidence of hepatic steatosis in addition to one of the following three criteria: overweight/obesity, presence of type 2 diabetes mellitus, or evidence of metabolic dysregulation. Hence, MAFLD is more reflective of the heterogeneous pathogenesis of metabolic fatty liver diseases than NAFLD.

Immediately after the reappraisal of the nomenclature, multiple studies have been carried out to better understand the epidemiologic impact of this new terminology and its differences from NAFLD. For example, in a US population-based study by Kim *et al*[13], it was found that MAFLD was associated with an increased risk of all-cause mortality after adjusting for metabolic risk factors, while NAFLD was not. Interestingly, insulin resistance and stage of fibrosis were predictors of increased liver mortality in NAFLD but not MAFLD whose liver-associated mortality is primarily driven by alcohol-associated liver disease[14]. However, an awareness of the differences between these conditions and their early recognition remains poor among general practitioners[15]. Given patients with fatty liver disease are usually asymptomatic, a high index of suspicion is required to make the diagnosis. Additionally, the clinical guidelines of MAFLD and NAFLD need to be updated on a rolling basis to keep up with the most recent management practices to prevent disease progression.

In this article, we will discuss the rationale and history behind the nomenclature change, as well as the core differences between MAFLD and NAFLD with respect to various clinical aspects in contemporary practice.

NOMENCLATURE AND HISTORY

"Fatty liver" was first described by Thomas Addison in 1836, who noted alcohol-related steatotic changes in liver histology[16,17]. A couple of decades later in 1857, George Budd noted similar histology in inactive, obese patients with a high-fat diet and alcohol intake[18]. Subsequently, Austin Flint observed a correlation between high carbohydrate intake with worsening steatosis and interval cirrhosis [19].

By the early 1900s, fatty liver changes unrelated to alcohol were well established but the mechanism of injury remained unclear. The role of diabetes as a risk factor for fatty liver began to be recognized by Pfluger in 1905 who noted an increase in hepatic steatosis in dogs who developed diabetes following total pancreatectomy[20]. These findings were later extrapolated to humans in 1936 when diet and newly discovered insulin were suggested as treatments for hepatomegaly secondary to steatosis in patients with juvenile diabetes[21].

In 1979, Adler and Schaffner developed a schema for fatty liver disease in overweight non- and light drinkers which included "fatty liver", "fatty hepatitis", "fatty fibrosis", and "fatty cirrhosis"[22]. A year later, fatty hepatitis would be designated "nonalcoholic steatohepatitis" (NASH) when Dr. Ludwig coins the term during characterizations of 20 Liver biopsies in primarily female patients with obesity and/or diabetes harboring lobular hepatitis, focal necrosis, and Mallory bodies on histology[1]. Not long after in 1986, NASH was included in the spectrum of "non-alcoholic fatty liver disease" NAFLD by Schaffner and Thaler[23,24].

Because the histology of alcohol-related and NAFLD steatosis is nearly indistinguishable from each other, NAFLD is a diagnosis of exclusion. Despite its name, NAFLD ironically includes patients with alcohol consumption of less than 14 and 7 drinks per week for men and women, respectively[25]. Further blurring the lines between NAFLD and alcoholic liver disease are studies recognizing that heavy alcohol drinkers who are obese are more likely to develop cirrhosis than non-obese heavy drinkers[25,26].

As more studies on NAFLD arose in the 21st century, NASH reappeared consistently as part of a syndrome including obesity, arterial hypertension, insulin resistance, dyslipidemia, and/or cardiovascular disease[27,28]. To better capture this syndrome, terms like "metabolic syndrome", "Syndrome X", "Insulin Resistance syndrome", and "the deadly quartet" were used[29]. The need for a more unified definition was reiterated in 2005, considering that the name "NAFLD" in no way highlighted the underlying metabolic etiologies, associated risk factors, or the phenotypic heterogeneity of the disease[30]. This paved the way for the introduction of the term "metabolic-associated fatty liver disease" in 2011 and was subsequently adopted in 2020 by international consensus[10,12]. Whereas NAFLD is defined as the presence of steatosis in > 5% of hepatocytes in the absence of other liver disease etiologies, MAFLD is defined by hepatic steatosis and components of the metabolic syndrome. MAFLD recognizes the positive determinants of the disease rather than defining the disease as the absence of other diseases, akin to the transition from “Non-Hepatitis A/Hepatitis B” to formally recognizing that entity as “Hepatitis C”[17]. Just as simultaneous alcohol-related liver disease and viral hepatitis can vary in disease behavior and prognosis from either entity alone, MAFLD can analogously exist with other liver diseases, including alcohol-related liver disease as patients with concomitant liver disease causes may have different outcomes than those of either disease apart[31-33].

EPIDEMIOLOGY

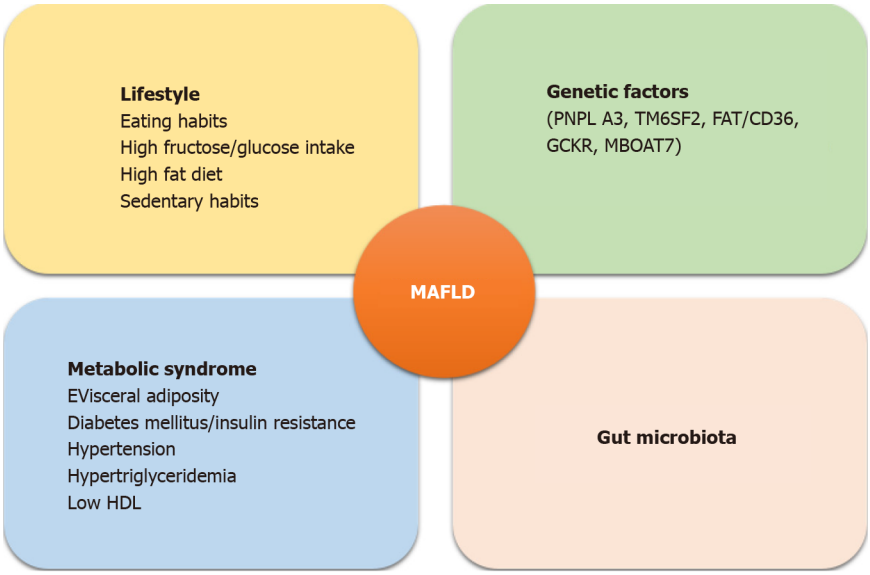
The adoption of the new inclusive nomenclature and diagnostic criteria for MAFLD[10] has called for multiple studies and[34-40] several meta-analyses estimating the prevalence of the disease under the new diagnostic criteria in the setting of rising numbers of patients with overweight, obesity and type 2 diabetes mellitus[41]. These studies have estimated a global prevalence of 24.2% to 39.22% for MAFLD, comprising half of the overweight and obese adults[42], compared to a 15.3% to 33.86% for NAFLD[43]. MAFLD and NAFLD patients share clinical and pathogenic features leading to similarities in their overall prevalence. However, there are differences based on the presence of other liver diseases (*i.e.*, alcoholic liver disease) that would still meet the criteria for MAFLD but not for NAFLD.

Compared to NAFLD, MAFLD is more likely to be diagnosed in Europe and Asia[44,45]. In addition, a non-statistically significant trend toward increased MAFLD in Hispanic ethnicity was reported[13]. Male sex, higher body mass index, lower high-density lipoprotein, higher triglyceride levels, and elevated aminotransferases carry a more significant correlation with MAFLD. Additionally, patients with MAFLD are more likely to have hypertension, diabetes mellitus, and chronic kidney disease[45]. Higher aminotransferases and the presence of advanced liver fibrosis in ultrasound elastography and liver biopsy are more common in patients with MAFLD+/NAFLD- when compared to a similar population with MAFLD-/NAFLD+. Mild alcohol consumption has been noted to be associated with a higher prevalence of significant fibrosis in those patients[46].

An analysis of the National Health and Nutrition Examination Survey (NHANES) database of the United States from 2017 to March 2020 found that the sample of patients with MAFLD had a higher prevalence of malignancies, coronary artery disease, myocardial infarction, heart failure, chronic pulmonary diseases, and psychiatric disorders, including sleep problems and depression when compared to the sample of patients with NAFLD; with the majority of these conditions being present in patients with more significant liver fibrosis[47].

PATHOPHYSIOLOGY AND RISK FACTORS

The exact pathophysiology of MAFLD\NAFLD remains largely unknown but a combination of genetic and environmental factors plays a crucial role (Figure 1). A contributing mechanism appears to be overnutrition and an increase in visceral adipose tissue[48]. Macrophages invade adipose tissue creating a pro-inflammatory state that promotes insulin resistance and excessive lipolysis enhancing the delivery of free fatty acids to the liver. An increased intrinsic lipogenesis overwhelms the liver's capacity to



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Figure 1 Summarizes the risk factors involved in the development of metabolic-associated fatty liver disease. HDL: High-density lipoprotein; MAFLD: Metabolic-associated fatty liver disease.

metabolize fatty acids resulting in dysfunction of the hepatocytes' mitochondria and endoplasmic reticulum[49]. This dysfunction creates excessive oxidation of fatty acids and production of reactive oxygen species leading to hepatocyte death which promotes further inflammation and a vicious cycle [50].

Risk factors

Role of gut microbiota: Gut microbiotas affect pro-inflammatory and anti-inflammatory balance in the liver by their effect on gut barrier function. Increased fructose intake leads to increased permeability of enteric cells. Fructokinase is an enzyme in the liver that is also highly expressed in the gut. Metabolism of fructose by fructokinase in the intestine can result in increased permeability of enteric cells' tight junctions. This leads to increased absorption of endotoxins into the portal circulation[51]. Endotoxemia activates the innate immune system and the resulting inflammation has been shown to have a role in the transition from steatosis to steatohepatitis and cirrhosis[52] Co-occurrence of fatty liver/NASH and alteration of gut microbiota and disruption of epithelial barrier do not prove causation. The cause or consequence relationship between gut microbiota and NAFLD remains unclear. Some authors have hypothesized that liver damage might precede alteration in gut microbiota and permeability of tight junctions of enteric cells[53].

Gut microbiota also plays an important role in the metabolism of carbohydrates. Gut microbacteria can ferment dietary sugars into alcohol that can enter the portal circulation increasing oxidative stress and inflammation in the liver[54]. Studies have shown that in patients with MAFLD/NAFLD, there are significantly more gut bacteria associated with increased alcohol levels in the blood as compared to obese controls. Furthermore, some bacterial species like Escherichia, Enterobacter, Proteobacteria, and Bacteroides were found to be higher in NASH patients as compared to healthy controls[55-57].

Genetic factors: PNPLA3 (patatin-like phospholipase domain containing 3) genetic variation has been associated with MAFLD/NAFLD independent of metabolic syndrome. This gene codes for I148M that hydrolyzes triglycerides in adipose tissue resulting in increased delivery of free fatty acids to the liver [58].

TM6SF2 (transmembrane 6 superfamily member 2) encodes E167K which is a lipid transporter on the endoplasmic reticulum. Genetic variation in this protein causes loss of function and increased deposition of triglycerides in the liver[59].

Upregulation of genes coding for SREBP1c (sterol regulatory binding protein-1c), chREBP (carbohydrate responsive element binding protein), and PPAR-c (peroxisome proliferator-activated receptor gamma) results in increased *de-novo* lipo-genesis[60].

Other genes associated with MAFLD/NAFLD, and NASH are GCKR, MBOAT7, FAT/CD36, IGFBP2, PGC1alpha, SIRT1, miR-122, and miR-34. HSD17B13 appears to have a protective role[61].

Lifestyle and dietary habits

There is a close association between eating habits, obesity, and NAFLD. Increased consumption of refined carbohydrates, animal proteins, soft drinks, a high-fat diet, and fructose are closely associated

with the development of MALFD/NAFLD[51]. Carbohydrates with high glycemic index led to increased liver glycogen and fat content and increased serum triglycerides. The predominant mechanism is increased de Novo Lipogenesis[62]. Saturated fatty acids and a diet high cholesterol in diet are associated with advanced fibrosis in MAFLD.

Patients with obesity and MAFLD/NAFLD have a more sedentary lifestyle[63,64]. Finally, another modifiable factor associated with advanced fibrosis in MAFLD/NAFLD is a smoking history of > 10 pack-years although the exact mechanism is unknown[65].

HISTOPATHOLOGY OF MAFLD/NAFLD AND NASH

The presence of > 5% steatotic hepatocytes on liver biopsy is considered the minimum histologic criteria to diagnose fatty liver disease. Macrovesicular steatosis is the most common pattern although a mixed macro/microvesicular steatosis is seen in some cases as well. Pure microvesicular steatosis is not common. In MAFLD/NAFLD, small areas of lobular and portal inflammation and lipogranulomatous changes can be seen but features of hepatocyte injury and fibrosis are absent. Steatosis in adults has predilection to start in acinar zone 3 (perivenular)[66]. During the histological examination, the involvement of hepatocytes is assessed in percentages: 0%-33%-mild, 33%-66%-moderate, and > 66% hepatocyte involvement is categorized as severe steatosis[67].

On the other hand, histological features of NASH include steatosis and more severe inflammation than mentioned above along with hepatocyte injury and fibrosis. Ballooning, apoptosis, and/or necrosis are typical features of hepatocyte injury. Ballooning is of particular importance in NASH as its presence has been associated with a more aggressive disease and higher progression to cirrhosis[68]. However, the recognition of hepatocyte ballooning has significant inter-observer variation[69]. Hepatic apoptosis appears as acidophil bodies on liver biopsy. The Acidophil body index (acidophil bodies per mm^2 of tissue) serves as further confirmation of NASH when the diagnosis is uncertain.

Fibrosis in NASH typically starts in zone 3 and has a "chicken wire" pattern which entails the deposition of fibrotic material along sinusoids of zone 3 and around hepatocytes. As the disease progresses, bridging fibrosis and features of macronodular or mixed cirrhosis are seen[70]. Other histological features that may be seen in the fatty liver include megamitochondria, iron deposition, glycogenated nuclei, and Mallory-Denk bodies[71].

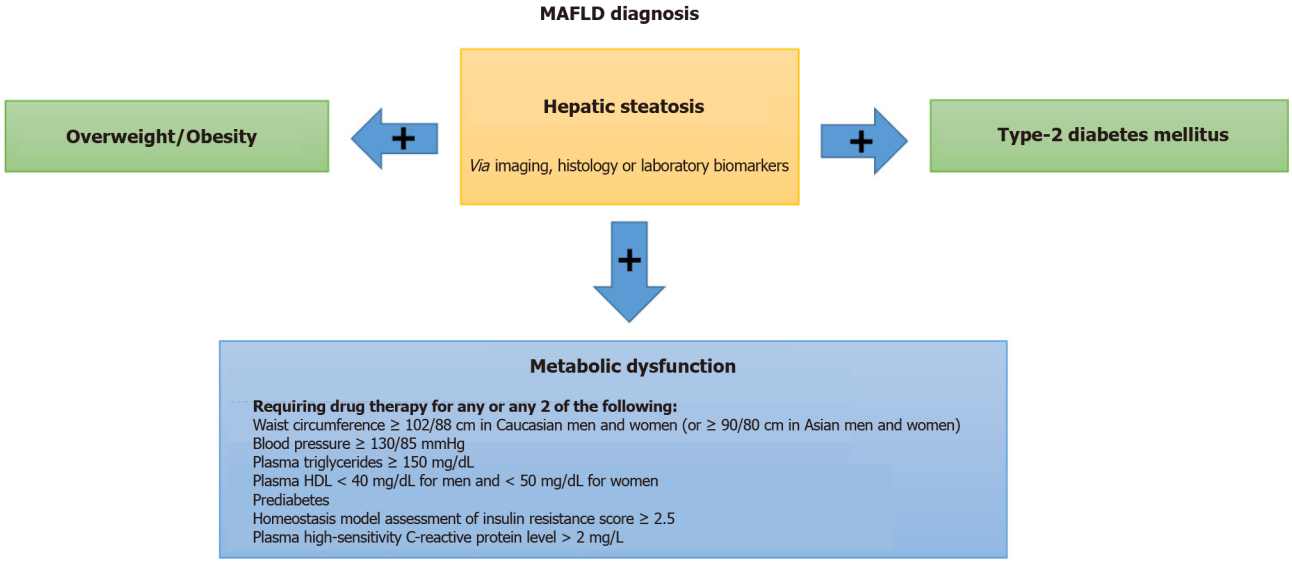
DIAGNOSIS

With recently published guidelines, experts have refined the criteria for the diagnosis of MAFLD. The new criterion involves the presence of hepatic steatosis in adults and the presence of one of the following: Type 2 diabetes mellitus, overweight/obesity, or metabolic dysregulation. Proposed diagnostic criteria for MAFLD are outlined in Figure 2.

Various modalities can be used to evaluate for the presence of hepatic steatosis including ultrasound, ultrasound elastography (*i.e.*, transient elastography, acoustic radiation force impulse imaging, strain elastography), computed tomography, magnetic resonance imaging (MRI), magnetic resonance spectroscopy, MRI-derived proton density fat fraction, histology and serum biomarkers (*i.e.*, fatty liver index, hepatic steatosis index, NAFLD liver fat score). Recently, ultrasound elastography has been increasingly used in clinical practice as compared to ultrasound. Ultrasound has limited sensitivity for detecting liver steatosis < 20% and performance may be suboptimal in patients with higher body mass index (BMI) > 40 kg/m^2[72-74].

Patients with normal body weight can still develop MAFLD. It was demonstrated in a recent study that patients with BMI < 23 kg/m^2 have the same disease severity on histology when compared to patients with BMI > 25 kg/m^2[75]. It is also known that metabolically unhealthy patients who are not obese and have MAFLD are at increased risk for cardiovascular morbidity and liver damage as compared to metabolically healthy individuals[76]. Moreover, hepatic fat can be an early indicator of metabolic dysfunction. Therefore, in the new criteria in addition to diabetes mellitus and overweight/obesity, patients with lean/normal weight with metabolic dysregulation are included. In these patients, the presence of at least two metabolic risk factors is needed for diagnosis. The various risks include waist circumference ≥ 102/88 cm in caucasian men and women, blood pressure ≥ 130/85 mmHg, serum triglycerides ≥ 150 mg/dL, serum high-density lipoprotein cholesterol < 40 mg/dL for men, and < 50 mg/dL for women, prediabetes, serum high-sensitivity C-reactive protein level > 2 mg/L and homeostasis model assessment of insulin resistance score ≥ 2.5. Patients who meet the criteria for MAFLD and also have one or other chronic liver condition causing fatty liver should be classified as having dual etiology (or more) fatty liver disease.

The experts have also suggested that disease severity in MAFLD should be defined by grade of activity and fibrosis stage similar to NAFLD and NASH. Patients without typical histology of steatohepatitis but have cirrhosis can be defined as MAFLD-related cirrhosis if there is past or present evidence of metabolic dysregulation risk factors for MAFLD with at least one of the following: MAFLD on a



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Figure 2 Proposed diagnostic criteria for metabolic-associated fatty liver disease are outlined. MAFLD: Metabolic-associated fatty liver disease; HDL: High-density lipoprotein.

previous liver biopsy or documentation of steatosis by hepatic imaging[77].

MANAGEMENT

Despite the high worldwide prevalence, significant healthcare burden, and costs, there are currently no FDA-approved treatments for NAFLD. The 2018 AASLD practice guidelines on the treatment of NAFLD are based on the following four principles: (1) Effective diet and lifestyle modifications to achieve weight loss; (2) Identification and correction of underlying cardiometabolic risk factors; (3) Pharmacological therapy primarily aimed at improving hepatic steatosis/fibrosis; and (4) Close monitoring and prevention of complications of NAFLD[78].

Obesity and diabetes mellitus constitute the major pathophysiological risk factors for NAFLD, and drugs that modify or alter glucose metabolism and/or body weight comprise the current mainstay therapies focused on improving clinical outcomes, such as the degree of hepatic inflammation and/or fibrosis[3]. Over the past several years, multiple potential treatment options have been extensively investigated in this regard. These include insulin sensitizers and glucose-lowering drugs (such as pioglitazone, glucagon-like peptide-1 receptor agonists (GLP-1RA), sodium-glucose co-transporter-2 (SGLT-2) inhibitors), antioxidants (such as vitamin E), lipid-lowering drugs (statins), farnesoid X activated receptor (FXR) agonists and others.

The following section focuses on the different available treatment strategies for NAFLD highlighting our understanding of key elements regarding therapy.

Lifestyle modifications

Lifestyle modifications in the form of diet, exercise, and weight loss remain the cornerstone and first-line recommendation for treating patients with NAFLD/MAFLD[79]. Weight loss has by far the best evidence thus far as an independent predictor for improvement in histopathological features of NASH. The best likelihood for sustained weight loss appears to be a combination of a hypocaloric diet (decrease in caloric intake by 500-1000 kcal/d) and moderate-intensity exercise[80-82]. While weight loss of a minimum of 7%-10% of body weight is required to improve a majority of histopathological features of NASH, losing body weight by at least 3%-5% results in improved hepatic steatosis[83]. These findings were observed in a 12-mo prospective study that revealed a dose-response curve with significant improvement in histopathology with a greater degree of weight loss. Patients who achieved greater than 10% weight loss had improvement in all features of NASH, including portal inflammation and fibrosis. Importantly, patients who lost at least 5% of body weight stabilized or improved fibrosis in 94% of the cases[84]. Other lifestyle modifications may result in benefits as physical activity of more than 150 min/wk or an increase in activity level by more than 60 min/wk have been associated with a decrease in serum aminotransferases independent of weight loss[85]. On the other hand, a Mediterranean diet containing low saturated fat and high polyunsaturated and monounsaturated fats is found to be beneficial[86].

Insulin sensitizers

Pioglitazone, a peroxisome proliferator-activated receptor agonist, is a thiazolidinedione derivative that modulates glucose and lipid metabolism. It ameliorates insulin resistance in addition to creating positive effects on vascular biology, adipose tissue function, and inflammation[80]. These unique properties of pioglitazone led to immense interest among researchers in exploring its potential role in patients with NAFLD/MAFLD. A single-center clinical trial performed almost a decade ago suggested that pioglitazone at a dose of 45 mg daily in addition to a hypocaloric diet improved histological findings of steatosis, ballooning necrosis, and inflammation. However, the degree of fibrosis was no different from that of the placebo group[87,88]. More recently, in another study by Belfort *et al*[89], pioglitazone treatment not only improved the NAFLD activity score and metabolic parameters but also caused significant regression of liver fibrosis. These effects were observed after 36 mo of therapy with no significant difference in adverse events except for net weight gain of > 5.0 kg when compared to patients who did not receive pioglitazone.

Apart from the diabetic population, pioglitazone has also proven to be quite effective in NASH patients without diabetes mellitus (DM). In the Pioglitazone *vs* Vitamin E *vs* Placebo for the Treatment of Nondiabetic Patients with Nonalcoholic Steatohepatitis (PIVENS) trial, a substantially higher percentage of patients who received pioglitazone achieved resolution of NASH. However, the primary endpoint of at least ≥ 2-point improvement in NAFLD activity score did not reach predetermined statistical significance[90]. Hence, although pioglitazone is advocated for type 2 diabetes mellitus (T2DM) patients with biopsy-proven NASH, its use in the non-diabetic population still remains a debate among hepatologists.

Antioxidants

A key mechanism of hepatocellular inflammation and injury in patients with NASH is oxidative stress. Vitamin E is an antioxidant that has been investigated in multiple studies as a potential treatment option for NAFLD. Initial studies demonstrated improved steatosis and inflammation with vitamin E administration but most of these were underpowered. Furthermore, it was challenging to compare data among the available studies largely due to significant heterogeneity regarding the dose and formulation of vitamin E, inclusion criteria, and concomitant use of other antioxidants or drugs.

More recently, the PIVENS clinical trial revealed that oral a-tocopherol, administered at a dose of 800 IU/d, results in considerable improvement in liver histology at 96 wk in non-diabetic biopsy-proven NASH patients[91]. These findings are substantiated by another US-based (TONIC) clinical trial that reported a significantly higher percentage of resolution of steatohepatitis in pediatric patients with NAFLD[92]. Despite similar convincing data from a few other studies, a high dose of vitamin E has been associated with increased all-cause mortality and a higher risk of prostate cancer with no improvement in fibrosis[93,94]. As such, the general consensus at this time includes consideration of vitamin E therapy in non-diabetic NAFLD/MAFLD patients following a patient-centered individualized approach. It is currently not recommended for treating NASH patients with DM or cirrhosis.

Statins

NAFLD/MAFLD are associated with metabolic syndrome that constitutes HTN, T2DM, dyslipidemia, and obesity. Current society guidelines recommend treating associated comorbidities in NAFLD patients in addition to treating the liver disease itself. Treatment with statins was associated with lower steatosis, inflammation, and fibrosis in NASH patients[95-98]. Moreover, patients who received statins had substantially lower cardiovascular mortality without any significant liver-related adverse events. A nationwide nested case-control study from South Korea including 11593409 patients from a nationwide database suggested that statins lower the risk of occurrence of NAFLD independent of accompanying T2DM[99]. In addition to the noted benefit in reducing the incidence of NAFLD, this study also highlighted that statin usage also prevented the progression to advanced fibrosis in patients with pre-existing fatty liver disease with an adjusted odds ratio of of 0.43; 95%CI 0.42–0.44). It is therefore reasonable to initiate anti-lipid therapy for patients that meet treatment criteria. While the concern for statin-induced hepatotoxicity prevails, severe liver injury from statins is extremely rare regardless of baseline elevation of transaminases or the presence of underlying chronic liver disease.

Gastrointestinal hormones (Incretins) and newer antidiabetic agents

Glucagon-like peptide (GLP)-1 is a gut-derived incretin hormone secreted in response to oral food intake. Glucagon-like peptide-1 receptor agonists (GLP-1RAs) constitute a novel class of well-established anti-diabetic drugs with encouraging data on the utility of these agents in treating obesity, preventing cardiovascular diseases, improving kidney function, and lowering mortality in patients with T2DM[99]. A growing body of evidence suggests that GLP-1RAs exert anti-NASH activity through several mechanisms such as increased insulin secretion, delayed gastric emptying, modulation of appetite, promoting fat redistribution, and reduced fat accumulation in the hepatocytes. A landmark UK-based 48-wk multicenter RCT (LEAN) revealed that NASH patients treated with liraglutide had a higher resolution of biopsy-proven steatohepatitis with no worsening of fibrosis when compared with placebo. Of those patients treated with liraglutide, 39% achieved resolution of NASH as opposed to only

9% in the placebo group. [Relative risk 4.3 (95%CI 1.0–17.7); P = 0.019]. These findings were supported by a Japanese pilot study (LEAN-J) where treatment with liraglutide was associated with significantly improved liver function and histological features in NASH patients with glucose intolerance[100].

Semaglutide, another GLP-1RA is currently approved for the treatment of T2DM and under extensive evaluation for weight loss[101]. Although semaglutide shares its mechanism of action with liraglutide, it has gained rapid recognition for its more pronounced metabolic effects with regard to improved glycemic control, reduced cardiovascular risk, and effective weight loss[102]. A placebo-controlled 72-wk trial suggested that once-daily subcutaneous semaglutide administered at a dose of 0.4 mg results in a higher likelihood of NASH resolution without worsening of underlying fibrosis in addition to a dose-dependent weight loss[103-105]. Despite these encouraging results, the rate of fibrosis regression was comparable between the treatment and placebo groups, and a higher number of patients treated with semaglutide experienced gastrointestinal side effects such as nausea and vomiting[106]. More refined data on histological outcomes and adverse effects will be required before the widespread use of semaglutide in routine clinical practice for the sole treatment of NAFLD/MAFLD.

Renal sodium-glucose co-transporter 2 (SGLT2) inhibitors are another class of anti-diabetic agents that work through the inhibition of glucose reabsorption in the kidneys in combination with enhanced urinary excretion of excess glucose. The consequent glucosuria results in lower blood glucose levels and eventual weight loss. A Malaysian open-label pilot study demonstrated that empagliflozin significantly improved steatosis, hepatocyte ballooning, and fibrosis in a small cohort of biopsy-proven NASH patients with T2DM. Interestingly, these effects were noted after a short duration of treatment (6 mo) and remained significant when compared with a historical placebo group at 48 wk[107]. Another meta-analysis of six randomized controlled trials including 309 patients by Xing *et al*[108] reported a positive effect of SGLT2 inhibitors in patients with NAFLD and T2DM. Patients treated with SGLT2 inhibitors achieved significant weight loss in addition to lower liver fat and improved alanine transaminase levels. Apart from the encouraging evidence on anti-NASH metabolic effects, several other studies have demonstrated improved cardiovascular outcomes in T2DM patients treated with SGLT2 inhibitors. Cardiovascular disease (CVD) currently remains the most common cause of death in patients with NAFLD, thus highlighting the added benefit of reduced cardiovascular deaths on the overall prognosis of NAFLD patients. Based on the aforementioned data, GLP-1 RAs, and SGLT2 inhibitors, either as monotherapy or combination therapy appear to be promising treatment options for NAFLD/MAFLD.

Dipeptidyl peptidase-4 (DPP-4) inhibitors such as sitagliptin, vildagliptin, and saxagliptin; slow the inactivation of incretin hormones such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) by selective inhibition of DPP-4 enzymes. As such, these agents indirectly increase insulin synthesis and lower glucagon levels through the prolonged action of GLP-1. Despite the observed benefit in early studies, these drugs have failed to significantly alter the histological profile in diabetic patients with NAFLD/MAFLD[109]. Possible explanations for the lack of clinical benefit postulated thus far in the literature include non-selective inhibition of both GLP-1 and GIP enzymes that could result in counterproductive effects on weight and fatty liver, questionable need for higher dosages in human models, and short study duration (3-6 mo). DPP-4 inhibitors are currently not recommended as therapies for patients with hepatic steatosis or steatohepatitis.

FXR agonists

The Farnesoid X receptor is a ligand-activated nuclear receptor that is a key regulator for bile acid signaling and metabolism. In recent years, FXR has gained considerable interest as a potential therapeutic target for the treatment of NASH. FXR activation leads to the inhibition of bile acid synthesis and increased conjugation, transport, and excretion of bile acids. These changes ultimately result in decreased cholestasis with protection of the liver from the deleterious effect of bile accumulation[110].

Obeticholic acid (OCA), a synthetic 6α-ethyl derivative of natural human bile acid, chenodeoxycholic acid regulates lipid and glucose metabolism through stimulation and upregulation of FXR activity. The FLINT trial for the treatment of NASH revealed that OCA administered at a dose of 25 mg daily improved multiple liver histology parameters in nearly half (45%) of the patients at 72 wk follow-up [111]. These findings were noted despite the study including a significant proportion of patients with T2DM and vitamin E non-responders. This landmark study underscored the clinical relevance of FXR agonists in improving hepatic insulin sensitivity and inhibition of lipogenesis in the NASH population. These findings were later corroborated by another phase 3 study (REGENERATE) that evaluated the efficacy of OCA in 2400 patients with NASH including 2100 patients with stage 2 or 3 Liver fibrosis. This study revealed that a significantly higher proportion of patients treated with either 10 mg or 25 mg of OCA achieved regression of fibrosis compared to placebo but there was no difference between the groups in regards to complete resolution of steatohepatitis[112]. While the most common adverse event was mild to moderate pruritis, the incidence of serious adverse events was similar across the groups.

Tropifexor is a novel non-bile acid agonist of FXR that has demonstrated potent *in vivo* activity in animal models. This drug is believed to be highly efficacious in upregulating FXR target genes even at very low doses and has currently progressed into clinical development. Several phase 2 human clinical trials on the safety and efficacy of Tropifexor in patients with NASH are under evaluation[113-115].

Bariatric surgery

Weight loss through lifestyle modifications is often challenging to achieve or sustain due to the substantial need for strict adherence and patient compliance. Bariatric surgery may be opted to achieve weight loss in select patients as it helps improve lipid metabolism and inflammatory pathways involved in the pathophysiology of NAFLD. National Institute of Health consensus criteria currently recommends bariatric surgery for patients with a BMI of 35 to 39.9 kg/m^2 with any severe obesity-related comorbidity such as T2DM, HTN, NAFLD/MAFLD, and/or NASH.

A systematic review appraising 29 studies by Bower *et al*[116] showed significant improvement in several histological (steatosis, hepatocyte ballooning, lobular inflammation, and fibrosis) and biochemical parameters of NAFLD following bariatric surgery. A more recent meta-analysis spanning 21 studies with a total of 2374 patients reaffirmed the noted benefit of bariatric surgery in patients with NAFLD with almost 88% of patients achieving improvement in steatosis and 30% having an improvement or resolution in liver fibrosis.

Apart from the long-term sustained weight loss, bariatric surgery also ameliorates NAFLD/MAFLD through multiple other mechanisms including but not limited to enhanced secretion of satiety hormones, variation in dietary habits, improvement in T2DM, alterations in bile acid homeostasis, modification of gut microbiome[116]. Further longitudinal controlled studies are needed to delineate the benefits and type of bariatric surgery as a therapy for those with NAFLD/MAFLD.

Although a few case series on bariatric surgery suggest an acceptable safety profile of these procedures in patients with cirrhosis, a vast majority of these studies included patients with well-compensated cirrhosis. More recently, evolving data demonstrated a modest but nonnegligible risk of complications following bariatric surgery in patients with more advanced cirrhosis. The common complications reported in such patients undergoing bariatric surgery include anastomotic leak, prolonged hospital stay, prolonged intubation, ileus, higher need for blood transfusion, and less commonly sepsis and fulminant hepatic failure[117-120]. As such, bariatric surgery is currently preferred for patients with Child A cirrhosis due to the acceptable risk of complications from surgical and hepatic factors[121-123].

Gut microbiome

Several gut microbiota is known to interact with carbohydrate and lipid metabolism by regulating homeostasis, immunity, and several metabolic pathways. Gut microbiome dysbiosis increases gut permeability thereby increasing exposure of hepatocytes to endotoxins that eventually can lead to hepatocyte inflammation and fibrosis[124]. Studies from animal models suggest that oral administration of prebiotics, probiotics, and synbiotics improves lipid metabolism, dysbiosis, insulin resistance, and hypercholesterolemia associated with hepatic inflammation by altering multiple genes involved in B-oxidation and lipogenesis.

A randomized controlled trial by Vrieze *et al*[125] suggested that FMT improved insulin resistance in Caucasian males with metabolic syndrome thus raising a potential therapeutic option for NAFLD/MAFLD. Needless to say, there is a dire need for more improved and standardized methods of gut microbiome analysis along with a better understanding of interactions between diet, dysbiosis, and environmental factors and their impact on the gut-liver axis to help devise effective and targeted treatment options.

Emerging therapies and future directives

Multiple other studies recently evaluated anti-fibrotic and anti-apoptotic therapies as unique treatment options for patients with NAFLD[126,127]. Albeit the intriguing preliminary results, these studies failed to show considerable clinical benefit in phase 2/3 clinical trials largely due to the limited utility of these drugs in the early stages of NAFLD. The introduction of MAFLD and its diagnostic criteria appear promising as it would potentially allow the incorporation of pharmacotherapeutic agents used in the treatment of metabolic syndrome at an earlier stage in the development of NAFLD.

OUTCOMES

Advanced fibrosis

If left untreated, patients with NAFLD are at risk of advanced fibrosis. A meta-analysis encompassing 11 studies with 411 patients with biopsy-proven NASH showed that 33.6% of patients have eventual fibrosis progression. The annual fibrosis progression rate in patients with NAFLD and stage 0 fibrosis at baseline was 0.07 stages (95%CI, 0.02-0.11 stages), compared with 0.14 stages in patients with NASH (95%CI, 0.07-0.21 stages). These findings correspond to 1 stage of progression over 14.3 years for patients with NAFLD (95%CI, 9.1-50.0 y) and 7.1 years for patients with NASH (95%CI, 4.8-14.3 y)[128]. While baseline demographics such as BMI, age, history of alcohol use, and T2DM play a major role in determining progression to advanced fibrosis, disease-related risk factors such as elevated baseline transaminases, presence of necroinflammation, ballooning degeneration, and Mallory hyaline on

histology are also known to contribute to disease progression[70,129-132].

Advanced fibrosis eventually leads to hepatic decompensation from the development of cirrhosis and end-stage liver disease (2.69 events per 100 person-years). Liver-related mortality is the third cause of death in patients with NAFLD[133].

Hepatocellular carcinoma

Cirrhosis secondary to NAFLD/MAFLD does confer a higher risk of hepatocellular carcinoma (HCC) than those without cirrhosis[134]. Interestingly, the presence of fatty liver disease also confers a higher risk of HCC even in the absence of cirrhosis. Past data suggests that patients with non-cirrhotic NAFLD have a modest risk of HCC when compared to the general population[135,136]. This risk is notably higher in male patients > 65 years with a smoking history, co-existing DM, and/or baseline elevation of ALT[137-144]. As such, performing routine periodic surveillance of these patients with abdominal imaging and measuring alpha-fetoprotein levels could be considered.

CVD

Cardiovascular disease is the most common cause of death among NAFLD/MAFLD patients followed by extrahepatic malignancies[145]. MAFLD is the hepatic manifestation of metabolic syndrome; as such, a vast majority of these patients have additional cardiometabolic risk factors. Indeed, when compared to NAFLD patients a nationwide database study from China demonstrated that MAFLD patients had an increased medium/high 10-year CVD risk according to Framingham risk score [1064 (29.92%) *vs* 1022 (26.37%), $P < 0.005$][146]. While steatosis confers a lesser risk of CVD compared to steatohepatitis, the overall individual risk of CVD ultimately stems from the combination of the stage of fatty liver disease and the presence of other cardiometabolic risk factors. Patients with steatohepatitis and /or advanced fibrosis and those with co-existing T2DM are considered special risk groups for significant cardiovascular events and mortality[147].

In addition to coronary atherosclerosis, NAFLD/MAFLD patients are at a higher risk of cardiac arrhythmias such as atrial fibrillation and ventricular arrhythmias. Furthermore, many NAFLD patients have left ventricular systolic and/or diastolic dysfunction, aortic valve sclerosis, and mitral calcifications [148-153]. While several studies have revealed a higher burden of cardiorenal disease in patients with MAFLD, a recent meta-analysis by Wen *et al*[147] evaluating ten studies suggested a 1.95 times higher incidence of CVD or CVD-related mortality in the MAFLD patients than in the control group[146,154, 155]. Therefore, a thoughtful risk assessment of CVD, evaluation for subclinical atherosclerosis, and presumptive diagnostic studies and interventions for high-risk patients are needed to lower the high global disease burden of CVD in NAFLD/MAFLD patients. Studies evaluating the association of NAFLD/MAFLD, and CVD are listed in Table 1 for further review.

CKD

Chronic kidney disease (CKD) is a worldwide public health problem affecting up to 10%-15% of the general population. More recently, a strong association between NAFLD/MAFLD and CKD has been established independent of commonly co-existing diseases such as obesity, HTN, and T2DM. A meta-analysis by Targher *et al*[156] suggested that NAFLD patients had a considerably higher risk of incident CKD than those without NAFLD. (OR 1.87; 95%CI 1.3-4.1). Few other studies also demonstrated similar findings even after adjustment for potential confounders such as age, sex, BMI, and other well-established metabolic risk factors[157]. Currently postulated etiologies include upregulation of the renin-angiotensin system and impairment of antioxidant defense. Other associated factors that link NAFLD/MAFLD and CKD include metabolic syndrome, platelet activation, dysbiosis, unhealthy eating habits, and aging[158-160].

Since the advent of the new diagnostic criteria for MAFLD, numerous studies have suggested a significant association of MAFLD with CKD, particularly in patients with concomitant DM. A nationwide cohort study evaluating 268946 patients by Jung *et al*[161] revealed a higher adjusted hazard ratio (aHR) for incident CKD in MAFLD patients when compared to those with NAFLD (1.18 (95%CI, 1.01-1.39; $P = 0.040$). Studies evaluating the association of NAFLD/MAFLD, and CKD are listed in Table 2 for further review.

Extrahepatic malignancies

Metabolic syndrome is a well-known risk factor for colorectal cancer. Consequently, several studies have suggested that NASH is independently associated with a heightened risk of colorectal adenomas and advanced colonic neoplasms. Of late, Fukunaga *et al*[162] suggested that MAFLD identifies colorectal adenomas more accurately than NAFLD (OR 3.191; 95%CI 1.494-7.070; $P = 0.003$), with a particularly high risk of colonic adenomas in individuals with non-obese MAFLD (OR 3.351; 95%CI 1.589-7.262; $P \leq 0.001$). Whether NAFLD/MAFLD directly contributes to colon cancer or if colon cancer occurs due to shared metabolic risk factors remains unclear. Patients with NAFLD are believed to have lower adiponectin levels that result in lesser endothelial cell apoptosis and increased proliferation of neoplastic cells, supporting the former possibility of a direct carcinogenic effect of steatohepatitis. Moreover, increased risk of other extrahepatic malignancies such as gastric cancer[163], pancreatic

Table 1 Studies evaluating the risk of cardiovascular disease in patients with nonalcoholic fatty liver disease and metabolic-associated fatty liver disease

Study	Number of patients	Type of study	Outcome measure	Results
Liang *et al* [168]	6873	Cohort Study with a 4.6 yr follow up	Associations of MAFLD and NAFLD with DM, CKD, and CVD	MAFLD was associated with higher risks of CVD (hazard ratio 1.44; 95%CI, 1.15-1.81); Similar associations were observed for NAFLD, except for a higher incidence of DM in MAFLD patients with HBV infection and excess alcohol consumption
Wang *et al* [169]	12183	Cross-sectional study (SPECT – China)	Compare the cardiovascular and renal burden between MAFLD and NAFLD patients	The odds ratio of previous CVD was higher in patients with MAFLD. Male 1.50 (1.22,1.85) *vs* 1.35 (1.1, 1.66); female 1.58 (1.33,1.87) *vs* 1.45 (1.22, 1.72)
Zhang *et al* [170]	19617	Nationwide database study	The burden of CKD and CVD in adults with MAFLD and NAFLD	The cardiorenal burden may be greater for MAFLD than for NAFLD
Lee *et al* [171]	8962813	Cohort study with a 10.1 yr follow up	Association of MAFLD and NAFLD with CVD	MAFLD patients have a higher risk of CKD when compared to NAFLD [1.43 (1.41–1.45) *vs* 1.09 (1.03–1.15)]
Yoneda *et al*[172]	2452949	Nationwide database study	Association of MAFLD and NAFLD with CVD	The incidence rates of CVD were 2.82 (95%CI 2.64-3.01) per 1000 person-yr in the NAFLD groups and 2.69 (95%CI 2.55-2.83) per 1000 person-years in the MAFLD groups
Guerreiro *et al*[173]	1233	Retrospective cross-sectional study	Compare CVR and risk of CVD between patients with NAFLD and MAFLD	In patients with MAFLD and NAFLD, CVR was intermediate/high (36.4 and 25.7%, $P = 0.209$) and CVD occurred in 20.1 and 12.8% ($P = 0.137$) of the cases, respectively, with no influence of liver injury severity
Zhang *et al* [158]	11673	Retrospective study	Compare the risk of CVD between patients with NAFLD and MAFLD	MAFLD was more significant than NAFLD in medium/high 10-yr CVD risk (according to Framingham risk score) [1064 (29.92%) *vs* 1022 (26.37%), $P < 0.005$]
Wen *et al* [147]	-	Meta-analysis	Investigate the risk of CVD incidence or CVD-related mortality in patients diagnosed with MAFLD and NAFLD	The incidence of CVD or CVD mortality was 1.95 times higher in the MAFLD group than in the control group. The risk of CVD or death from CVD was significantly higher in the MAFLD-only group than in the NAFLD-only group, with an RR of 2.57 (95%CI 1.41–4.71; $I^2 = 78\%$, $P = 0.002$)
Guo *et al* [174]	12794	Cohort study	Study the relationship between MAFLD and incident CVD	The incidence of CVD in the patients with MAFLD was significantly higher than that in the non-MAFLD patients (18.38% *vs* 9.02%, $P \leq 0.001$; aHR = 1.37, 95%CI = 1.20-1.56)
Moon *et al* [175]	8919	Cohort study	Effect of MAFLD on future mortality and CVD using a prospective community-based cohort study	T2DM in MAFLD increased the risk of both mortality (HR, 2.07; 95%CI, 1.52 to 2.81) and CVD (HR, 1.42; 95%CI, 1.09 to 1.85)
Zou *et al* [176]	513	Cross-sectional study	Prevalence of MAFLD and its relationship with CVD risks in RA patients	RA patients with MAFLD had a higher rate of CVD events (17.3% *vs* 9.2%) and a higher proportion of high estimated 10-yr CVD risk (55.5% *vs* 26.1%) than those without

MAFLD: Metabolic dysfunction associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease; FL: Fatty liver; FLD: Fatty liver disease; CVD: Cardiovascular disease; CVR: Cardiovascular risk; CKD: Chronic kidney disease; T2DM: Type 2 Diabetes Mellitus; RA: Rheumatoid Arthritis; HR: Hazard ratio; RR: Relative risk; aHR: Adjusted hazard ratio; CI: Confidence Interval; OR: Odds ratio.

cancer[164], esophageal cancer[165], breast cancer[166], and prostate cancer[167] are noted in patients with NAFLD.

Others

Other commonly associated conditions in patients with NAFLD/MAFLD include gastroesophageal reflux disease, obstructive sleep apnea syndrome, psychological dysfunction, hypothyroidism, growth hormone deficiency, and polycystic ovarian syndrome. Despite the substantial research and evidence revealing an association between NAFLD/MAFLD and the aforementioned extrahepatic complications, there are no standardized screening recommendations for these conditions in these patient populations. Careful surveillance and proactive treatment of these extrahepatic complications might improve overall outcomes, morbidity, and mortality in patients with NAFLD/MAFLD.

Table 2 Studies evaluating the risk of chronic kidney disease in patients with non-alcoholic fatty liver disease and Metabolic dysfunction associated fatty liver disease

Study	Number of patients	Type of Study	Outcome measure	Results
Sun *et al*[177]	12571	Cross-sectional study	Association between MAFLD and NALFD with CKD	MAFLD patients had lower GFR (74.96 ± 18.21) and higher prevalence of CKD (29.6%)
Liang *et al* [168]	6873	Cohort Study with a 4.6 yr follow up	Associations of MAFLD and NAFLD with T2DM, CKD, and CVD	MAFLD was associated with a higher risk of CKD (RR 1.64; 95%CI, 1.39-1.94). Similar associations were observed for NAFLD, except for a higher incidence of DM in MAFLD patients with HBV infection and excess alcohol consumption.
Deng *et al* [178]	4869	A cross-sectional study from the NHANES database 2017 – 2018	Association between MAFLD and CKD	Higher prevalence of CKD in MAFLD subjects than in non-MALFD subjects (22.2% *vs* 19.1%, $P = 0.048$)
Wang *et al* [169]	12183	Cross-sectional study (SPECT – China)	Compare the cardiovascular and renal burden between MAFLD and NAFLD patients	OR of CKD was higher in males with NAFLD [CKD: 1.44 (1.05, 1.96) *vs* 1.56 (1.14, 2.12)] than those with MAFLD
Su *et al*[179]	5594	Cross-sectional study	Association between MAFLD and CKD	MAFLD was independently associated with an increased risk of CKD [odds ratio (OR): 1.35, 95%CI: 1.09-1.67]. MAFLD with T2DM had significant associations with increased risk of CKD (OR: 2.85, 95%CI: 2.24-3.63), as well as increased eGFR and UACR
Hu *et al*[180]	15010	Cross-sectional study	Association between MAFLD and CKD	MAFLD was significantly associated with a higher CKD prevalence (OR 1.715, 95%CI 1.389-2.117, $P < 0.001$). MAFLD alone was not an independent risk factor for CKD
Hashimoto *et al*[181]	27371	Cross-sectional study	Association between FLD and MAFLD with CKD	MAFLD was associated with the risk of incident CKD [adjusted hazard ratio 1.24 (1.14-1.36), $P < 0.001$], whereas FLD without MD was not [1.11 (0.85-1.41), $P = 0.433$]
Zhang *et al* [170]	19617	A retrospective nationwide cohort study	Renal burdens in adults with MAFLD and NAFLD	The cardiorenal burden may be greater for MAFLD than for NAFLD
Jung *et al* [161]	268946	A retrospective nationwide cohort study	Association between MAFLD and NALFD with CKD	The adjusted hazard ratio (aHR) for incident CKD in MAFLD was 1.18 (95%CI, 1.01-1.39; $P = 0.040$) compared to those with NAFLD
Tanaka *et al* [182]	13159	Retrospective single-center study	Associations of FL, NAFLD, and MAFLD with the development of CKD	MAFLD [HR (95%CI): 1.12 (1.02-1.26), $P = 0.027$], but not FL or NAFLD, was an independent risk factor for incident CKD

MAFLD: Metabolic dysfunction associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease; FL: Fatty liver; FLD: Fatty liver disease; CVD: Cardiovascular disease; CVR: Cardiovascular risk; CKD: Chronic kidney disease; T2DM: Type 2 diabetes mellitus; HR: Hazard ratio; RR: Relative risk; aHR: Adjusted hazard ratio; CI: Confidence Interval; OR: Odds ratio.

CONCLUSION

The change in nomenclature has impacted the current understanding of fatty liver disease and stimulated more interest from the research community to better understand and treat this silent but deadly condition. As a limitation of our review, it only provides information about what is known and has been published to date as data on MAFLD is emerging. As new evidence becomes available, practitioners will be better able to tackle this disease and prevent its deleterious complications.

FOOTNOTES

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REVIEW

Emerging concepts in the care of patients with cirrhosis and septic shock

Jose Victor Jimenez, Guadalupe Garcia-Tsao, Saad Saffo

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Abstract

Septic shock impacts approximately 6% of hospitalized patients with cirrhosis and is associated with high rates of morbidity and mortality. Although a number of landmark clinical trials have paved the way for incremental improvements in the diagnosis and management of septic shock in the general population, patients with cirrhosis have largely been excluded from these studies and critical knowledge gaps continue to impact the care of these individuals. In this review, we discuss nuances in the care of patients with cirrhosis and septic shock using a pathophysiology-based approach. We illustrate that septic shock may be challenging to diagnose in this population in the context of factors such as chronic hypotension, impaired lactate metabolism, and concomitant hepatic encephalopathy. Furthermore, we demonstrate that the application of routine interventions such as intravenous fluids, vasopressors, antibiotics, and steroids should be carefully considered among those with decompensated cirrhosis in light of hemodynamic, metabolic, hormonal, and immunologic disturbances. We propose that future research should include and characterize patients with cirrhosis in a systematic manner, and clinical practice guidelines may need to be refined accordingly.

Key Words: Cirrhosis; Septic shock; Intravenous fluids; Vasopressors; Antibiotics; Steroids

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Core Tip: Septic shock is an important cause of morbidity and mortality among hospitalized patients with cirrhosis. In turn, the pathophysiology of cirrhosis impacts both the diagnosis and management of septic shock in meaningful ways. However, patients with cirrhosis have been traditionally underrepresented in clinical trials for septic shock, leading to critical knowledge gaps. The optimal care of these patients depends on achieving an understanding of the current limitations and implementing strategies for future research to address these shortcomings.

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INTRODUCTION

Among hospitalized patients with cirrhosis, approximately one-third develop sepsis and 6% develop septic shock[1]. Historically, due to unacceptably high mortality rates, individuals with cirrhosis and septic shock were generally considered poor candidates for admission to the intensive care unit (ICU). However, over the past three decades, the findings of randomized controlled trials (RCTs) have led to incremental progress in the management of septic shock, resulting in decreased mortality[2]. Although patients with cirrhosis were underrepresented in these trials, recent epidemiologic studies suggest parallel improvement in survival among this subset, indicating that management in the ICU is warranted[3-6]. While patients with compensated cirrhosis may respond to the same interventions and may have comparable outcomes to those without cirrhosis[7], patients with decompensated cirrhosis and clinically significant portal hypertension have marked local and systemic hemodynamic aberrations and hepatic functional impairment that may profoundly impact their management and prognosis. Consequently, the care of these patients should be appropriately tailored based on their unique pathophysiology. This review highlights the salient aspects of the management of septic shock among patients with cirrhosis and identifies critical knowledge gaps for future research.

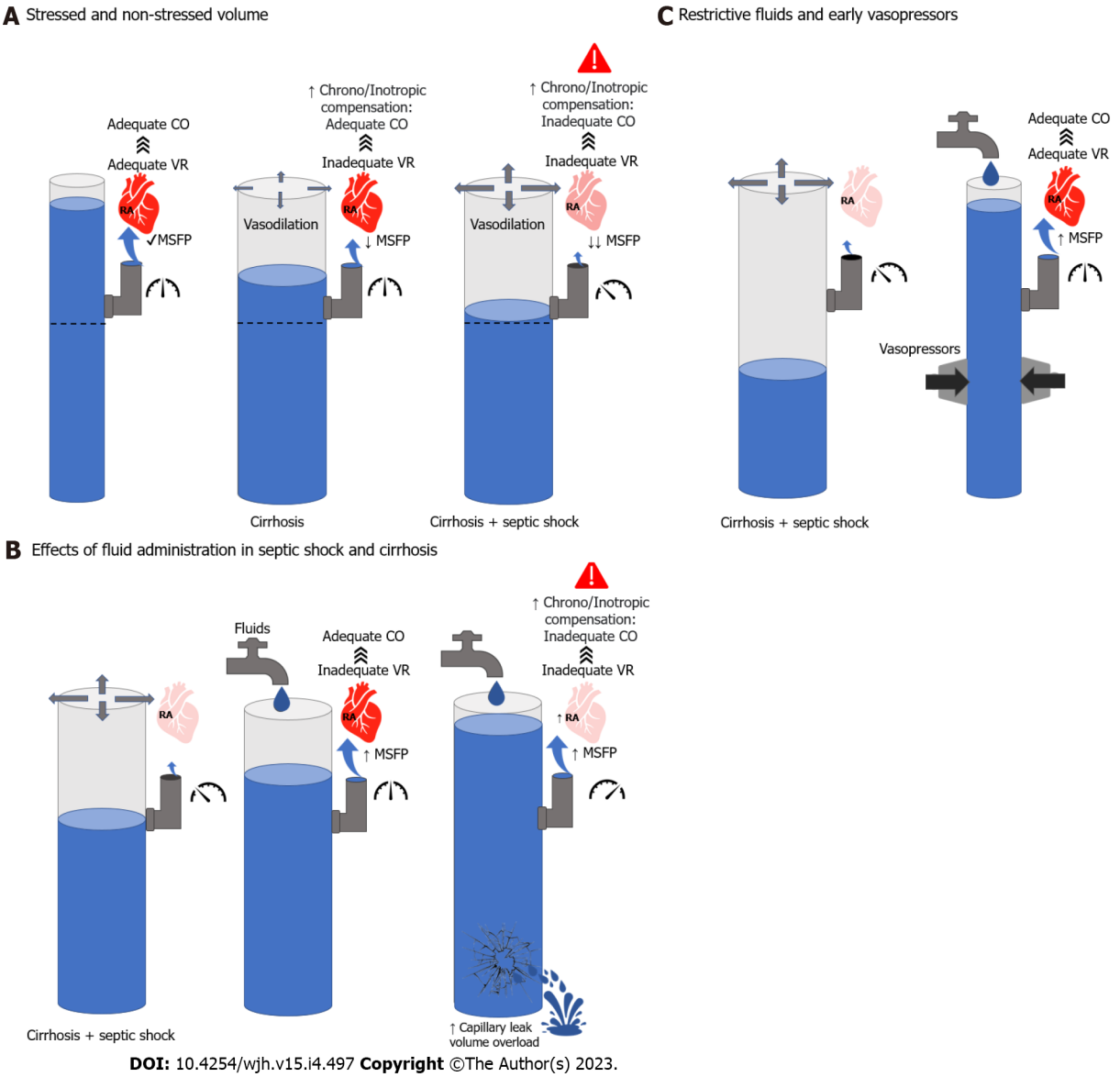
PATHOPHYSIOLOGY OF PORTAL HYPERTENSION

Portal hypertension occurs as a result of increased resistance in the hepatic vasculature and nitric oxide (NO)-mediated splanchnic and peripheral arteriolar vasodilation. Together, decreased systemic vascular resistance and increased splanchnic pooling contribute to a state of decreased effective circulating volume. This results in the activation of neurohumoral mechanisms aimed at maintaining adequate tissue perfusion, including beta-adrenergic signaling and the renin-angiotensin-aldosterone system. In patients with portal hypertension, these mechanisms increase cardiac contractility and promote salt and water retention[7]. When a severe infection ensues, macrovascular[8,9] and microvascular[10] vasodilatory effects are exaggerated, further decreasing the effective circulating volume and potentiating the neurohumoral response (Figure 1A).

Irrespective of the etiology of liver disease, 50% of patients develop cirrhotic cardiomyopathy (CCM) as a byproduct of the neurohumoral mechanisms aimed at maintaining the effective circulating volume [11,12]. CCM can manifest with diastolic and/or systolic dysfunction, limiting further augmentation in cardiac contractility in response to hemodynamic stress. Likewise, CCM predisposes to anasarca if excess fluids are administered during resuscitation (Figure 2). Decreased oncotic pressures secondary to hypoalbuminemia and increased hydrostatic pressures secondary to portal hypertension enhance capillary leak.

REPRESENTATION OF CIRRHOSIS IN SEPTIC SHOCK TRIALS

Although individual RCTs investigating diagnostic and therapeutic interventions for the management of septic shock have yielded controversial results, mortality has declined significantly over time. A possible explanation for the lack of benefit observed in trials is the frequent use of overall survival as the primary outcome. This endpoint may be suboptimal in the ICU setting, where the risk of death could be attributed to multiple competing causes[13,14]. In this context, isolated interventions are less likely to influence survival. Heterogeneity in patient selection and disease characteristics and the effects of confounding interventions are additional factors that may impact study results. A neutral association in a RCT may represent benefits for a particular subgroup of patients and harm to another[15]. To



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Figure 1 Hemodynamic considerations in the management of cirrhosis and septic shock. A: *Left*; Normal mean systemic filling pressures (MSFP) leading to adequate venous return and cardiac output (CO). *Middle*; vasodilation in cirrhosis leading to lower MSFP and inadequate venous return (VR). However, compensatory mechanisms are able to maintain adequate CO. *Right*; further vasodilation leading to lower MSFP and inadequate VR. In this case, neurohumoral and cardiac compensation are not enough to maintain CO; B: *Left*; cirrhosis and septic shock pathophysiology. *Middle*; Effects of adequate volume resuscitation leading to increased MSFP. In the context of normal filling pressures, this will increase VR and CO. *Right*; Excessive fluid resuscitation will lead to high filling pressures which will decrease VR and CO. In addition, it may lead to volume overload and capillary leak; C: *Left*; cirrhosis and septic shock pathophysiology. *Right*; adjuvant effect of fluids and vasopressors on MSFP, VR and CO without leading to volume overload. CO: Cardiac output; MSFP: Mean systemic filling pressures; RA: Right atrium; VR: Venous return.

adequately interpret treatment effects among subpopulations such as cirrhosis, large pragmatic trials are required. Unfortunately, most contemporary septic shock trials have either underrepresented, excluded, or mischaracterized patients with cirrhosis (Table 1), limiting the potential applicability of common interventions in this patient population. In some cases, small RCTs of patients with cirrhosis have yielded conflicting results in comparison to those that excluded cirrhosis, leading to controversies in the care of these patients (Figure 3). Throughout the remainder of this review, we will highlight both evidence-based principles and areas of uncertainty.

MANIFESTATIONS OF SHOCK IN CIRRHOSIS

Shock is a state of tissue hypoxia. It occurs when tissue oxygen demands cannot be met by the circulatory system or when tissue oxygen extraction is impaired, leading to cellular dysfunction[16]. It should be considered in patients who develop hypotension with additional clinical or biochemical findings of hypoperfusion, including altered mental status, acute kidney injury (AKI), or lactic acidosis

Table 1 Patients with liver disease in randomized controlled trials of sepsis and septic shock

Trial	Intervention	*n*	Liver disease present (%)	Cirrhosis excluded	Comments
Rivers (2001)	EGDT *vs* Standard	263	61 (23)	No	
ProMISe Trial (2014)	EGDT *vs* Standard	1260	22 (1.8)	No	
ARISE Trial (2014)	EGDT *vs* Standard	1600	83 (5)	No	
ProCESS (2014)	EGDT *vs* Standard	1341	11 (0.8)	No	
ANDROMEDA-SHOCK (2019)	CRT *vs* Lactate clearance	424	0 (0)	Yes	Excluded Child B and C
SMART Study (2018)	Balanced crystalloids *vs* 0.9% NS	15802	180 (11)	No	
BaSICS Trial (2021)	Balanced crystalloids *vs* 0.9% NS and Slow *vs* Fast bolus	11052	266 (2.4)	No	
PLUS Study (2022)	Balanced crystalloids *vs* 0.9% NS	5037	NR	No	
Classic Trial (2022)	Restrictive *vs* Liberal fluids	1554	NR	No	
SAFE Trial (2004)	4% Albumin *vs* 0.9% NS	6997	NR	No	
ALBIOS Study (2014)	20% Albumin + Crystalloids *vs* Crystalloids alone	1818	27 (1.4)	No	Excluded cirrhotic patients with cirrhosis and ascites
VASST Trial (2008)	Vasopressin *vs* NE	778	88 (11)	No	Excluded Na < 130 mEq/L and irreversible disease with less than six-month survival
VANISH Trial (2016)	Vasopressin *vs* NE - AKI	409	14 (4)	No	Factorial design (vasopressin/hydrocortisone)
ATHOS-3 (2018)	Angiotensin-II *vs* Placebo	344	NR	Yes	Excluded MELD > 30
CENSER (2019)	Early NE *vs* Placebo	310	27 (9)	No	
CORTICUS Trial (2008)	Hydrocortisone *vs* Placebo	499	40 (8)	No	
ADRENAL Trial (2018)	Hydrocortisone *vs* Placebo	3800	NR	No	
APROCCHSS Trial (2018)	Hydrocortisone + Fludrocortisone *vs* Placebo	1241	NR	Yes	Excluded Child C

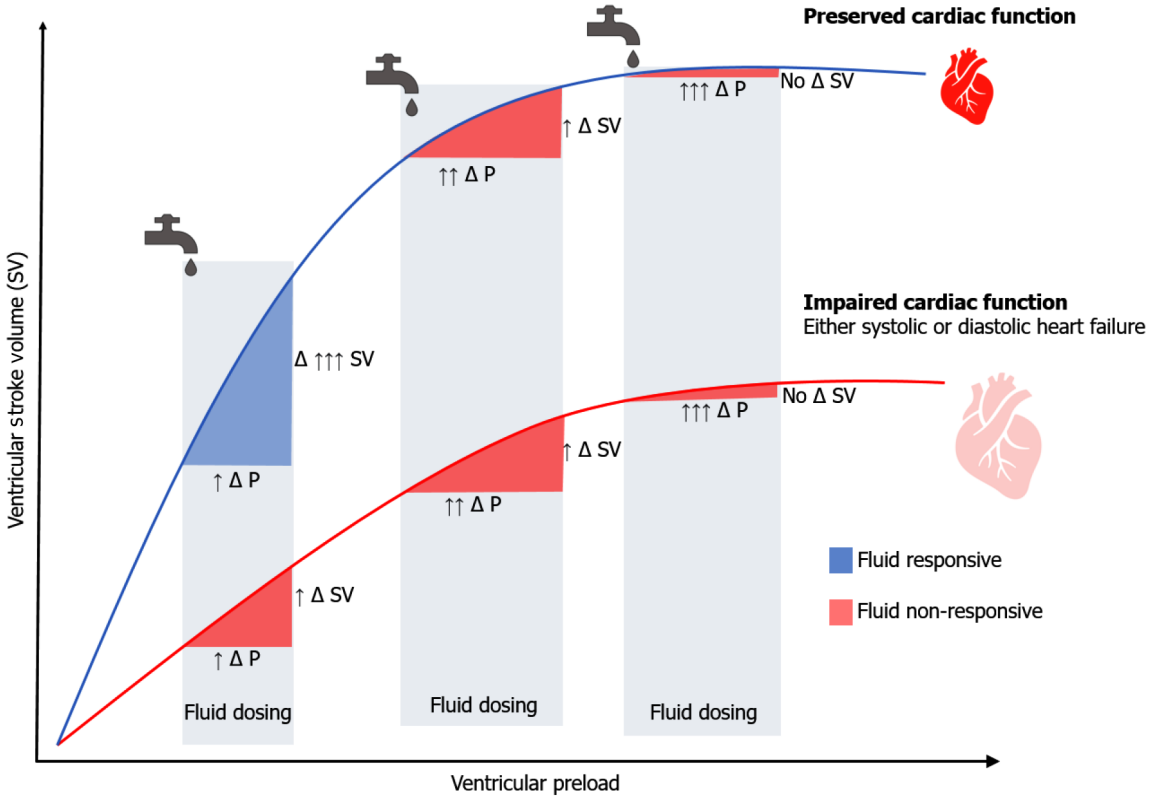
AKI: Acute kidney injury; CRT: Capillary refill time; EGDT: Early goal-directed therapy; NE: Norepinephrine; NR: Not reported; NS: Normal saline.

[17]. Of the various subtypes, septic shock is most common among patients with cirrhosis[18]. It represents a dysregulated immune response to an infection, leading to systemic inflammation, vasodilation, and organ impairment[19].

In patients with cirrhosis and clinically significant portal hypertension, a low mean arterial pressure (MAP) is often present without overt signs and symptoms of hypoperfusion[20]. The ATTIRE trial[21] which included hospitalized patients with decompensated cirrhosis, defined hemodynamic dysfunction as a MAP < 60 mmHg rather than 65 mmHg, illustrating the point that a fixed MAP may not strictly reflect adequate tissue perfusion. Patients with advanced cirrhosis and a chronic state of systemic vasodilation have adaptive autoregulatory mechanisms to maintain perfusion to vital organs despite MAPs < 65 mmHg[22] whereas patients with early cirrhosis, metabolic syndrome, and chronic hypertension may develop tissue hypoperfusion despite MAPs 65 mmHg[23].

Therefore, in addition to assessing blood pressure, a determination of shock relies on assessing perfusion markers. In this respect, it is important to note that the clinical manifestations of hypoperfusion may be less reliable in cirrhosis. For instance, the neurological window for hypoperfusion might represent a diagnostic dilemma in cirrhosis, especially in patients with a history of hepatic encephalopathy (HE). In patients with new or unexplained HE, there should be a high index of suspicion for sepsis or septic shock. Similarly, skin mottling and other skin perfusion signs have lower sensitivity in patients with cirrhosis due to sustained peripheral vasodilation[24].

Another marker of hypoperfusion is type A hyperlactatemia. It occurs when lactate is produced under anaerobic conditions by lactate dehydrogenase[25] and is also confounded in cirrhosis in the context of altered lactate production and clearance. Septic shock is associated with normal to high tissue oxygen delivery but impaired oxygen extraction. Although tissue hypoxia may be present, direct clinical correlation with serum lactate levels may be unreliable in some instances[26-29]. However, peak lactic

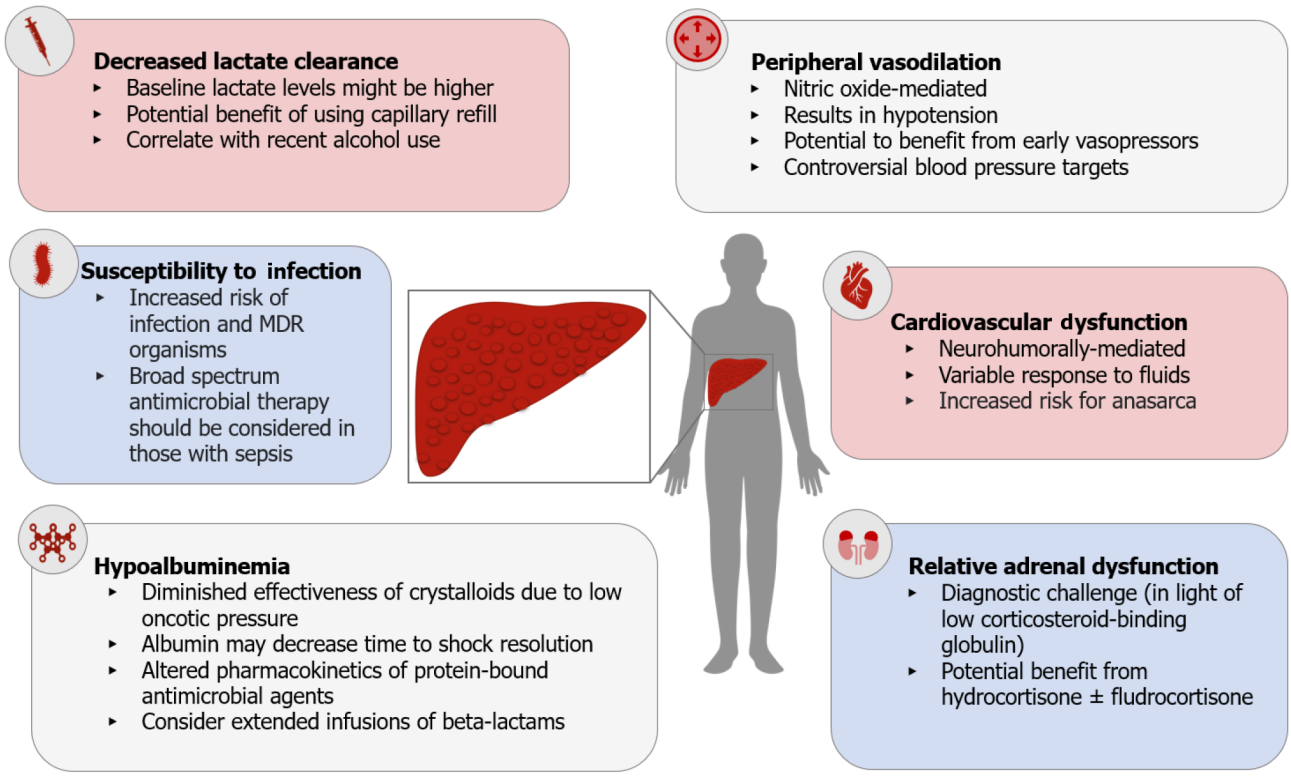


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Figure 2 Frank-Starling curves in septic shock. Every fluid bolus will lead in a change in pressure (Δ P) and a change in stroke volume (Δ SV). The effect of fluids on cardiac output among patients with normal (upper curve) and impaired (lower curve) myocardial function is depicted. Even among patients with normal myocardial reserve, excess fluid administration may significantly increase pressure without significantly increasing stroke volume, which may ultimately lead to anasarca. Δ P: Change in pressure; Δ SV: Change in stroke volume.

acid values and trends have prognostic significance[30]. The contemporary view of hyperlactatemia in septic shock relies on the observation that increased lactate production is driven by beta-adrenergic stimulation, otherwise referred to as stress hyperlactatemia[31]. Stress hyperlactatemia is believed to be a compensatory response to sepsis-induced vasodilation. In a stable hemodynamic state, patients with cirrhosis and more severe liver disease [*i.e.*, those with decompensated disease and/or higher Child-Turcotte-Pugh (CTP) scores] have increased adrenergic tone and higher serum lactic acid values[32]. Because the liver provides up to 70% of the lactate clearance from the body[22], its disproportionate accumulation in patients with cirrhosis and critical illness is not surprising[33,34]. In a propensity score matched analysis accounting for potential confounding factors, Cheng *et al*[35] demonstrated that patients with cirrhosis had higher lactate levels. The difference was particularly robust in those with decompensated cirrhosis (4.08 mmol/L in patients with decompensated cirrhosis who survived *vs* 2.48 mmol/L in patients without cirrhosis who survived and 7.16 mmol/L in patients with decompensated cirrhosis who died *vs* 5.93 mmol/L in patients without cirrhosis who died). Similarly, Drolz *et al*[36] analyzed the predictive value of arterial lactate levels and clearance in critically ill patients with cirrhosis, demonstrating that values greater than 5 mmol/L were independently associated with 28-d mortality, and models such as the model for end-stage liver disease-lactate (MELD-LA) score have incorporated lactate values for prognostication[37]. Higher cutoffs for lactate levels have also been described in critically ill patients with cirrhosis and AKI[38] and in acute liver injury[39]. In the recent Baveno consensus conference, the criteria for futility in patients with variceal hemorrhage included lactate > 12 mmol/L[40]. Finally, it is important to note that, in patients with alcohol use disorder, ethanol oxidation decreases nicotinamide adenine dinucleotide (NAD^+) thereby altering the NAD^+/NADH ratio and shifting pyruvate metabolism toward lactate production. Although its impact on lactate levels appears to be modest[41], clinicians should consider the effects of alcohol use on lactate metabolism[42].

These cumulative data suggest that, although lactate remains useful as a predictor of mortality, the cutoff for normality may be higher in cirrhosis. Venous lactate levels > 2 mmol/L should raise suspicion for shock, but a multimodal approach that accounts for other signs and symptoms of organ hypoperfusion is warranted. In decompensated cirrhosis, a higher threshold (> 4 mmol/L) may be considered [35]. In patients without other signs of hypoperfusion, lactate elevations may indicate progressive physiologic stress and may correlate with poor prognoses but are not necessarily indicative of shock.



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Figure 3 Pathophysiologic changes in cirrhosis that impact the management of septic shock. MDR: Multi-drug resistant.

This concept has important therapeutic implications.

FLUID RESUSCITATION

The initial management of sepsis is based on a practical evidence-based approach endorsed by the Society of Critical Care Medicine and the European Society of Intensive Care Medicine. The Surviving Sepsis Campaign (SSC) guidelines clarify best practices relating to critical aspects of care, including fluid resuscitation, vasopressor, antibiotic use, and steroid use, and hemodynamic monitoring, among other things. In the general population, the timely implementation of some components of this bundle are associated with improved outcomes[43].

Since inception, the SSC continues to suggest the use of at least 30 mL/kg of crystalloids within the first three hours as the initial management of patients with suspected septic shock[44], regardless of initial volume status or degree of volume responsiveness. However, the strength of the recommendation was downgraded from strong to weak in 2021 given the lack of robust data to support aggressive fluid resuscitation[45].

Septic shock is characterized by arteriolar vasodilation and venous pooling, further complicated by hypovolemia due to poor oral intake, insensible losses, and capillary leak in the context of endothelial dysfunction. The rationale for fluid administration is to increase the venous volume and augment the effective circulating volume. If right- and left-sided cardiac filling pressures are not elevated, the use of intravenous fluids may improve cardiac preload. On the dependent (steep) portion of the Frank-Starling curve, increased preload will ultimately augment cardiac output. However, if intravenous fluids are given in the setting of elevated cardiac filling pressures, or if the myocardium lacks inotropic reserve, fluids may not increase cardiac output (Figure 2). On the contrary, venous congestion, interstitial edema, and ineffective gas exchange will ensue (Figure 1B). Critically ill patients who develop anasarca have increased mortality for every liter of positive fluid balance[46,47]. Those with cirrhosis have an even greater risk for complications in light of decreased oncotic pressures and impaired cardiac reserve. Therefore, intravenous fluid therapy should be carefully administered, and as for any other medication, the type, dose, and duration need to be considered.

The type of intravenous fluids used for shock resuscitation are typically classified as crystalloids or colloids. Crystalloids include normal (0.9%) saline or balanced solutions such as Lactated Ringer's, Plasma-Lyte, and Hartmann's solution. Normal saline is the most ubiquitous worldwide, but its use associated with renal dysfunction[48], hyperchloremic metabolic acidosis, and decreased survival[49]. RCTs have demonstrated a potential benefit in favor of balanced crystalloid solutions in comparison to

normal saline in critically ill patients, particularly when large volumes are necessary. Although patients with cirrhosis were underrepresented in these trials[50,51], there is no physiologic rationale against the use of balanced crystalloid in this population. Rather, in light of the risk for kidney injury, the use of hyperchloremic solutions should be limited in patients with cirrhosis.

As a result of the endothelial damage that occurs in sepsis, crystalloids remain in the intravascular compartment for minutes, whereas colloids, such as albumin, remain for up to three hours. In addition, the pleiotropic properties of albumin led to its use in critically ill patients, though RCTs have demonstrated mixed results[52-54]. The ALBIOS trial studied the addition of 20% albumin to crystalloids in hypoalbuminemic patients with severe sepsis or septic shock[54]. Although survival, length of stay, and organ failure scores did not improve, albumin use was associated with higher MAPs, lower net fluid balance, and decreased time to vasopressor or inotrope discontinuation. In a post-hoc analysis of only patients with septic shock, those randomized to the albumin arm had a 6.3% absolute reduction in 90-d mortality (RR 0.87, 95%CI 0.77-0.99; P = 0.03). However, less than 2% of the subjects had liver disease and patients with advanced cirrhosis were excluded from the trial. In the recent FRISC trial[55], investigators compared the use of 5% albumin with 0.9% saline in patients with advanced cirrhosis (mean CTP score of 12 and MELD-sodium score of 33) and sepsis-induced hypotension. The authors found improved hypotension reversal (primary outcome, defined as achieving a MAP ^{3} 65 mmHg at three hours), lower lactate levels, and resolution of tachycardia in the albumin arm. At one week, 43.5% of the patients in the albumin arm were alive in comparison to 38.3% in the normal saline arm (P = 0.03). Similarly, in the recent ALPS study[56] higher rates of short-term septic shock reversal were found using 20% albumin in comparison to Plasma-Lyte. Although albumin use was also associated with more rapid lactate clearance and lower rates of renal replacement therapy, there was no difference in mortality. One in every five patients in the albumin arm required discontinuation of the colloid due to pulmonary edema, most commonly among those with pneumonia. The safety concern of pulmonary edema with the rapid infusion of 20% albumin was also observed in the ATTIRE[21] and CONFIRM[57] studies. Thus, albumin may be effective for shock reversal in patients with cirrhosis, but due to the increased risk for pulmonary complications, close monitoring for volume overload is warranted, specifically in patients with AKI, lung disease, and higher MELD scores.

Finally, the volume of fluid administered also matters. Although no study has directly compared the initial 30 mL/kg of crystalloids to smaller volumes, recent studies have attempted to address the impact of volume. The CLASSIC trial compared restrictive (median 1798 mL) to liberal (median 3811 mL) fluid strategies for resuscitation after an initial administration of one liter of crystalloids[58]. The authors found no differences in 90-d mortality. However, the study provides valuable data regarding the safety of restrictive fluid resuscitation, which could be particularly useful in patients prone to develop volume overload, such as those with cirrhosis. The results of the CLOVERS trial, which tested a similar hypothesis[59] are pending, though the trial was terminated for futility after an interim analysis demonstrated no differences in 90-d survival between both groups. Regardless, it should be noted that weight-based fluid strategies should be reconsidered in some patients, especially those with underlying obesity or marked anasarca. In principle, individualizing fluid resuscitation is an essential principle as requirements and tolerance to fluids vary substantially among individuals[60].

VASOPRESSORS

The application of a restrictive fluid strategy hinges on the early use of vasopressors. Vasopressors target the vasodilatory physiology of septic shock by restoring vascular tone and mobilizing the pooled volume of blood to the heart (Figure 1C). Vasopressors consist of catecholamines such as norepinephrine, epinephrine, dopamine, or phenylephrine and non-catecholamines like vasopressin analogs and angiotensin II. In part, they increase the tone of the vascular bed in patients with septic shock *via* effects on alpha-1 (catecholamines), V1 (vasopressin analogs), or angiotensin II receptors (angiotensin II). The early use of vasopressors leads to a faster resolution of shock[61], whereas delay is associated with increased mortality[62]. In fact, there is an approximately 5.3% increased risk of death for every hour of delay[63]. However, common adverse effects of vasopressors include digital and splanchnic ischemia in a dose-dependent manner[61,62]. Catecholamine-based vasopressors can also lead to cardiac arrhythmias and ischemia due to their effect on beta-1 receptors[64-66].

Based on head-to-head RCTs comparing different adrenergic vasopressors, the SSC recommends norepinephrine as the first line vasopressor for the management of septic shock[45]. Nonetheless, most of these RCTs included less than 10% of patients with liver disease (Table 1). Multiple trials have demonstrated the benefit of vasopressin analogs in hepatorenal syndrome[67-70], a functional manifestation of end-stage portal hypertension characterized by systemic vasodilation and renal vasoconstriction, often precipitated by infections. In this setting, a recent network meta-analysis suggested that terlipressin may be more beneficial than norepinephrine[70]. Terlipressin, is a vasopressin analogue with greater affinity for V1 receptors. It has been proposed as an alternative vasopressor in septic shock. In a small RCT, Choudhury *et al*[71] compared the use of norepinephrine and terlipressin in patients with cirrhosis and septic shock. The authors observed higher rates of shock

resolution, lower incidence of variceal bleeding, and improved time to vasopressor discontinuation with the use of terlipressin. However, a subsequent RCT in patients without cirrhosis showed a higher incidence of adverse events such as digital ischemia when compared to norepinephrine with no improvement in mortality or organ failure resolution[72]. To date, norepinephrine remains the first line vasopressor in patients with septic shock who do not respond to fluids. Although the results of the VASST[73] and VANISH[74] trials did not demonstrate that the early use of vasopressin improved mortality or renal outcomes, respectively, low-dose vasopressin remains the agent of choice after norepinephrine in septic shock because of its relatively favorable side-effect profile and possible pleiotropic effects[75]. Though it may be reasonable to consider vasopressin analogues such as terlipressin in some patients with cirrhosis, there is currently insufficient data to support their use over vasopressin[76-78]. The efficacy of new non-catecholamine based vasopressors such as angiotensin II might be limited in the setting of cirrhosis as hypoalbuminemia was a negative predictor for response in the ATHOS-3 trial[79].

GOALS OF RESUSCITATION

Although the trigger for initiating resuscitation in septic shock is well defined, the endpoint is less clear. The goal of resuscitation is to augment tissue perfusion. However, the resolution of organ dysfunction lags behind the sufficiency of resuscitation, which often leads to excess fluid administration[80]. The ideal targets for adequate resuscitation and the type of monitoring necessary continue to be heavily debated topics.

With the publication of the Rivers study[81], early goal-directed therapy (EGDT) became the cornerstone for the management of septic shock. In this single-center trial, the use of EGDT decreased mortality compared to standard therapy. EGDT consisted of a protocol for the administration of crystalloids, vasopressors, inotropes, and blood products to achieve specific hemodynamic goals (blood pressure, central venous pressure, central venous oxygen saturation, and hemoglobin levels). However, two decades later, three multicenter RCT demonstrated that EGDT does not improve outcomes[82-84] but leads to higher hospitalizations costs[85]. Of note, these trials included a small number of patients with chronic liver disease and cirrhosis (Table 1). Currently, the goals of septic shock resuscitation are to reverse derangements in the very same components that define it. This includes achieving an adequate MAP, improving the signs/symptoms of skin, renal and brain hypoperfusion, and decreasing lactate levels.

First, the SSC guidelines recommend targeting a goal MAP of 65 mmHg over higher values. This recommendation is based on the results of the SEPSISPAM trial which compared high (80-85 mmHg) *vs* low (65-70 mmHg) MAPs. The investigators found no difference in mortality. However, they observed a higher incidence of new-onset atrial fibrillation and lower rates of renal replacement therapy in the high MAP group[23]. The latter was observed only in those with chronic hypertension, suggesting that a personalized target for MAP must be considered, particularly in patients with chronic adaptive mechanisms of autoregulation to higher MAPs.

Of interest, among patients with chronic hypotension, Gershengorn and colleagues demonstrated a robust association between baseline low systolic blood pressure, prolonged use of vasopressors, and increased length of stay[86]. Whether these observations are a consequence of clinicians aiming for unrealistic MAP goals in chronically hypotensive patients or a reflection of more severe disease is unclear. Recently, the results of the 65 trial demonstrated the safety of more liberal MAP goals (60-65 mmHg) in patients with distributive shock[87]. Although these trials did not include patients with cirrhosis, they provide reassurance that lower conventional goals can achieve adequate oxygen delivery and may be adequate targets. Notably, however, as the etiology of cirrhosis has shifted to patients with metabolic syndrome, more patients with chronic arterial hypertension will present with septic shock.

Second, the SSC recommends guiding resuscitation to decrease serum lactate[45]. As mentioned in the previous sections, its use has limitations, particularly in the setting of cirrhosis. However, a decrease in lactate levels after initial resuscitation is associated with improved outcomes, even among patients with cirrhosis[88-91]. In a RCT, Jansen and colleagues tested a lactate-guided strategy for resuscitation based on lactate clearance which led to a significant decrease in mortality. The authors pursued a 20% decrease every two hours for the initial eight hours of management. Interestingly, the levels of lactate within groups were not significantly different, suggesting that perhaps closer monitoring with timely interventions for persistent hypoperfusion rather than lactate clearance is more consequential[91]. Given the caveats of lactate kinetics[92], which are impacted by hepatic clearance[93] and stress-induced production, new alternatives have been proposed.

The ANDROMEDA-SHOCK trial compared the use of capillary refill time (CRT) normalization to lactate clearance in patients with septic shock[94]. The authors demonstrated a non-significant trend towards improved 28-d mortality among the CRT group (HR 0.75, 95%CI 0.55 to 1.02; P = 0.06). Individuals randomized to the CRT arm did receive less fluids and had improvement in organ-dysfunction at 72 h. Zampieri *et al*[95] performed a Bayesian re-analysis of the data, finding a possible mortality benefit with the use of CRT. CRT is now recommended in the SSC guidelines as it offers an

alternative for resuscitation targets, especially in patients in whom lactate clearance is impaired, such as in patients with cirrhosis.

HEMODYNAMIC MONITORING

Whether lactate clearance, CRT, or alternative markers are used as targets for adequate resuscitation, clinicians should assess whether their interventions are achieving the desired effect. Only half of patients with septic shock are volume-responsive during initial resuscitation, around 30% after two hours, and less than 20% after four hours[96]. The hemodynamic response to fluid administration can be assessed by dynamic tests that evaluate whether an increase in preload increases cardiac output[97,98].

Multiple RCTs have demonstrated the feasibility of using fluid responsiveness markers to monitor and guide fluid resuscitation. Their use led to a reduction in the amount of volume administered[99] and need for renal replacement therapy[100], albeit with no effect on survival[101]. Unfortunately, they have not been validated in patients with cirrhosis. Moving forward, the application of tools like point-of-care ultrasonography may help optimize fluid resuscitation in patients with cirrhosis, but studies are necessary to determine the parameters that are most applicable.

ANTIBIOTICS

Early antibiotic administration provides the greatest survival benefit in septic shock. Every hour of delay in their administration conveys an increased risk for mortality[102], even within the first six hours [103]. The SSC guidelines recommend the initiation of antibiotics within one hour of the diagnosis of sepsis with a particular emphasis on patients with shock, for which every hour of delay conveys a 7% increase in mortality[45]. Although the rapid initiation of antimicrobial therapy is essential, the adequacy of coverage and pharmacokinetics are also important in patients with cirrhosis.

Appropriate antibiotic initiation involves administering the drug most likely to eradicate the suspected organism while avoiding unnecessary antibiotic-associated toxicities and exposures that predispose to the development of multi-drug resistant (MDR) organisms[104,105]. In patients with cirrhosis, up to a third of bacterial infections are now due to resistant organisms[106], and these infections are associated with dismal prognoses[107]. Therefore, the choice of empiric treatment, specifically in septic shock, should account for local epidemiology and individual risk factors for MDR infections. Recent hospitalization, nosocomial infection, prior health-care exposure, ICU admission, and recent antibiotics use (within 90 d) predispose to MDR infections in patients with cirrhosis[108,109]. In individuals with these risk factors, broad spectrum antibiotics tailored to local antibiograms and site of infection are warranted, followed by de-escalation within 48-72 h, based on laboratory data and clinical status. Unfortunately, up to 50% of cases of sepsis are associated with insufficient or negative culture data, which complicates both antimicrobial de-escalation and the detection of resistant strains[110]. Rapid diagnostic techniques, which rely on molecular methods such as polymerase chain reaction, are now available for the identification of pathogens and resistance genes. They have been shown to be efficient and effective in isolating the cause of sepsis[111]. Their use is associated with improved antibiotic selection, decreased antimicrobial use[112], shortened hospital stays, and in the case of bloodstream infections, improved mortality[113]. When available, these techniques should be used to optimize the treatment of sepsis. Finally, although the prevalence of fungal infections is variable, patients with cirrhosis have functional defects in neutrophils function that increase the likelihood of infections due to *Candida* and *Aspergillus* species. In general, fungal infections should be strongly considered in patients with abdominal sepsis, exposure to broad spectrum antibiotics or steroids, parenteral nutrition, prolonged ICU stay[114], and ACLF[115].

In cirrhosis, altered pharmacokinetics and pharmacodynamics modify the efficacy of antimicrobial agents. For highly protein-bound antibiotics such as ceftriaxone, aztreonam, or carbapenems, hypoalbuminemia increases the unbound fraction and increases its clearance[116], resulting in lower drug levels over the minimal inhibitory concentration (MIC). For antibiotics such as beta-lactams, for which efficacy depends on the time over the MIC, this may lead to treatment failure[117]. In patients with hypoalbuminemia the use of ertapenem is associated with a fivefold increase in mortality, which is not observed with lower protein-bound carbapenems such as meropenem or imipenem[118]. Furthermore, patients with ascites have an increased volume of distribution, which may result in decreased peak concentrations of antibiotics, especially those which distribute extracellularly[119]. In the case of spontaneous bacterial peritonitis, a common source of sepsis among hospitalized patients with cirrhosis, peritoneal antibiotic penetration is an essential concept. While some agents like cephalosporins, fluoroquinolones, and meropenem[120-122] achieve high concentrations in ascitic fluid, others such as aminoglycosides and tigecycline have reduced penetration[123,124]. The use of continuous or extended infusions of beta-lactams increases the duration of antibiotic levels over the MIC and lead to higher cure rates and decreased mortality in RCTs among patients without cirrhosis[125-127]. In a secondary analysis of the BICHROME study, Bartoletti *et al*[128] compared extended infusions *vs* bolus dosing of carbapenems or

piperacillin/tazobactam in patients with cirrhosis who had bloodstream infections. The authors found that extended infusions were associated with improved mortality and higher rates of hospital discharge. Currently, the use of prolonged infusions of beta-lactams is recommended in patients with sepsis and septic shock[45].

ADRENAL DYSFUNCTION

Corticosteroids provide anti-inflammatory counterbalance to the dysregulated inflammatory response. They counteract vasodilatation by acting on endothelial glucocorticoid receptors[129], potentiate catecholamine effects, and contribute to volume retention. Therefore, hydrocortisone is recommended for the treatment of septic shock refractory to norepinephrine (at doses > 0.25 mg/kg/min)[45], although multiple RCTs have yielded conflicting data about their efficacy[130-134]. In 2018 the results of the most recent trials ADRENAL[135] and APROCCHSS[136] were published. In the former, the investigators tested the administration of continuous intravenous hydrocortisone against placebo for seven days in patients with septic shock. Although the authors did not observe a mortality benefit, time to shock reversal, length of ICU stay, and mechanical ventilation duration were all reduced in the hydrocortisone group[137]. In the APROCCHSS trial, investigators compared bolus intravenous hydrocortisone plus oral fludrocortisone to placebo, demonstrating improved survival and faster shock resolution[136]. Based on these mixed results, Pirracchio *et al*[138] used data from these RCTs in a machine learning model to explore the individual treatment effect of corticosteroids based on individual estimates of benefit. The authors found that corticosteroid administration based on risk modeling yielded benefit compared to a treat-all-or-none approach. However, the impact of the presence or absence of cirrhosis was not assessed.

The number of patients with cirrhosis in studies evaluating the role of steroids in septic shock is low (Table 1). Nonetheless, 50%-80%[139-141] of patients with advanced cirrhosis have normal baseline cortisol secretion but impaired response to stress; a state called relative adrenal insufficiency (RAI). In stable patients with cirrhosis, RAI is diagnosed and managed according to the adrenal response to ACTH stimulation[142], but in critically ill patients, its use to characterize and manage RAI is discouraged[143].

The high prevalence of RAI would suggest a clear benefit in favor of corticosteroids among patients with cirrhosis, but the evidence for their efficacy is mixed. In a prospective observational study, Ferná ndez *et al*[144] demonstrated that corticosteroids conferred improved survival and faster shock resolution. In a small RCT, Arabi *et al*[145] noted improvements in shock resolution but no survival benefit. Patients treated in the corticosteroid arm had a higher incidence of shock relapse, which supports the notion of unmasked RAI. A higher incidence of gastrointestinal bleeding was observed in the Arabi trial, but this was not replicated in larger observational studies[146]. Despite the mixed evidence, SSC guidelines currently recommend the use of corticosteroids in patients with refractory shock[45].

CONCLUSION

The management of patients with cirrhosis and septic shock is largely based on data extrapolated from RCTs of patients without cirrhosis. However, in light of key differences in pathophysiology, basic interventions may be associated with different outcomes in this subset. Although the SSC guidelines have streamlined and improved the management of septic shock in the general population, these recommendations must ultimately be individualized for patients with cirrhosis using evidence-based strategies. In light of the growing impact of cirrhosis on the care of critically ill patients, future research in septic shock should focus on including and accurately characterizing this population in an effort to overcome critical knowledge gaps.

FOOTNOTES

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Ablative strategies for recurrent hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and is the fifth leading cause of cancer death worldwide and the third leading cause of all diseases worldwide. Liver transplantation, surgical resection and ablation are the three main curative treatments for HCC. Liver transplantation is the optimal treatment option for HCC, but its usage is limited by the shortage of liver sources. Surgical resection is considered the first choice for early-stage HCC, but it does not apply to patients with poor liver function. Therefore, more and more doctors choose ablation for HCC. However, intrahepatic recurrence occurs in up to 70% patients within 5 years after initial treatment. For patients with oligo recurrence after primary treatment, repeated resection and local ablation are both alternative. Only 20% patients with recurrent HCC (rHCC) indicate repeated surgical resection because of limitations in liver function, tumor location and intraperitoneal adhesions. Local ablation has become an option for the waiting period when liver transplantation is unavailable. For patients with intrahepatic recurrence after liver transplantation, local ablation can reduce the tumor burden and prepare them for liver transplantation. This review systematically describes the various ablation treatments for rHCC, including radiofrequency ablation, microwave ablation, laser ablation, high-intensity focused ultrasound ablation, cryablation, irreversible electroporation, percutaneous ethanol injection, and the combination of ablation and other treatment modalities.

Key Words: Hepatocellular carcinoma; Recurrence; Ablative therapy; Thermal ablation

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Core Tip: Despite the tremendous efforts in the fight against hepatocellular carcinoma, there is still no way to prevent its recurrence. Intrahepatic recurrence can be treated by repeated resection and ablation, and there are many studies showing the advantages and disadvantages of each treatment method. For tumors ≤ 3 cm in diameter, there is no significant difference between surgery resection and radiofrequency/microwave ablation treatment. Non-thermal ablation treatment has clearer borders but a higher postoperative recurrence rate. Percutaneous ethanol injection has comparable efficacy to radiofrequency ablation for small tumors. Multiple recurrences require combined systemic therapy.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer death worldwide[1]. Up to 20% of HCC patients relapse within 2 years after liver transplantation and 1/3 of post liver transplantation patients with recurrent HCC (rHCC) experienced late recurrence (> 5 years after liver transplantation)[2]. Nearly half of patients with early-stage HCC experienced recurrence after Hepatic resection[3]. The local recurrence rate in patients with HCC treated radiofrequency ablation (RFA) varied between 18.2% and 46.6%[4].The incidence of intrahepatic and extrahepatic recurrence had been reported to be approximately 70% eventually[5,6]. Thus, the treatment and management of HCC recurrence is very important.

rHCC can be divided into two types: Oligo recurrence and disseminated recurrence[7,8]. Patients with oligo recurrence were consider for radical treatment, including surgery and RFA[9]. Whereas patients with disseminated recurrence received palliative treatment or supportive care only[9]. The curative approaches for intrahepatic rHCC include salvage liver transplantation, repeat resection and RFA. Salvage liver transplantation has been proposed to be the optimal option but have precluded its extensive application because of the shortage of organ donors and the strict selection criteria for patients [10-12]. In addition, because of progressive liver dysfunction, the presence of multiple tumors, various tumor sites, and intraperitoneal adhesions, only 20% patients with recurrence are eligible for repeated resection[13,14].

Ablation has the advantages of minimal invasiveness, fewer complications and good repeatability [15]. Ablative therapy is a locoregional treatment that can be used alone or in combination with other treatment modalities[16]. Monotherapy includes RFA, microwave ablation (MWA), laser ablation (LA), high-intensity focused ultrasound (HIFU) ablation, cryoablation (CRA), irreversible electroporation (IRE), and percutaneous ethanol injection (PEI). Combination therapy includes RFA/MWA-transcatheter arterial chemoembolization (TACE) and RFA-PEI.

RADIOFREQUENCY ABLATION

RFA generates 400-500 kHz radiofrequency current through the distal end of the uninsulated part of the puncture needle, which causes high-frequency friction of water molecules in the tumor tissue and local high temperature, leading to co-degeneration necrosis and protein degeneration in the tumor tissue[4, 17,18]. RFA is one of the curative treatment modalities for early-stage HCC with advantages of safety, tolerability, ease of operation, and cost-effectiveness[4]. Previous study has demonstrated that RFA provided similar long-term survival rates for isolated HCC of 5 cm or less, regardless of whether the treatment was initial or salvage therapy[19]. Indications of RFA for rHCC are the same as those for initial HCC, including single nodule < 5 cm in diameter or less than 3 nodules with the largest diameter < 3 cm, and without vascular invasion nor extrahepatic metastasis[15].

There were no significant differences in overall survival (OS), re-recurrence rate, distant progression-free survival rate, local progression-free survival rate, nor complications between RFA and repeated resection in early-staged rHCC[16,20]. Another recent randomized controlled trial showed no statistically significant difference in OS and repeat recurrence–free survival (RFS) between repeated resection and RFA in early-staged rHCC[21]. Additionally, in patients with rHCC diameter greater than 3 cm or alpha-fetoprotein level greater than 200 ng/mL, local disease control and long-term survival may be better with repeated resection[21]. Moreover, thermal ablation is superior to repeated resection in safety, such as significantly shorter average hospital stay, less risk of intraoperative blood transfusion, and less invasive[16,22,23].

The main contraindications of RFA are severe bleeding diathesis (platelet count less than 50000/μL), hemostatic compromise, decompensated ascites, jaundice and the presence of metallic devices such as pacemakers[24]. Relative contraindications are lesions near the gastrointestinal tract, biliary system and heart. RFA should also be avoided for tumors within 1 cm proximal to the hepatic portal tract[24].

MICROWAVE ABLATION

MWA causes cell death by increasing the temperature of tumor tissue caused by electromagnetic energy deposition in the tumor[25]. The advantages of MWA over RFA are as follows: (1) MWA uses electromagnetic wave energy without grounding poles, so it does not cause skin burns and has no taboos to metals[26]; (2) The electromagnetic field of the MWA causes rapid and uniform heating of the tissue, creating a more uniform and predictable ablation zone with less time; and (3) MWA provides faster heating and higher temperature, so MWA is suitable for perivascular, subcapsular lesions and those adjacent to bile duct[27-29]. Previous study has demonstrated no significant difference in OS nor disease-free survival (DFS) between MWA and surgical resection[30]. Meanwhile, the meta-analysis demonstrated that MWA was associated with shorter operation time, less amount of blood loss in operation, and less complications when compared to surgical resection[30].

The complications of MWA and RFA are similar, such as bleeding, liver abscess, hemothorax, colon perforation and bile duct stenosis[31].

LASER ABLATION

LA is a procedure based on laser devices that convert heat energy into light energy and generate heat with tissues to cause cell death[32] and was firstly described in 1983 for the treatment of liver tumors [33].

A randomized controlled trial confirmed LA should be considered a viable treatment option for HCC ≤ 20 mm, given lower incidence of complications than the RFA group and comparable primary technique efficacy rate and RFS rate[34]. Traditional thermal ablation techniques (RFA and MWA) are considered less effective than TACE in obtaining a complete response for solitary large HCC ≥ 40 mm [35-37]. A recently published retrospective case-control study indicated that multifiber LA approach was more effective than TACE by achieving a complete tumor ablation and reducing the recurrence rates[38]. However, LA is rarely used and has been superseded by MWA or RFA in many centers partly because LA requires a high level of equipment and its results need to be confirmed by randomized controlled trials[39].

HIGH-INTENSITY FOCUSED ULTRASOUND ABLATION

HIFU ablation is a non-invasive ablation mode using an ultrasound frequency of 0.8-3.5 MHz focused through intact skin on a distant therapeutic transducer[40,41]. Compared with RFA, HIFU has the following advantages: (1) HIFU is an *ex vivo* conformal therapy without invasiveness; (2) Tumor seeding is unlikely to occur in HIFU; and (3) No direct puncture of target tumor[42].

HIFU is currently used mainly for palliative treatment of advanced HCC[43]. There was only one retrospective study showed that the OS of HIFU was slightly higher than that of RFA, but the DFS was lower than that of RFA, and the procedure-related morbidity was lower after a median follow-up time of 27.9 mo of patients with rHCC[44]. Notably, this study was retrospective in nature and had a small sample size.

The main limitation to the clinical application of HIFU is the long ablation time required. Other challenges are the difficulty in precise localization and monitoring, and the difficulty in transmitting ultrasonic energy through the covering bone structure to the lesions behind the ribs[45]. The main systemic changes of HIFU are fever, supraventricular tachycardia, hypertension. The local complications are skin burns, pain, mild impairment of hepatic function and mild hematuria[46].

CRYOABLATION

CRA uses extremely low temperatures to directly cause intracellular and extracellular ice crystal formation and lytic agent deformation, leading to cell dehydration and rupture. Vascular injury leads to ischemic hypoxia indirectly destroying tumor tissue. CRA has several potential advantages over RFA: (1) Multiple probes can be used simultaneously to produce a large puck; (2) the size and shape of the puck produced by cryotherapy can be easily seen by intraoperative computed tomography (CT), magnetic resonance imaging, or ultrasound; and (3) relatively painless procedure compared to thermal-

based ablation, which can be performed under local anesthesia rather than the general anesthesia required for radiofrequency ablation[47].

The efficacy of CRA for HCC has been demonstrated by a large study cohort including 1595 patients with 2313 tumors[47]. The complete response (CR) rates were 81.2% (1893/2313), 99.4% (780/784), 94.4% (1622/1719), and 45.6% (271/594) in all tumors, tumors < 3 cm, tumors < 5 cm, and tumors > 5 cm, respectively. The CR rate was high than that for RFA that ranged from 50% to 80% in HCCs of 3 to 5cm[48]. At present, the application of CRA in the treatment of rHCC has not been reported.

The most common complications of CRA are postoperative pain, postoperative fever, transient elevations of alanine aminotransferase, hepatic hemorrhage, liver and pleural abscesses and cryoreaction (chills, fever, tachycardia, tachypnea and temporary renal damage, *etc.*)[49,50]. CRA is recommended as first-line therapy for tumors < 5 cm. For tumors > 5 cm, CRA can reduce tumor burden [47].

IRREVERSIBLE ELECTROPORATION

IRE works by generating high voltage (> 640 V/cm) and high intensity (> 20 A) electrical pulses of short duration (70–100 μs) which render the cellular bilipid membrane of the cells permanently irreversible porous[51,52]. IRE is a good option for patients who cannot undergo surgery, thermal ablation surgery, or whose tumors are close to important structures[51].

A recent meta-analysis reported an OS of 81.3% at 12 mo, 61.5% at 2 years, and 40.9% at 3 years; PFS was reported as 64.2% at 12 mo and 49.1% at 2 years[53]. Since RFA and MWA are preferred in tumors located at "non-risk" locations and IRE is used in "high location", the efficacy of IRE cannot be directly compared with RFA and MWA in a clinical setting[53].

The major complications are liver abscess, hemorrhage, fever, mild pleural effusion, mild hemoperitoneum, subcapsular hematomas, atrial fibrillation and partial portal vein thrombosis[54-57].

PERCUTANEOUS ETHANOL INJECTION

Injection of ethanol caused dehydration and necrosis of tumor cells accompanied by thrombosis in small vessels to kill tumor tissue[58]. A matched case-control comparative analysis showed that the OS of PEI is comparable to RFA in patients with HCC smaller than 1.5 cm[59]. The major limitation of PEI is significantly higher local recurrence than RFA[60,61]. Interestingly, recent studies have shown that the combination of PEI with RFA in the treatment of HCCs provides comparable OS rates and RFS.

The mechanisms of RFA-PEI are as follows: (1) RFA enhances the ablative effect of ethanol due to its low boiling point (78.3 °C); (2) Ethanol embolizes small vessels to reduce the heat-sink effect; (3) Ethanol distributes to RFA enabled areas (or difficult-to-treat areas); (4) Ethanol diffuses beyond the RFA ablation zone to establish a safety margin; and (5) An ethanol makes the tissue around the electrode less prone to carbonization and further thermal conduction[62-64].

A retrospective study enrolled 271 patients to compare combined RFA-PEI with hepatic resection in the treatment of resectable solitary HCC with 2.1-5.0 cm diameter[65]. RFA-PEI had higher OS rates at 1, 3, and 5 years and RFS rates at 1, 3, and 5 years over hepatic resection in the treatment of solitary HCCs, especially for those with 2.1-3.0 cm in diameter. Additionally, RFA-PEI was superior to hepatic resection in major complication rates, length of hospital stay and cost. A meta-analysis showed that for tumors with 3-5 cm in diameter, the 2-year OS was slightly higher in the RFA-PEI than in the RFA group[66]. There were another two studies showed significant clinical improvements in the combination group in terms of the 1-/1.5-/2-/3-/5-year OS[67,68]. Furthermore, post-procedural major complications and pain did not significantly differ between the RFA-PEI groups and RFA groups[69]. A retrospective study found that the combined RFA-PEI group had comparable OS and RFS to repeat hepatectomy for elderly patients with small rHCC after hepatectomy, but with shorter hospital stays and lower rates of major complications and non-tumour-related deaths[70]. In summary, combined therapy with RFA-PEI is suitable for 2 to 3 cm lesions with liver function compensation.

ABLATION COMBINED WITH TACE

Iodized oil and gelatin sponge particles used in TACE can increase RFA- or MWA-induced coagulation necrosis by going through multiple arterio-portal communications. TACE enhances heat transfer in RFA or MWA treatment by blocking hepatic arterial blood flow and reducing perfusion-mediated hepatic blood flow cooling (heat-sink effect)[4,24]. It has been improved in: (1) Minimizing heat loss due to the heat-sink effect; (2) increasing the area of coagulative necrosis; (3) producing more thorough necrosis within the mass; and (4) enlarging the ablation margin, destroying the satellite lesion[4,24,71]. In addition, the digital subtraction angiography technique during TACE helps to detect multiple small

tumors and subsequent eradication these tumors[72].

A recent study by Li *et al*[73] that included 3000 cases of HCC showed the OS rate and CR rate of the TACE-RFA group was significantly higher than the TACE alone group[73]. Another review presented that TACE-RFA combined therapy and surgical resection had a similar 1-year OS rate, 3-year OS rate, 1-year RFS rate, and 3-year RFS rate for early HCC[74]. However, the 5-year OS rate and 5-year RFS rate were lower in patients with TACE-RFA than in those with surgical resection. Furthermore, there were two studies found that TACE-RFA treatment is superior to RFA used alone in OS and RFS[75,76]. A recent study demonstrated that for HCC patients with microvascular invasion (MVI) and rHCC up to three nodules smaller than 3 cm within 2 years, TACE-RFA could achieve better secondary RFS than repeated resection or RFA alone, while RFA alone had survival benefits comparable to repeated resection in other rHCC patients with small recurrence[77]. There was a study that selected 186 patients who underwent TACE-RFA (n = 107) or repeated resection (n = 79) for rHCC with a diameter < 5 cm [72]. It showed fewer complications and shorter hospital stays in the TACE-RFA group than in the repeated resection group, and there were no significant differences in OS nor RFS.

A recent study conducted by Zaitoun *et al*[78] screened 278 patients with HCC 3-5 cm, and patients were randomized into three groups: 90 underwent TACE (group 1); 95 underwent MWA (group 2); and 93 underwent combined therapy (group 3)[78]. Their study found that group 3 had the significantly lower recurrence rate after 12 mo, and the significantly higher OS and mean progression-free survival than groups 1 and 2. Therefore, the combination of thermal ablation with TACE therapy is an optimal choice for patients with HCC tumors > 3 cm[78,79].

RFA or MWA can be performed the same day or less than 2 wk after TACE[75,78]. The most common complications of TACE-RFA are gastrointestinal bleeding, abscess, liver failure, liver infarction[71].

PERCUTANEOUS VS LAPAROSCOPIC TECHNIQUES

Thermal ablation can be performed safely using percutaneous or laparoscopic techniques. RFA was generally applied to HCC patients who could not endure repeated resection of the tumor or were not eligible for liver transplantation. RFA is commonly used to eliminate percutaneous tumors and is the most appropriate method for HCC masses far from the intestine, bile duct, ureter, or diaphragm[80]. In contrast, the laparoscopic RFA (LRFA) procedure requires general anesthesia, so the patient is more cooperative, the ablation boundaries are clearer, and the ablation can reach deeper. Therefore, laparoscopic RFA performed better than PRFA in the deep-seated liver cancers, such as subphrenic lesions[81].

According to Kwak *et al's* study[82] on subphrenic HCC, the local tumor progression rate of the LRFA group was significantly lower than that of the PRFA group, the cumulative OS rate of the LRFA group was significantly higher than that of the PRFA group, and there was no statistical difference in DFS rate between the two groups[82]. Another study showed that laparoscopic MWA seemed to have a tendency to be more effective than percutaneous MWA in the treatment of subcapsular HCC[83]. However, the laparoscopic approach has a higher rate of postoperative complications than the percutaneous approach [80,82,83]. Consequently, LRFA can be a valuable treatment option for subphrenic and subcapsular HCC if accessible using the laparoscopic approach.

ABLATION VS OTHER LAGICAL TREATMENT

Apart from ablation, non-operative local treatment of HCC includes TACE, stereotactic body radiation therapy (SBRT) and Proton beam radiotherapy (PBT). Many articles have shown that RFA has long-term benefits comparable to repeat hepatectomy (RH) for tumours less than 3 cm[16,20,21,84]. The study showed that RFA has better OS and RFS advantages than TACE for rHCCs in both ≤ 3 cm and > 3 cm lesions[84]. RFA and SBRT showed considerable therapeutic benefit for rHCC ≤ 3 cm, better OS but lower RFS rate for rHCC > 3 cm[84]. A prospective randomized study showed that the LPFS rate of PBT was comparable to that of RFA observed in rHCC patients with ≤ 2 tumor (s) of < 3 cm[85].

The 2-year OS was slightly higher in the RFA-PEI than in the RFA group, and current evidence was difficult to draw a definite conclusion regarding the therapeutic management in terms of local recurrence free proportion and complete tumor necrosis. However, TACE-RFA is comparable to RH in both OS and RFS, and has a lower complication rate and hospital stay than RH. Therefore, in patients with liver function compensation, TACE-RFA local therapy may be considered as a preferred option.

The use of percutaneous fusion imaging-guided RFA is effective and safe for the treatment of subcentimeter rHCC[86]. However, PRFA was not feasible in 34.3% (72/210) of sub-centimeter rHCCs primarily due to poor lesion conspicuity[87]. And fusion imaging with or without CEUS does not always satisfactorily locate sub-centimeter rHCC. Cone-beam CT-guided TACE can also be used as an alternative local therapy for subcentimeter rHCC due to its high technical feasibility and detectability [88].

Table 1 Application of various minimally invasive treatments in hepatocellular carcinoma

Tumour size	Patients condition	Treatment
Sub-centimeter	Percutaneous tumors	PRFA
	Local ablation therapy is not feasible	TACE
< 3 cm	Percutaneous tumors	PRFA
	Subphrenic and subcapsular tumors	LRFA
	Perivascular tumors	MWA
	Can't endure thermal ablation	CRA
	MVI (+)	TACE-RFA
3-5 cm	With liver function compensation	TACE-RFA
	Liver failure	MWA, CRA

PRFA: Percutaneous radiofrequency ablation; LRFA: Laparoscopic radiofrequency ablation; TACE: Transarterial chemoembolization; MWA: Microwave ablation; CRA: Cryoablation; MVI: Microvascular invasion.

CONCLUSION

This review methodically describes the treatment of rHCC by various ablation procedures in recent years. Moreover, this study compares the indications, advantages and survival analysis of various ablative treatments. Therefore, we summarize how to choose the appropriate ablation therapy for different rHCC patients (Table 1).

RFA, MWA and CRA can be considered recommended as first-line treatment for rHCC < 3 cm in diameter. RFA is currently the most widely used, PRFA can be used in percutaneous tumors and LRFA can be used in subphrenic and subcapsular tumors, while MWA is more recommended for patients with perivascular lesions and TACE-RFA could be consider for patients with MVI (+). CRA is an option for patients who are not candidates for thermal ablation. For patients with 2 to 3 cm lesions with liver function compensation, PEI-RFA can be selected. TACE combined with RFA/MWA provided better overall and disease-free survival than TACE alone. For tumors with diameter ranging from 3 to 5 cm, MWA, CRA, and TACE-RFA are recommended. Remarkably, TACE-RFA is a better choice for patients with 3-5 cm rHCC with liver function compensation. For tumors > 5 cm in diameter, local ablation can reduce the tumor burden as a bridging therapy during the waiting period for liver transplantation, or as palliative treatment for recurrence after liver transplantation. In conclusion, the treatment decisions were individualized requires a professional liver surgeon to consider the patient performance status, liver function, and recurrent tumor status.

FOOTNOTES

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MINIREVIEWS

Therapeutic possibilities of gut microbiota modulation in acute decompensation of liver cirrhosis

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Abstract

The formation of liver cirrhosis (LC) is an unfavorable event in the natural history of chronic liver diseases and with the development of portal hypertension and/or impaired liver function can cause a fatal outcome. Decompensation of LC is considered the most important stratification variable for the risk of death. It is currently postulated that decompensation of LC occurs through an acute (including acute-on-chronic liver failure) and non-acute pathway. Acute decompensation of LC is accompanied by the development of life-threatening complications, characterized by an unfavorable prognosis and high mortality. Progress in understanding the underlying molecular mechanisms has led to the search for new interventions, drugs, and biological substances that can affect key links in the pathogenesis of acute decompensation in LC, for example the impaired gut-liver axis and associated systemic inflammation. Given that particular alterations in the composition and function of gut microbiota play a crucial role here, the study of the therapeutic possibilities of its modulation has emerged as one of the top concerns in modern hepatology. This review summarized the investigations that describe the theoretical foundations and therapeutic potential of gut microbiota modulation in acute decompensation of LC. Despite the encouraging preliminary data, the majority of the suggested strategies have only been tested in animal models or in preliminary clinical trials; additional multicenter randomized controlled trials must demonstrate their efficacy in larger patient populations.

Key Words: Liver cirrhosis; Acute decompensation; Pathogenesis; Therapy; Gut microbiota; Modulation

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Core Tip: Given that particular alterations in the composition and function of gut microbiota play a crucial role in the pathogenesis of acute decompensation in liver cirrhosis (LC), this review summarized the investigations that describe the theoretical foundations and therapeutic potential of gut microbiota modulation in acute decompensation of LC. Despite the encouraging preliminary data, the majority of the suggested strategies have only been tested in animal models or in preliminary clinical trials. Additional multicenter randomized controlled trials must demonstrate their efficacy in larger patient populations.

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INTRODUCTION

The formation of liver cirrhosis (LC) is an unfavorable event in the natural history of chronic liver diseases and with the development of portal hypertension and/or impaired liver function can cause a fatal outcome. During LC, there is a compensated stage, which is usually asymptomatic and characterized by preserved quality of life and a median survival exceeding 12 years, and a decompensated stage associated with the occurrence of life-threatening complications, in which a median survival drops to 2-4 years. Accordingly, the decompensation of LC is considered the most important stratification variable for the risk of death[1]. Decompensation of LC presents as acute decompensation in a portion of patients with the development of one or more major complications and is accompanied by high mortality. In many other patients, its characteristic signs are usually slow-progressing ascites or mild grade 1-2 hepatic encephalopathy or jaundice not requiring hospitalization[2].

The European Association for the Study of the Liver (EASL)-CLIF Consortium CANONIC study established diagnostic criteria for acute-on-chronic liver failure (ACLF) and introduced the concept of acute decompensation as a distinct clinical presentation of decompensation of LC defined by the acute development of more than one major complication: (1) Acute (for less than 2 wk) development of grade 2 or 3 ascites. This may be the first or a new episode of ascites; (2) Acute hepatic encephalopathy, which is manifested by a sudden change in the mental status of a patient with no previous cognitive impairment and no signs of acute neurological disease. This may be the first or a new episode of hepatic encephalopathy; (3) Acute gastrointestinal bleeding from the upper and/or lower gastrointestinal tract of any etiology; and (4) Spontaneous bacterial peritonitis (SBP), spontaneous bacteremia, urinary tract infection, pneumonia, cellulite, as well as any other type of acute bacterial infection[3].

The cause of acute decompensation of LC can be both exogenous factors (*e.g.*, bacterial infections, alcohol abuse, *etc*) and endogenous factors (*e.g.*, progressive liver disease, translocation of intestinal bacterial immunogenic material to the systemic circulation)[4]. Its most severe form (ACLF) according to the definition of the American College of Gastroenterology is a potentially reversible condition in patients with chronic liver disease with or without LC that is associated with the potential for multiple organ failure and mortality within 3 mo in the absence of treatment of the underlying liver disease, liver support, or liver transplantation. The severity of organ failure may be assessed by the EASL-CLIF sequential organ failure assessment score or North American Consortium for the Study of End-Stage Liver Disease organ failure score[5].

The first investigation derived from the PREDICT study group of the EASL-CLIF Consortium uncovered that acute decompensation of LC without ACLF is a heterogeneous condition with three different clinical courses and two major pathophysiological mechanisms: Systemic inflammation and portal hypertension. The first clinical course includes patients who develop ACLF and have an extremely high short-term mortality rate, termed pre-ACLF. The second clinical course includes patients with unstable decompensated LC who require frequent hospitalizations unrelated to ACLF and is associated with a lower mortality risk than pre-ACLF. The third clinical course includes patients with stable decompensated LC who rarely require hospital admission and have a much lower 1-year mortality risk.

Each clinical course of acute decompensation of LC differs significantly regarding the grade of systemic inflammation and the severity of portal hypertension. A high grade of systemic inflammation at admission with exacerbation during follow-up is observed in pre-ACLF. A low grade of systemic inflammation at admission with subsequent steady course is observed in patients with unstable decompensated LC. A low grade of systemic inflammation at admission with subsequent improvement is observed in patients with stable decompensated LC. A high grade of portal hypertension is observed in patients with unstable decompensated LC. A low grade of portal hypertension is observed in pre-ACLF and stable decompensated LC[6].

The aim of the second investigation derived from the PREDICT study group of the EASL-CLIF Consortium was to analyze and characterize the precipitants leading to acute decompensation of LC without ACLF or with ACLF. Of all the potential precipitants explored, only four (proven bacterial infections, severe acute alcoholic hepatitis, gastrointestinal bleeding associated with shock, and toxic encephalopathy) fulfilled the diagnostic criteria of precipitants. Proven bacterial infections and severe alcoholic hepatitis were present in the absolute majority (> 96%) of the patients. However, no precipitating event could be identified in two-thirds of acute decompensation of LC without ACLF patients and in one-third of acute decompensation of LC with ACLF patients. These data suggest that acute decompensation of LC without ACLF develops more frequently in the context of endogenous mechanisms (*e.g.*, progressive liver disease, bacterial translocation). The prevalence and number of precipitants increased with the severity of the acute decompensation sub-phenotype form stable decompensated LC/unstable decompensated LC to pre-ACLF and ACLF, which were also directly related to clinical course severity and short-term mortality in patients with acute decompensation of LC. These data, therefore, strongly suggest that specific preventive and therapeutic approaches for these precipitants must improve outcomes in decompensated LC[7].

Current therapeutic strategies in acute decompensation of LC provide the removal of the precipitants, the treatment of specific complications, as well as intensive monitoring and support of vital body functions[8]. Liver transplantation may be a successful treatment option for some of the most severe ACLF patients, but its implementation is usually associated with high costs and worse survival compared to "standard" elective surgery[9].

Progress in understanding the underlying molecular mechanisms has led to the search for new interventions, drugs, and biological substances that can affect key links in the pathogenesis of acute decompensation in LC, for example the impaired gut-liver axis and associated systemic inflammation [10]. Given that particular alterations in the composition and function of gut microbiota play a crucial role here, this review summarized the investigations that describe the theoretical foundations and therapeutic potential of gut microbiota modulation in acute decompensation of LC.

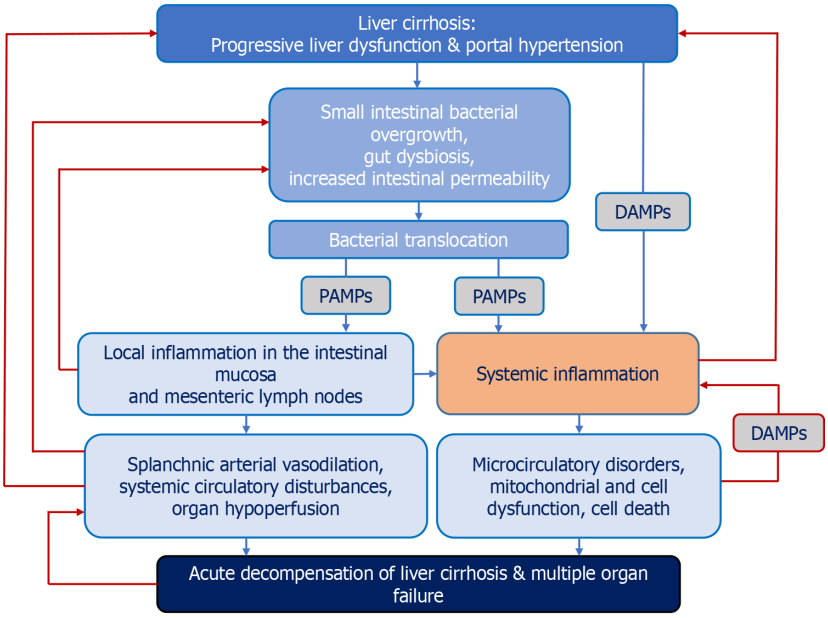
LITERATURE SEARCH

This review provided an overview of the current knowledge of the therapeutic possibilities of gut microbiota modulation in acute decompensation of LC. The PubMed and Embase databases, the Web of Science platform, the Google Scholar retrieval system, the Cochrane Database of Systematic Reviews, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/), and the reference lists from related articles were used to search for relevant publications. Articles corresponding to the aim of the review were selected for 2003–2023 using the keywords: "liver cirrhosis," "acute decompensation," "acute-on-chronic liver failure," "pathogenesis," "therapy," "gut microbiota," and "modulation." The investigations that described the theoretical foundations and therapeutic potential of gut microbiota modulation in acute decompensation of LC were included.

SYSTEMIC INFLAMMATION AS A MAIN DRIVER OF ACUTE DECOMPENSATION IN LC

One of the leading hypotheses in recent years suggests that the main driver of acute decompensation and concomitant multiple organ failure in LC is systemic inflammation. Its cause may be the "spill over" of damage-associated molecular patterns, cytokines, and immune regulatory cells from the chronically inflamed liver and potential other inflamed tissue sites to the systemic circulation. Additionally, pathogen-associated molecular patterns (PAMPs), namely bacterial and bacterial products (in particular, the lipopolysaccharides of the cell wall of Gram-negative bacteria), due to pathological translocation from the intestinal lumen through the portal vein, reach the cirrhotic liver where they may be ineffectively cleared by Kupffer cells or shunted through vascularized septae into the systemic circulation, contributing to systemic inflammation (Figure 1). Over time, immune tolerance may develop, which is characterized by accumulation in the systemic circulation of immune cells with immune suppressive or immune regulatory properties, along with high serum levels of proinflammatory and anti-inflammatory cytokines, damage-associated molecular patterns, and PAMPs. Additionally, with further disease progression to ACLF, cells with a reduced capacity to repel microbial challenges appear in the systemic circulation, which increases the risk of infectious complications and sepsis[11].

Once the first episode of acute decompensation of LC develops, systemic inflammation follows a chronic course, with transient episodes of reactivation caused by exogenous proinflammatory factors or to bursts of bacterial translocation. Repeated episodes of acute decompensation during the clinical course of decompensated LC develop in the setting of reactivation of the immune system. The prognosis of patients with acute decompensation of LC associated with moderate, non-progressive systemic inflammation depends on the severity of portal hypertension. Patients with severe portal hypertension frequently develop an unstable clinical course, requiring frequent hospital readmission, and significant



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Figure 1 Endogenous mechanisms of acute decompensation and concomitant multiple organ failure in liver cirrhosis. DAMPs: Damage-associated molecular patterns; PAMPs: Pathogen-associated molecular patterns.

short-term and long-term mortality. In contrast, if portal hypertension is moderate, systemic inflammation improves after the episode of acute decompensation of LC, patients develop a benign stable course, and long-term mortality is low[12].

THE ROLE OF GUT MICROBIOTA IN THE PATHOGENESIS OF ACUTE DECOMPENSATION IN LC

The alteration of gut microbiota composition in acute decompensation of LC creates prerequisites for disruption of the gut-liver axis, and bacterial translocation contributing to systemic inflammation is based on small intestinal bacterial overgrowth (SIBO), gut dysbiosis, and increased permeability of the intestinal epithelial barrier[13].

SIBO, which is characterized by an excessive number of bacteria in the small intestine (≥ 10^3 colony-forming units/mL) with a predominance of Gram-negative aerobic and anaerobic species, occurs in about half of the patients with LC, but the mechanism of its development has not been definitively established. One of the possible reasons may be the slowing down of orocecal transit[14]. However, the causal relationship between these pathophysiological conditions remains unclear. In some studies, a more pronounced slowing down of orocecal transit was observed in patients with Child-Turcotte-Pugh (CTP) class B and C LC with hepatic encephalopathy, which was explained by the autonomic neuropathy, metabolic derangements due to portosystemic shunting, and SIBO[15].

Because gastric acid is an important barrier that prevents bacterial colonization of the stomach and small intestine, it is assumed that proton pump inhibitor therapy may promote SIBO through chronic acid suppression and subsequent hypochlorhydria. However, a meta-analysis of 19 studies demonstrated that proton pump inhibitor therapy was significantly associated with a moderately increased risk of SIBO (odds ratio: 1.71, 95% confidence interval: 1.20-2.43)[16]. The immune system also plays a role in the genesis of SIBO, as evidenced by the high prevalence of SIBO in patients who have immunodeficiency. Besides, immunoglobulin A content on the duodenum and jejunum mucosa has been shown to be significantly increased in patients with SIBO[17].

Gut dysbiosis in LC is manifested by an unfavorable change in the balance of autochthonous species of microorganisms with a reduction in symbiont bacteria belonging to the Firmicutes phylum (*e.g.*, the Ruminococcaceae and Lachnospiraceae families, *etc*.) and growth in pathobiont bacteria of the Bacteroidetes and Actinobacteria phyla[18]. These changes largely depend on the etiology of LC and are aggravated in its decompensated stage. For example, they were the most significant in patients with alcoholic LC, who had the highest content of bacteria of the Enterobacteriaceae and Halomonadaceae families and the lowest content of bacteria of the Lachnospiraceae, Ruminococcaceae, and Clostridiales Incertae Sedis XIV families, which was accompanied by an exorbitant level of endotoxemia[19]. Note that the bacteria of the Enterobacteriaceae family express a powerful immunostimulating endotoxin and are the main pathogens involved in the pathogenesis of SBP[20]. In a study by Shu *et al*[21], in patients

with hepatitis B virus-related LC, the most common phyla of bacteria were Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia, together constituting 92.1% of the total number of microorganisms studied. Patients with compensated LC had a high relative abundance of bacteria of the genus *Faecalibacterium spp*. and the Ruminococcaceae family, whereas with decompensated LC, bacteria of the genus *Streptococcus spp*. and the Enterobacteriaceae family prevailed.

In a prospective study by Solé *et al*[22], patients with LC had a significant decrease in gene and metagenomic species diversity compared to healthy subjects. This was associated with disease stages and was especially noticeable in patients with ACLF and persisted after antibiotic therapy. ACLF was accompanied by a significant increase in *Enterococcus spp*. and *Peptostreptococcus spp*. and a decrease in some autochthonous bacteria. Gut microbiome alterations correlated with the model for end-stage liver disease (MELD) and CTP scores and multiple organ failure and were associated with some complications, especially hepatic encephalopathy and infections. Additionally, the gut microbiome predicted 3-mo survival with good stable predictors. Functional analysis showed that patients with LC had enriched pathways related to ethanol production, γ-aminobutyric acid metabolism, and endotoxin biosynthesis.

According to a study by Philips *et al*[23] pathogenic genera of bacteria in gut microbiota, in particular, *Leptotrichia spp*., *Neisseria spp*., and *Erwinia spp*., were predominant in patients with decompensated LC with infection, and their survival correlated with the presence of microorganisms with high functional propionate metabolism, for example, bacteria of the genus *Megamonas spp*.

Bajaj *et al*[19] developed a quantitative index to describe microbiome alterations accompanying LC based on the ratio of "good *vs* bad" taxa abundance [cirrhosis dysbiosis ratio (CDR)]. It is designed to predict the course of LC and assess the risk of possible complications. CDR is calculated using the formula:

CDR = [Lachnospiraceae (%) + Ruminococcacceae (%) + Veillonellaceae (%) + Clostridiales Incertae Sedis XIV (%)]/[*Bacteroides spp.* (%) + Enterobacteriaceae (%)].

The authors found that a low CDR was associated with death and organ failure within 30 d.

In a prospective study by the North American Consortium for the Study of End-Stage Liver Disease, including hospitalized patients with LC across North America, the CDR was lower in subjects who developed ACLF, especially among those with renal failure. Taxa belonging to the Proteobacteria phylum (Enterobacteriaceae, Campylobacteriaceae, and Pasteurellaceae) and Firmicutes phylum (Enterococcaceae and Streptococcaceae) were associated with the development of negative outcomes, whereas other Firmicutes members (Lachnospiraceae and Clostridiales) reduced the risk of negative outcomes. Changes in the microbiota were associated independently on logistic regression analyses with extrahepatic organ failure, transfer to intensive care, ACLF, and death[24].

To study the influence of gut dysbiosis on prognosis in LC, Maslennikov *et al*[25] modified CDR by placing "bad" bacteria in the numerator and "good" bacteria in the denominator (MDR)[25]:

MDR = [Bacilli (%) + Proteobacteria (%)]/[Clostridia (%) + Bacteroidetes (%)].

Their case-control study included 48 patients with LC and 21 healthy controls. Patients with an MDR greater than the median indicated the group with severe dysbiosis. The other patients were in the non-severe dysbiosis group. The follow-up period was 4 years. The mortality rate of patients with severe dysbiosis was significantly higher than that of patients with non-severe dysbiosis. The abundance of Enterobacteriaceae, Proteobacteria, and Lactobacillaceae was increased and the abundance of Firmicutes and Clostridia was reduced in the deceased patients compared with survivors. The abundance of Bacilli, Enterococcaceae, and Lactobacillaceae was higher and the abundance of Clostridia was lower in those who died during the 1st year of follow-up compared with those who survived the 1st year. The abundance of Enterobacteriaceae and Proteobacteria was higher in those who died in the second to 4th year of follow-up compared with survivors.

The cause of gut dysbiosis in LC is not fully understood. One of the key theories explains its presence by depletion of the pool of bile acids due to a decrease in their synthesis and secretion by hepatocytes. Bile acid synthesis is regulated mainly through the activation of nuclear receptors, in particular the farnesoid X receptor (FXR), which also induces genes affecting intestinal permeability and inflammation, preventing bacterial translocation in experimental LC[26]. Bile acids have both direct and indirect antimicrobial effects through the modulation of FXR activity, which is important for the homeostasis of the epithelial and gut-vascular barrier. Colonic microbial groups are responsible for the deconjugation and 7α-dehydroxylation of bile acids, and it is hypothesized that the presence of microbe toxic bile acids (particularly deoxycholic acid) in the intestine is a factor that keeps undesirable microbial populations under control[27]. The insufficient content of primary bile acids in feces decreased in 7α-dehydroxylating bacteria belonging to the Firmicutes phylum, especially the genera *Blautia spp*. and *Ruminococcus spp*. Their deficiency in the small intestine causes overgrowth of proinflammatory pathogenic bacteria belonging to the Proteobacteria phylum, in particular the Enterobacteriaceae family, which induces the release of markers of intestinal inflammation and exacerbates necroinflammatory changes in liver tissues. This triggers a positive feedback mechanism leading to additional inhibition of bile acid synthesis[28]. On the contrary, oral administration of conjugated bile acids to rats with a model of CCl4-induced LC and ascites significantly reduced the bacteria in the ileum (especially *Escherichia coli* and *Enterococcus spp*.) to levels comparable to those in healthy rats, decreased the SIBO, bacterial translocation, and endotoxemia[29].

Increased permeability of the intestinal epithelial barrier is associated with both gut dysbiosis[30] and microcirculatory disorders in LC that change the barrier properties of the intestinal mucosa, which include mechanical, biological, immune, and chemical protection factors[31]. Intestinal mucosa and intercellular junctions among epithelial cells form a layer that allows selective passage of the toxins and bacterial products. Intestinal epithelial cells produce mucus, which forms a thick layer on the mucosa and prevents bacterial translocation. Mucous secretions are rich in immunoglobulin A, which neutralizes toxins and microorganisms and prevents their adhesion and colonization. Bile acid secretion also plays a role in intestinal permeability by affecting the intestinal mucosa and by neutralizing endotoxin[32].

In LC, the thickness of the intestinal mucosa is decreased with the loss of mucus-producing goblet cells. The ultrastructural changes of the mucosa, contributing to increased permeability of the intestinal epithelial barrier, are characterized by atrophy, edema, and inflammatory infiltration of the lamina propria, fibromuscular proliferation, expansion of the space between neighboring cells, a reduction in the number of short but thicker microvilli, and a decrease in the villi/crypt ratio. These disorders are associated with a diminution in the expression of the tight junction proteins, including occludin and claudin-1, in the intestinal mucosa. Additionally, irregularities of the glandular epithelia, loss of the normal cylindrical shape, edematous villi, and loosening of the mucous membrane were revealed. High levels of lipid peroxidation markers in enterocytes led to mitochondrial dysfunction and cellular instability[33].

The increased stimulation of gut-associated lymphoid tissue leads to the persistent activation of monocytes, dendritic cells, and T lymphocytes both in the intestinal mucosa and mesenteric lymph nodes and to the consequent production of proinflammatory and anti-inflammatory cytokines. Local inflammatory disorders can be a trigger for systemic inflammation because of immune cells entering the systemic circulation. This is facilitated by a violation of the production of intestinal antimicrobial peptides, in particular α-defensins and Reg3 lectins, a decrease in the ability to bind and neutralize bacterial endotoxin by albumin, lipopolysaccharide-binding protein, high-density lipoproteins, low-density lipoproteins, very low-density lipoproteins, chylomicrons, apolipoproteins, as well as dysfunction of the immune system in patients with LC[34].

The persistence of systemic inflammation leads to a progressive failure of the immune response similar to a condition of immunodeficiency. The immune dysregulation in patients with LC can be defined as a "dysbiotic immune-inflammatory disorder" characterized by abnormal local (gut and liver) and systemic inflammation, triggered by an impaired immune response to gut-derived antigens. The main feature of cirrhotic dysbiotic immune-inflammatory disorder is a perpetual immunologic activation, which involves all the immune cells (neutrophils, monocytes, T and B lymphocytes) that exhibit activation and costimulatory markers.

At the molecular level, the recognition of PAMPs by Toll-like receptors activates MyD88-dependent and MyD88-independent signaling pathways, leading to the activation of nuclear factor kB, production of inflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and interferon-β], chemokines [keratinocyte chemoattractant (CXCL1), MIP-2 (CXCL2), MCP-1 (CCL2), RANTES (CCL5), MIP-1α (CCL3), and MIP-1β (CCL4)], nitric oxide, and reactive oxygen species[35].

The association of Toll-like receptor gene polymorphisms with a decrease in the inflammatory response was established, which further increases the load of circulating bacterial antigens that modulate the immune response and contribute to the development of complications[36]. Cytosolic NOD-like receptors (NLRs) are also involved in this process. The NLRP3 inflammasome formed after the oligomerization of the NLRP3 protein activates caspase 1, which cleaves pro-IL-1β and pro-IL-18, followed by the formation of proinflammatory cytokines IL-1β and IL-18[37].

A cascade of molecular events arising from dysbiotic immuno-inflammatory disorders leads to the enhanced phagocytic activity, vascular endothelial injury, synthesis of acute phase proteins by the liver, chemotaxis of leukocytes to the sites of inflammation (mainly the liver), and activation of leukocytes at the systemic level[38]. This in itself worsens bacterial translocation and contributes to the formation of a vicious circle, which can aggravate the pathological process associated with acute decompensation of LC and predispose to the development of its characteristic complications.

GUT MICROBIOTA AS A POTENTIAL TARGET FOR PROPHYLAXIS AND THERAPY OF ACUTE DECOMPENSATION IN LC

In accordance with the current recommendations of the EASL, one of therapeutic strategy that prevents disease progression in patients with decompensated LC should be aimed to improve the microbiome abnormalities and bacterial translocation to ameliorate the impaired gut-liver axis[39]. In this regard, a potential target for therapy may be the gut microbiota, which is the main regulator of bacterial translocation and systemic inflammation[40].

Antibiotics

The use of non-absorbable or poorly absorbable oral antibiotics is an obvious solution aimed at countering bacterial translocation. They affect the gut microbiota with rare side effects and a favorable long-term safety profile and are recommended as primary and secondary prevention of bacterial infections and treatment of hepatic encephalopathy in patients with decompensated LC[41].

Selective decontamination of the intestine with norfloxacin can contribute to a significant reduction in bacterial translocation. In a study by Albillos *et al*[42], this was manifested by a reduction in the serum levels of lipopolysaccharide-binding protein, soluble CD14, proinflammatory cytokines TNF-α, IL-12 and interferon-γ, as well as the metabolite nitric oxide. In a multicenter, randomized, prospective, double-blind, placebo-controlled trial in parallel groups (NORFLOCIR), including 291 patients with CTP class C LC, the administration of norfloxacin at a dose of 400 mg once daily for 6 mo significantly decreased the incidence of any and Gram-negative bacterial infections without growth infections caused by *Clostridium difficile* or multiresistant bacteria and an increase in survival in patients with ascites fluid protein concentrations < 15 g/L[43].

At the same time, long-term use of norfloxacin increased gut microbiota resistance to fluoroquinolones[44]. Given this, a semisynthetic broad-spectrum antibiotic rifaximin was proposed as an alternative. Rifaximin belongs to the family of naphthalene-ringed ansamycins (rifamycins group) and has a low risk of bacterial resistance. In a randomized controlled trial (RCT) involving 36 patients with decompensated LC with ascites and mean values of the MELD score of 12 ± 3.9, after 4 wk of treatment with rifaximin at a dose of 550 mg twice a day, circulating markers of inflammation, including TNF-α, IL-6, IL-10, IL-18, stromal cell factor-1α, transforming growth factor β1, and high sensitivity C-reactive protein, were unaltered. Rifaximin altered abundance of bacterial taxa in blood marginally, only a decrease in the Pseudomonadaceae family was observed. In feces, rifaximin decreased the bacterial richness but did not affect the particular species[45].

In an observational study involving 30 patients with decompensated LC after 4 wk of treatment with rifaximin at a dose of 1200 mg/d, there was an improvement in hyperammonemia and cognitive dysfunction, although no significant changes in the serum proinflammatory cytokine levels were observed. Rifaximin reduced the serum levels of ammonia, bacterial endotoxin, soluble CD163, and the D-mannose receptor. At the same time, the serum proinflammatory cytokine levels remained the same. Gut microbial analysis revealed that the richness and complexity of species were unchanged, while the abundance of the genus *Streptococcus spp*. after treatment with rifaximin was reduced[46].

The current literature data do not provide a clear answer as to which of the antibiotics is more effective in preventing bacterial translocation in patients with decompensated LC. Nevertheless, in a randomized, double-blind, placebo-controlled trial by Kulkarni *et al*[47], primary prophylaxis with oral norfloxacin (400 mg/d for 30 d) effectively prevented bacterial infections in patients with ACLF. Furthermore, a systematic review and meta-analysis of 17 RCTs showed that rifaximin is useful for both primary and secondary prevention of SBP, whereas norfloxacin daily and alternate norfloxacin and rifaximin are useful for primary prophylaxis[48].

Probiotics

A scientific basis for the use of probiotics in the treatment of liver diseases is their ability to correct gut dysbiosis, elevate the production of short-chain fatty acids, and reduce the increased permeability of the intestinal epithelial barrier[49]. The therapeutic potential of probiotics in LC has been studied in both experimental and clinical studies. For example, oral administration of a combined probiotic VSL#3 containing 8 different strains (*Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*) to rats with different models of LC led to stabilization in the intestinal epithelial barrier, reduction of bacterial translocation, and decrease in severity of endotoxemia and systemic inflammation [50,51]. Oral administration of probiotics *Bifidobacterium pseudocatenulatum* CECT7765 to mice with a model of CCl4-induced LC was accompanied by an improvement in the integrity of the intestinal epithelial barrier and prevented bacterial translocation[52]. It also induced a morphologic, phenotypic, and functional transitional change towards an anti-inflammatory profile in blood-derived and ascitic fluid macrophages from patients with CTP class C LC as well as Kupffer cells from rats with a model of bile duct ligation-induced LC[53]. The combined use of probiotics containing *Clostridium butyricum* and *Bifidobacterium infantis* in patients with hepatitis B virus-related LC and minimal hepatic encephalopathy significantly decreased the pathogenic bacteria of the genus *Enterococcus spp*. and the Enterobacteriaceae family in gut microbiota as well as reduced the circulating levels of bacterial translocation markers and decreased the permeability and damage of the intestinal epithelial barrier[54].

Some RCTs have studied the effect of probiotics on gut microbiota in patients with LC. In one of them, the administration of the probiotic beverage Yakult 400, which contains *Lactobacillus casei* strain Shirota, twice a day during the first half of the 4-wk study contributed to the normalization of gut microbiota and improved liver function in patients with CTP class A alcoholic LC[55]. This probiotic was safe and effective in patients with cirrhosis (CTP score ≤ 10) who took it three times daily for 6 mo. It significantly reduced the plasma monocyte chemotactic protein-1, plasma IL-1β (alcoholic LC), IL-17a, and macrophage inflammatory protein-1β (non-alcoholic LC) compared to the placebo group. At the

same time, no significant differences in intestinal permeability, bacterial translocation, or metabolomic profile were observed[56].

Bajaj *et al*[57] showed that the administration of the probiotic *Lactobacillus rhamnosus GG* for 8 wk to patients with LC (mean values of the MELD score of 8.6 ± 2.2) and minimal hepatic encephalopathy was safe and well tolerated and reduced the serum levels of bacterial endotoxin and TNF-α, decreased the relative abundance of Enterobacteriaceae family, and increased the Clostridiales Incertae Sedis XIV and Lachnospiraceae families, without changes in cognitive dysfunction.

Daily intake for 6 mo of a probiotic powder containing eight different bacterial strains (*Bifidobacterium bifidum W23*, *Bifidobacterium lactis W52*, *Lactobacillus acidophilus W37*, *Lactobacillus brevis W63*, *Lactobacillus casei W56*, *Lactobacillus salivarius W24*, *Lactococcus lactis W19*, and *Lactococcus lactis W58*) by patients with cirrhosis (CTP score < 12) had a beneficial effect on immune function, but no effect on the permeability of the intestinal epithelial barrier and bacterial translocation was observed[58]. In addition, it increased the relative abundance of bacteria of the species *Faecalibacterium prausnitzii*, *Syntrophococcus sucromutans*, *Bacteroides vulgatus*, and *Alistipes shahii* and the genus *Prevotella spp*. compared to the placebo group. At the same time, the relative abundance of bacteria of the species *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, and *Lactobacillus casei* remained unchanged[59].

Thus, although in most studies the probiotic use in LC is associated with an improvement in gut microbiota profile, data concerning their impact on permeability of the intestinal epithelial barrier, bacterial translocation, and systemic inflammation are scarce and contradictory.

Fecal microbiota transplantation

In recent years, numerous studies have demonstrated the therapeutic possibilities of fecal microbiota transplantation (FMT) from healthy donors to patients with chronic liver diseases[60]. It is assumed that the effectiveness of FMT is associated with the creation of a competitive environment in the intestine due to non-pathogenic microorganisms and their production of antimicrobial substances, such as bacteriocins. In addition, the positive effect of donor fecal material on the gut virome and microbiota, the metabolism of short-chain fatty acids and some bile acids, as well as various immune mechanisms is not excluded[61]. Much attention has been paid to the use of FMT to treat hepatic encephalopathy in LC. At the same time, the issues of its effectiveness, safety, and tolerability, as well as methods of administration of donor fecal material (using enemas, colonoscopy, or in encapsulated form), the type and number of transplanted microorganisms necessary to obtain a positive result are discussed[62].

In the first open-label RCT involving 10 patients with LC (MELD score < 17) and recurrent hepatic encephalopathy, three frozen-then-thawed FMT units (90 mL total) instilled by enema and retained for 30 min eliminated antibiotic-induced dysbiosis. All patients showed an improvement in cognitive dysfunction, which may have been associated with an increase of the relative abundance of bacteria of the Lactobacillaceae and Bifidobacteriaceae families[63]. With further follow-up (12.9 ± 2.9 mo), no cases of hepatic encephalopathy were detected, and only 1 patient from this cohort required hospitalization. Microbiological analysis of the gut microflora showed an increase of relative abundance of bacteria of the Burkholderiaceae family and a decrease in the relative abundance of bacteria of the Acidaminococcaceae family, while the relative abundance of bacteria of the Lactobacillaceae and Bifidobacteriaceae families did not differ from the placebo group[64].

In a phase I RCT, the administration of 15 capsules with donor fecal microbiota to 10 patients with LC (MELD score < 17) and recurrent hepatic encephalopathy had a positive effect on cognitive dysfunction was safe and well tolerated. After 30 d of monitoring, there was an improvement in duodenal mucosal microbial diversity with higher Ruminococcaceae and Bifidobacteriaceae families and lower Streptococcaceae and Veillonellaceae families (P = 0.01). Reduction in the Veillonellaceae family was seen post-FMT in sigmoid (P = 0.04) and stool (P = 0.05). Duodenal E-cadherin (P = 0.03) and defensin A5 (P = 0.03) increased, while the IL-6 (P = 0.02) and serum levels of lipopolysaccharide-binding protein (P = 0.009) reduced post-FMT[65].

An important problem of FMT is the risk of severe infection transmission, which is especially significant in patients with weakened immunities[66]. For this reason, the United States Food and Drug Administration published a list of minimum requirements for screening and testing of fecal microbiota donors for the presence of multidrug resistant microorganisms in 2019[67].

The coronavirus disease 2019 pandemic has raised concerns about the possible transmission of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in FMT. Although the genetic material of SARS-CoV-2, including the live virus, has been detected in the feces of patients with a new coronavirus infection even after the elimination of respiratory symptoms[68], no actual cases of infection through donor fecal material were reported. Stool testing for SARS-CoV-2 is not currently widely available. Nevertheless, experts advocate screening donors for the presence of symptoms of a new coronavirus infection with quarantine of their fecal material during further monitoring of the disease [69].

Obeticholic acid

Obeticholic acid is a semisynthetic bile acid that, in addition to bacteriostatic activity, is an agonist of FXR and thus can modulate the gut microbiota. For example, oral administration of obeticholic acid to rats with a model of CCl4-induced LC reduced the intestinal content of the genus *Enterococcus spp*.[70]

and decreased the bacterial translocation[71]. Besides, obeticholic acid prevented the increased expression of monocyte chemoattractant protein-1 following stimulation with TNF-α and lipopolysaccharides or TNF-α alone in liver sinusoidal endothelial cells and Kupffer cells in rats with a model of thioacetamide-induced LC[72]. In these studies, obeticholic acid had a beneficial effect on the production of antimicrobial peptides by ileum epithelial cells, the expression of the tight junction proteins, intestinal inflammation, and liver fibrosis.

At present, the therapeutic potential of obeticholic acid in LC has been primarily studied in experimental models, and for safety reasons its use in patients with decompensated LC is still considered premature.

Carbon nanoparticles

The newly developed carbon-based enterosorbent Carbalive™ (Yaq-001, Yaqrit Limited, United Kingdom) has a high absorption capacity for bacterial toxins and may be a new strategy to counteract changes in gut microbiota and translocation of bacterial products in patients with decompensated LC. It is non-absorbable carbon nanoparticles with a tailored bimodal distribution of porous domains within the macroporous range (> 50 nm) and microporous range (< 2 nm) and a vast surface area.

The biological significance of this is that in addition to binding smaller mediators such as indoles, acetaldehyde, *etc* carbon granules exhibit rapid adsorption kinetics for larger molecular weight factors, for example bacterial endotoxin, exotoxins, and cytokines. Yaq-001 was found to reduce liver injury, portal pressure, and lipopolysaccharide-induced reactive oxygen species production in an *in vivo* model of LC and ACLF[73]. Yaq-001 significantly increased the relative abundance of symbiont bacteria belonging to the Firmicutes phylum and decreased the relative abundance of pathobiont bacteria belonging to the Bacteroidetes phylum in fecal samples from rodents with a model of bile duct ligation-induced LC, despite the absence of a direct effect on bacterial growth kinetics[74]. In the first phase II multicenter, randomized, double-blind, placebo-controlled trial (CARBALIVE:634579), 14 patients with decompensated LC with diuretic-responsive ascites and mean MELD scores of 12.4 ± 0.9 received 4 g of Yaq-001 for 12 wk. Yaq-001 was safe, well tolerated, contributed to the restoration of intestinal eubiosis, and by affecting the permeability of the intestinal epithelial barrier weakened the severity of endotoxemia and systemic inflammation[75].

CONCLUSION

Given that particular alterations in the composition and function of gut microbiota play a crucial role in the pathogenesis of acute decompensation in LC, the study of the therapeutic possibilities of its modulation has emerged as one of the top concerns in modern hepatology. Despite the encouraging preliminary data, the majority of the suggested strategies have only been tested in animal models or in preliminary clinical trials; additional multicenter RCTs must demonstrate their efficacy in larger patient populations.

FOOTNOTES

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MINIREVIEWS

Morphological aspects of small-duct cholangiopathies: A minireview

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Abstract

The biliary system consists of intrahepatic and extrahepatic bile ducts lined by biliary epithelial cells (cholangiocytes). Bile ducts and cholangiocytes are affected by a variety of disorders called cholangiopathies, which differ in aetiology, pathogenesis, and morphology. Classification of cholangiopathies is complex and reflects pathogenic mechanisms (immune-mediated, genetic, drug- and toxin-induced, ischaemic, infectious, neoplastic), predominant morphological patterns of biliary injury (suppurative and non-suppurative cholangitis, cholangiopathy), and specific segments of the biliary tree affected by the disease process. While the involvement of large extrahepatic and intrahepatic bile ducts is typically visualised using radiology imaging, histopathological examination of liver tissue obtained by percutaneous liver biopsy still plays an important role in the diagnosis of cholangiopathies affecting the small intrahepatic bile ducts. To increase the diagnostic yield of a liver biopsy and determine the optimal therapeutic approach, the referring clinician is tasked with interpreting the results of histopathological examination. This requires knowledge and understanding of basic morphological patterns of hepatobiliary injury and an ability to correlate microscopic findings with results obtained by imaging and laboratory methods. This minireview describes the morphological aspects of small-duct cholangiopathies pertaining to the diagnostic process.

Key Words: Bile duct; Cholangiocytes; Cholangiopathy; Cholangitis; Liver biopsy; Histopathology

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Core Tip: A wide range of non-neoplastic and neoplastic conditions affects the small intrahepatic bile ducts. Cholangiopathies account for significant morbidity and mortality and represent an important indication for liver transplantation in adult and paediatric populations. Pathogenesis of most cholangiopathies is complex, and likely involves both environmental and genetic factors. Understanding the underlying pathogenetic mechanisms, knowledge of basic morphological patterns, and an ability to correlate microscopic findings with results obtained by imaging and laboratory methods are important steps in forming an overall clinical picture and selecting the optimal therapeutic approach in patients with biliary diseases. Our minireview addresses some important morphological aspects of small-duct cholangiopathies relevant to the diagnostic process, and also provides a brief overview of the most clinically significant conditions in light of recent progress.

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INTRODUCTION

The biliary system is a three-dimensional complex of developmentally, anatomically, and functionally different ductal structures. The extrahepatic bile ducts are composed of the right and left hepatic ducts, their confluence, the common hepatic duct, and the common bile duct. The intrahepatic biliary tree is divided according to size into the large intrahepatic bile ducts (area and segmental) and the small intrahepatic bile ducts (interlobular and septal).

Septal bile ducts (> 100 μm in diameter) are lined by tall columnar cells with basal nuclei supported by a fibrous duct wall (Figure 1A). Interlobular bile ducts (15-100 μm) are thin-walled structures lined by cuboidal epithelial cells resting on a basal membrane (Figure 1B). Interlobular bile ducts are connected to a complex network by ductules (< 15 μm diameter), lined by low cuboidal cells, and the canals of Hering, which are partly composed of the biliary epithelium and partly of hepatocytes.

In humans, biliary epithelial cells (cholangiocytes) account for 3%-5% of the total liver cell mass. Cholangiocytes of the intrahepatic biliary tree are derived from the endodermal cells of the hepatic primordium, while epithelial cells lining the extrahepatic bile ducts derive from the caudal portion of the hepatic diverticulum[1,2].

Biliary epithelial cells along with a variety of receptors and transporters play important roles in the secretion and reabsorption of water, electrolytes, bile acids, and many other bile compounds. As well as modifying bile composition, cholangiocytes secreting mucin and HCO_3^- are important elements in the biliary self-defence system. In addition, biliary epithelial cells are involved in a wide range of physiological processes, including intracellular and intercellular signalling, liver regeneration, and immune-mediated reactions[3,4].

Bile ducts and cholangiocytes can be affected by a variety of disorders generally referred to as cholangiopathies. Under pathological circumstances, cholangiocytes can undergo morphological and functional changes, which differ according to the size of the affected segment of the biliary tree. Large bile duct disorders are primarily diagnosed by radiology imaging and liver biopsy is generally not required. However, in the case of small bile duct injuries, imaging methods are not adept at detecting alterations of the biliary tree. Therefore, histomorphological examination should be considered a relevant part of the diagnostic process.

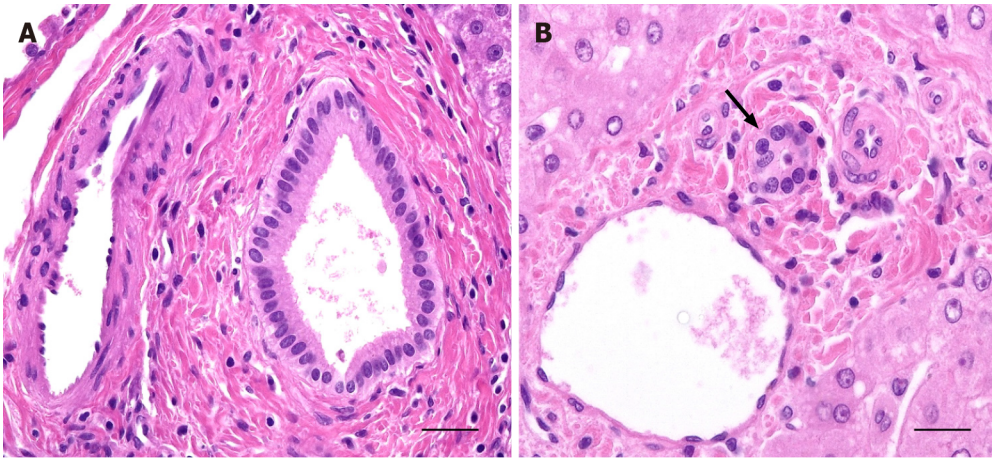
To increase the diagnostic yield of a liver biopsy and arrive at an optimal therapeutic approach, the results of histopathological examination must be interpreted by the presiding clinician. This requires knowledge and understanding of the basic morphological patterns of hepatobiliary injury.

This minireview briefly addresses some important morphological aspects of small-duct cholangiopathies relevant to the diagnostic process, and also provides a brief overview of the most clinically significant conditions in light of recent progress.

CLASSIFICATION OF CHOLANGIOPATHIES

Biliary diseases are classified according to aetiological aspects, pathogenic mechanisms, and the predominant morphological pattern of bile duct injury.

Based on the underlying pathogenic mechanism, cholangiopathies can be classified as follows[5,6]: (1) Immune-mediated cholangiopathy; (2) genetic cholangiopathy; (3) drug- and toxin-induced cholangiopathy; (4) ischaemic cholangiopathy; (5) infectious cholangiopathy; and (6) neoplastic cholan-



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Figure 1 Histology of small intrahepatic bile ducts. A: Septal bile duct lined by a columnar epithelium supported by a fibrous wall; B: Interlobular bile duct (arrow) lined by cuboidal epithelia. Haematoxylin and eosin; bar corresponds to 50 μm.

giopathy. From a morphological point of view, diseases that target bile ducts and cholangiocytes are categorised as either cholangitis (inflammatory bile duct injury) or cholangiopathy (non-inflammatory bile duct injury). Based on the predominant inflammatory cell type and the presence of associated periductal fibrosis, cholangitis can assume a suppurative (neutrophilic) form, predominantly infective, or a non-suppurative form, *e.g.* lymphocytic, pleomorphic, granulomatous, or sclerosing (Table 1)[5,6].

The most clinically important patterns of non-inflammatory bile duct lesions are ductal plate malformation, bile duct loss and ductopenia (vanishing bile duct syndrome), and – in a broader context – neoplastic biliary lesions[5].

GENERAL MORPHOLOGICAL ASPECTS OF SMALL-DUCT CHOLANGIOPATHIES

Several common features of cholangiopathies mainly affecting the small bile ducts have been observed. Considering that early histomorphological changes are often subtle and focal, they can be easily overlooked in a percutaneous liver biopsy. Early alterations secondary to impaired bile flow include focal mild portal oedema accompanied by mild periductal inflammation. Periportal ductular proliferation is commonly detectable at the periphery of some portal areas. Focal and mild juxtaportal copper and copper-associated protein depositions due to prolonged cholestasis can be highlighted by Schmorl's reaction and orcein or rhodanine staining. And although these changes are not pathognomonic, they can indicate a diagnosis of early-stage chronic cholangiopathy[7,8].

Morphological features of prolonged periportal bile salt accumulation (cholate stasis) can be accompanied by biliary interface activity, a complex reaction composed of periportal ductular proliferation with mild neutrophilic inflammation, aberrant cytokeratin 7 expression in hepatocytes (cholangiolar or ductular metaplasia), and varying degrees of fibrosis (Figure 2). Late stages of the disease are characterised by prominent cholate stasis with oedema and "feathery" degeneration of periportal hepatocytes containing Mallory-Denk hyaline inclusions and depositions of copper and copper-associated protein (Figure 2). This results in the formation of a signature "halo" effect at the peripheries of portal areas and septa (Figure 3A). Biliary-type fibrosis can also progress, with a typically uneven distribution of fibrous changes in the liver tissue. Periportal changes may be accompanied by bilirubinostasis in liver lobules, predominantly in the centrilobular zone (zone 3)[7,8,9].

Some cholangiopathies are associated with progressive small bile duct destruction and loss. Hepatic ductopenia, an uncommon but potentially serious cholestatic liver disease, is defined as a loss of the interlobular bile ducts in at least 50% of portal areas[7,10,11]. Ten portal tracts in a liver biopsy specimen are usually considered sufficient for an evaluation of bile duct loss. Vanishing bile duct syndrome is a rare condition in which progressive bile duct loss results in chronic cholestasis and ductopenia[10,11]. A heterogeneous group of conditions, comprising immune-mediated processes, drug- and toxin-induced hepatobiliary injury, ischaemia, infections, and some hereditary diseases, has been implicated in bile duct destruction (Table 2)[11,12].

Table 1 Morphological classification of cholangiopathies

Pattern of biliary injury		Disease
Cholangitis	Neutrophilic	Infection, sterile
	Lymphocytic	PBC, GVHD, allograft rejection
	Pleomorphic	AIH, PBC, DILI
	Granulomatous	PBC, sarcoidosis, DILI
	Sclerosing	PSC, IgG4-sclerosing cholangitis, DILI
Bile duct loss and ductopenia		See Table 2
Ductal plate malformation		See Table 3
Neoplastic		BilIN, IPN, cholangiocarcinoma

PBC: Primary biliary cholangitis; GVHD: Graft-versus-host disease; AIH: Autoimmune hepatitis; DILI: Drug induced liver injury; PSC: Primary sclerosing cholangitis; BilIN: Biliary intraepithelial neoplasia; IPN: Intraductal papillary neoplasia.

Table 2 Conditions associated with ductopenia

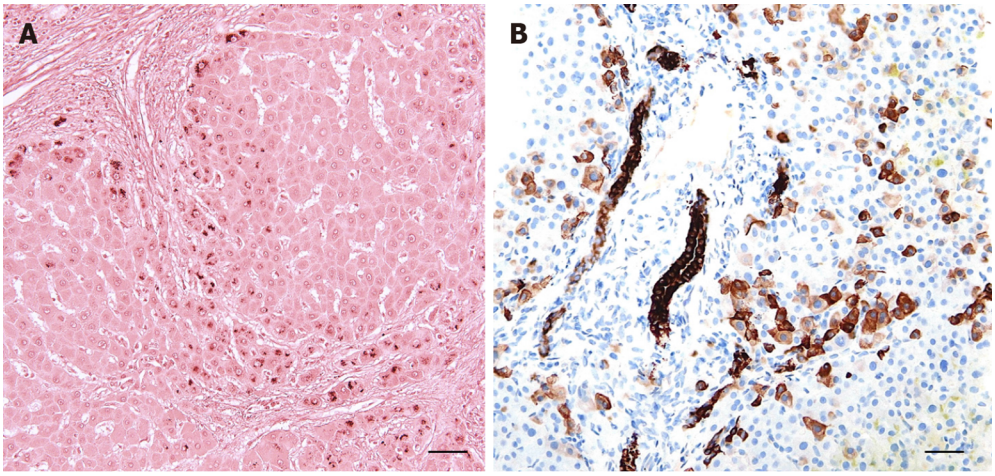
Infantile and childhood diseases	Bile duct paucity (syndromic/non-syndromic)
	Extrahepatic biliary atresia
	Progressive familial intrahepatic cholestasis
	Fibropolycystic disease
	Alpha-1 antitrypsin deficiency
Immune-mediated diseases	Primary biliary cholangitis
	Primary sclerosing cholangitis
	Chronic graft-versus-host disease
	Chronic hepatic allograft rejection
	Hepatic sarcoidosis
Vascular diseases	Ischaemic cholangiopathy
	Portal biliopathy
Infectious diseases	Ascending cholangitis
	Protozoal and parasitic infestations
Drug- or toxin-induced biliary injury	
Neoplastic diseases	Hodgkin's lymphoma
	Langerhans cell histiocytosis
	Systemic mastocytosis
Idiopathic	Idiopathic adulthood ductopenia

MORPHOLOGY OF SELECTED SMALL-DUCT CHOLANGIOPATHIES

The major histomorphological features of various small-duct cholangiopathies are briefly discussed below.

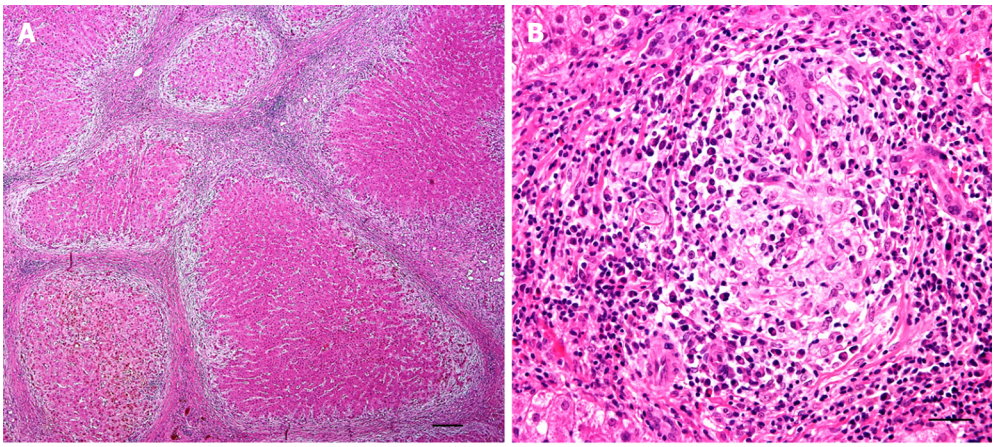
Immune-mediated bile duct injury

Primary biliary cholangitis: Primary biliary cholangitis (PBC) is an autoimmune disease that selectively affects the small intrahepatic bile ducts. Typical clinical features of PBC include marked female preponderance, middle-to-elderly age range, frequent association with other autoimmune disorders, pruritus, and skin hyperpigmentation. Increased activity of serum alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), elevated serum IgM, and the presence of M2-type antimitochondrial antibodies (AMA) are also commonly observed[13-15].



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Figure 2 Morphology of chronic cholestasis. A: Orcein-positive periseptal depositions of copper-binding protein in hepatocytes; B: Aberrant cytokeratin 7 immunoexpression on periportal hepatocytes. Bar corresponds to 100 μm (A) and 50 μm (B).



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Figure 3 Primary biliary cholangitis. A: Biliary-type cirrhosis with a signature "halo" at the peripheries of parenchymal nodules; B: Detail of portal tract with lymphoplasmacytic infiltrate and granulomatous destruction of the interlobular bile duct. Haematoxylin and eosin; bar corresponds to 500 μm (A) and 50 μm (B).

According to the classic histological systems proposed by Scheuer and Ludwig, microscopical changes are categorised into the following four stages[16,17]: Stage 1: Focal, destructive, lymphocytic and/or granulomatous cholangitis of the interlobular bile ducts (florid ductal lesion) with inflammation generally confined within portal tract boundaries (Figure 3B); Stage 2: Ongoing bile duct injury with portal tract expansion due to periportal ductular proliferation (biliary interface activity), inflammatory interface activity, and periportal fibrosis; Stage 3: Bridging portoportal fibrosis with persistent biliary interface activity and ongoing small bile duct loss; Stage 4: Cirrhosis with ductopenia and chronic cholestasis (cholate stasis) discernible by a periportal "halo" (Figure 3A).

The above classic staging systems are simple and easily reproducible. However, they do not reflect all of the morphological features of disease activity and progression that should be considered when determining the optimal therapeutic approach. In addition, the heterogeneous distribution of diagnostic changes in the liver parenchyma and the potential for sampling errors during percutaneous needle biopsy reduce their applicability and practical use. These shortcomings have been overcome by a novel complex system applicable to needle liver biopsy specimens proposed by Nakanuma *et al*[18,19]. Three components – fibrosis, bile duct loss, and chronic cholestasis – are used in this PBC staging system, while necroinflammatory activity of PBC is graded based on chronic cholangitis and hepatitis activity [18,19].

Apart from portal and portal-parenchymal interface changes, small cell changes in periportal hepatocytes and nodular regenerative hyperplasia (NRH) can occur during non-cirrhotic stages of PBC. NRH may explain the hepatomegaly in PBC and the development of clinically significant portal hypertension long before cirrhosis develops[20,21].

The following PBC variants have been recognised: AMA-negative PBC (autoimmune cholangitis) represents 5% of PBC cases. Subtle differences in the clinical, laboratory, and immunological features of AMA-negative PBC have been reported in a number of studies. However, the basic histopathological findings and responses to ursodeoxycholic acid are similar to those observed in AMA-positive patients [13,14].

PBC-AIH overlap syndrome is characterised by the features of both PBC and autoimmune hepatitis coexisting in a single patient. Diagnostic criteria for PBC-AIH overlap syndrome have been proposed by Chazouillères *et al*[22], while other guidelines have been issued by the International Autoimmune Hepatitis Group. These include the presence of at least two of three diagnostic features of PBC (1-3) and at least two of three diagnostic features of AIH (4-6)[23]: (1) GGT ≥ 5× upper limit of normal (ULN) or ALP ≥ 2× ULN; (2) positive AMA; (3) florid bile duct lesion on histology; (4) increased alanine transaminase (ALT) levels ≥ 5× ULN; (5) serum IgG levels ≥ 2× ULN or positive anti-smooth muscle antibody (SMA); and (6) moderate or severe lymphocytic interface activity on histology.

Nevertheless, as opposed to genuine overlap syndrome, most cases are best regarded as either PBC with unusually prominent inflammatory activity (PBC with hepatitis features) or as classical AIH with PBC-like bile duct injury.

Rare cases of overlapping features involving PBC and primary sclerosing cholangitis (PSC), IgG4-related sclerosing cholangiopathy, and sarcoidosis have been reported in the medical literature[14,22].

Small-duct PSC

PSC is a chronic cholestatic condition characterised by non-specific inflammatory fibrosis in bile duct walls of all sizes, with irregular areas of stricturing and beading on cholangiography. Unlike PBC, PSC can occur in infancy and childhood, has a male preponderance, and is closely associated with inflammatory bowel disease (IBD). An increased risk of hepatobiliary malignancy in PSC patients has also been observed[13,14,24,25].

PSC typically involves extrahepatic and large intrahepatic bile ducts with variable involvement of small ducts. Nonetheless, in 5%–10% of cases, the disease is confined to the small intrahepatic bile ducts (small-duct PSC or sdPSC)[24,25,26]. The aetiopathogenesis of sdPSC is poorly understood. A study by Naess *et al*[27] evaluated sdPSC components in a subset of patients with and without concomitant IBD. They found that patients with sdPSC and IBD may represent precursor lesions indicative of classic PSC, while sdPSC patients without IBD could point to a different biliary disease process.

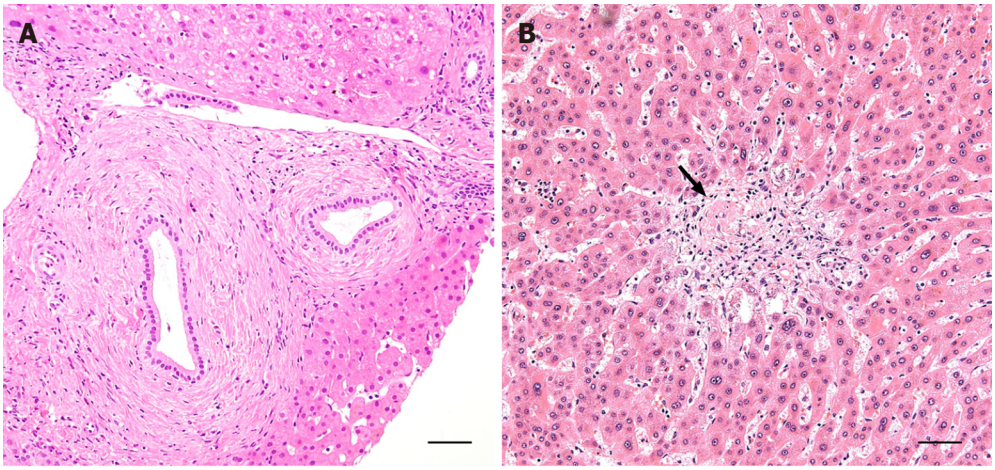
Since a cholangiography is negative in sdPSC, histopathological evaluation of liver tissue specimens is usually required to establish a diagnosis. Although most studies have reported that sdPSC has a similar histopathological picture to large-duct PSC, its pathognomonic features may be subtle and randomly distributed within the liver tissue[7,24,26]. The distinctive features of PSC are fibro-obliterative lesions characterised by 'onion-skin' periductal fibrosis and replacement of the bile duct by a dense acellular fibrous scar (Figure 4). Biliary changes are frequently associated with mild portal tract oedema, low-grade periductal inflammatory infiltrate, and periportal ductular proliferation accompanied by juxtaportal copper and copper-binding protein deposition. As the disease advances, fibrous expansion of portal areas may progress to bridging septal fibrosis and eventual biliary-type cirrhosis[7,24,26].

Histopathological changes in PSC are divided into four stages (1-4) based on the same classification systems as proposed for PBC (see above).

Autoimmune sclerosing cholangitis (PSC-AIH overlap syndrome) is a variant syndrome characterised by features of sclerosing cholangitis and AIH serology[15,23,28]. Importantly, biliary features detected by histopathological examination may be the first indication for biliary tree imaging in patients originally diagnosed with uncomplicated AIH. The syndrome is more common in children and adolescents, with these groups also displaying higher prevalence of sdPSC[7,15,28].

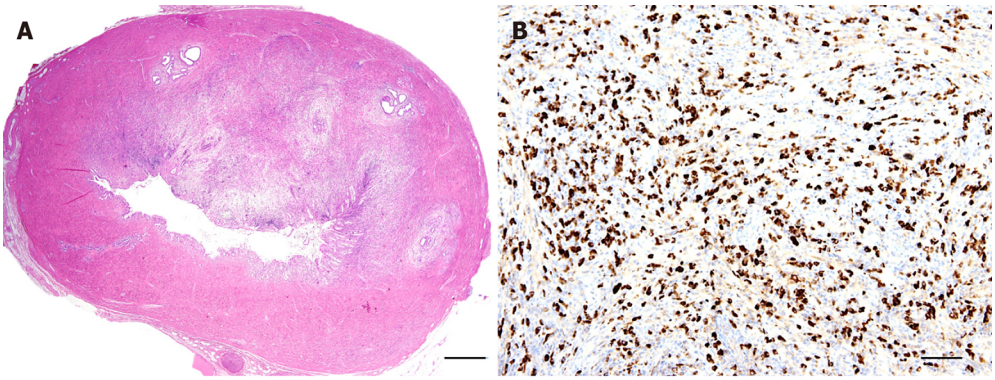
IgG4-related sclerosing cholangitis

IgG4-related sclerosing cholangitis is now considered a biliary manifestation of systemic IgG4-related disease. This condition is commonly associated with IgG4-related lesions in other organs, most often with type 1 autoimmune pancreatitis[29,30]. The affected extrahepatic, hilar and perihilar ducts show diffuse and uniform tube-like thickening on gross examination. Histological characteristics of large duct lesions are transmural inflammation, obliterative phlebitis, and heavy lymphoplasmacytic infiltration with a predominance of IgG4-positive cells and abundant eosinophilic leukocytes (Figure 5)[29,30]. In up to 30% of patients, intrahepatic disease involving the small bile ducts is detectable by liver biopsy. Morphological findings include plasma cell-rich portal inflammation and portal fibrosis, interlobular bile duct damage, lobular inflammation, and features of cholestasis with periportal copper-associated protein deposition. A distinctive portal-based fibroinflammatory nodule composed of inflammatory cells and fibroblasts was observed in 50% of liver biopsies obtained from patients with IgG4-related sclerosing cholangitis[30,31,32]. While differentiating the condition from PSC by needle biopsy is challenging, the presence of more than ten IgG4+ plasma cells on liver biopsy can serve as a diagnostic aid[30,31].



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Figure 4 Primary sclerosing cholangitis. A: Concentric periductal lamellar fibrosis; B: Complete fibrous obliteration of the interlobular duct (arrow). Haematoxylin and eosin; bar corresponds to 100 μm.



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Figure 5 Morphology of IgG4-related sclerosing cholangiopathy. A: Marked fibroinflammatory thickening of the bile duct wall; B: Increased numbers of IgG4-positive plasma cells. Haematoxylin and eosin (A), IgG4 immunohistochemistry (B). Bar corresponds to 1000 μm (A) and 100 μm (B).

Rejection cholangiopathy and biliary lesions in graft-versus-host disease

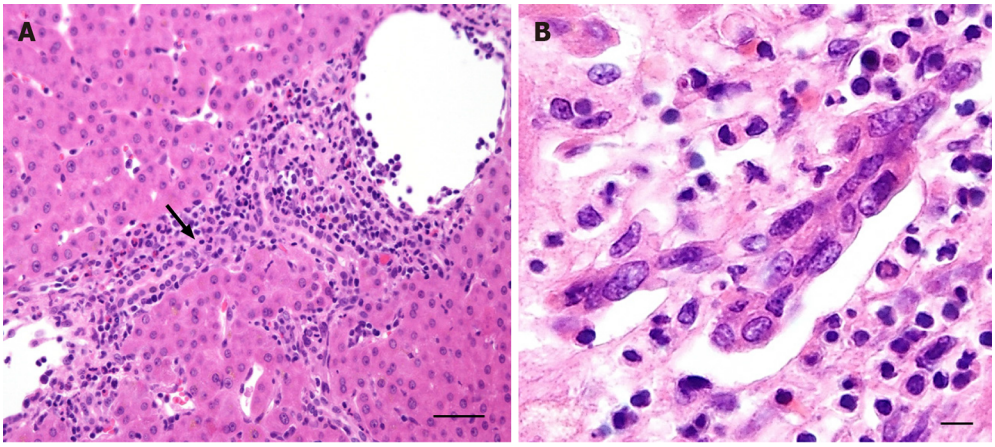
Cholangiocytes and small interlobular bile ducts are targets of an immune reaction associated with liver allograft rejection and graft-versus-host disease (GVHD)[5,33].

Biliary lesions in acute T cell-mediated rejection are characterised by periductal and intraepithelial lymphocytic infiltration, degenerative changes to biliary epithelial cells with cytoplasmic vacuolisation, increased N:C ratios, disordered nuclear polarity, and eventual luminal disruption (Figure 6A). Senescence-related changes to biliary epithelial cells (eosinophilic cytoplasmic transformation, uneven nuclear spacing, nuclear hyperchromasia) and progressive interlobular bile duct loss indicate chronic (ductopenic) rejection (Figure 6B)[33-35].

Immune-mediated small bile duct injury, degenerated dysplastic-like cholangiocytes, and progressive bile duct destruction are the most characteristic histological features of GVHD, a frequent complication of haematopoietic cell transplantation. Lymphocytic infiltration of the biliary epithelium in GVHD is usually minimal to mild[36].

GENETIC CHOLANGIOPATHIES

Genetic factors are variably implicated in most cholangiopathies. In addition to ductal plate malformation (hereditary fibropolycystic disease), neonatal sclerosing cholangitis, and various conditions within the spectrum of familial intrahepatic cholestasis, cholangiopathy can also occur in cystic fibrosis, a multisystemic disease caused by a homozygous or compound heterozygous mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene[37]. Alpha-1 antitrypsin deficiency (AATD) is an autosomal recessive storage disease caused by pathogenic mutations in the *SERPINA1* gene. Besides pulmonary emphysema, AATD can manifest as a progressive cholestatic liver



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Figure 6 Rejection cholangiopathy. A: Inflammatory damage of interlobular bile duct (arrow) in acute T cell-mediated rejection; B: Senescence-related changes of the interlobular bile duct in chronic rejection. Haematoxylin and eosin; bar corresponds to 50 μm (A) and 25 μm (B).

disease involving hypoplasia of the small bile ducts, mostly occurring in paediatric patients[38].

Ductal plate malformation

Ductal plate malformation is a common feature in ciliopathy-associated liver diseases, characterised by a spectrum of biliary hamartomatous lesions, segmental bile duct dilatations and cysts. All of the above conditions stem from aberrant remodelling of the embryonal ductal plate (Figure 7A)[39,40]. Liver disease is often associated with the involvement of other organs, mainly the kidneys. Over the past decade, a number of genes (and their corresponding protein products) involved in several of these disorders have been identified (Table 3)[40,41].

Alagille syndrome

Alagille syndrome (ALGS) is an autosomal dominant multisystem disorder accompanied by severe cholestasis due to congenital maldifferentiation of the intrahepatic bile ducts. The syndrome is caused by mutations in the *JAG1* gene, which encodes the intercellular signalling protein JAGGED1 (type 1 ALGS), and, rarely, the *NOTCH2* gene (type 2 ALGS)[42,43]. Common manifestations include paucity of the intrahepatic bile ducts with obstructive-type cholestasis, skeletal abnormalities (butterfly-like vertebrae), congenital heart disease (usually peripheral pulmonary stenosis), ocular anomalies, and facial dysmorphism[42,44].

ABCB4 deficiency-associated cholangiopathy

ATP-binding cassette (ABC) subfamily B member 4 (ABCB4), also known as multidrug resistance protein 3 (MDR3), is a membrane-associated transport protein. Almost exclusively expressed in the liver, ABCB4 is principally involved in biliary phospholipid secretion[45]. The *ABCB4* alteration has deleterious effects on the hepatobiliary system, mostly caused by toxic bile with a potent detergent and lithogenic profile. The morphological spectrum of biliary lesions connected with ABCB4/MDR3 deficiency varies widely. The most commonly associated conditions are gallbladder disease-1 (GBD1), also known as low phospholipid-associated cholelithiasis (LPAC) syndrome, secondary sclerosing cholangitis (characterised by fibro-obliterative lesions and progressive small bile duct loss), and adult biliary-type liver fibrosis/cirrhosis[46,47].

GBD1 (LPAC syndrome) should be suspected in patients with symptomatic cholelithiasis and at least one of the following criteria: under 40 years of age at onset of symptoms; recurrence after cholecystectomy; intrahepatic sludge or microlithiasis with spindle-shaped dilatations of the intrahepatic bile ducts on cholangiography; familial history of cholelithiasis in first-degree relatives. Histologically, cholesterol crystal aggregates and small cholesterol stones are typically observed in bile ducts[46,48].

Neonatal sclerosing cholangitis

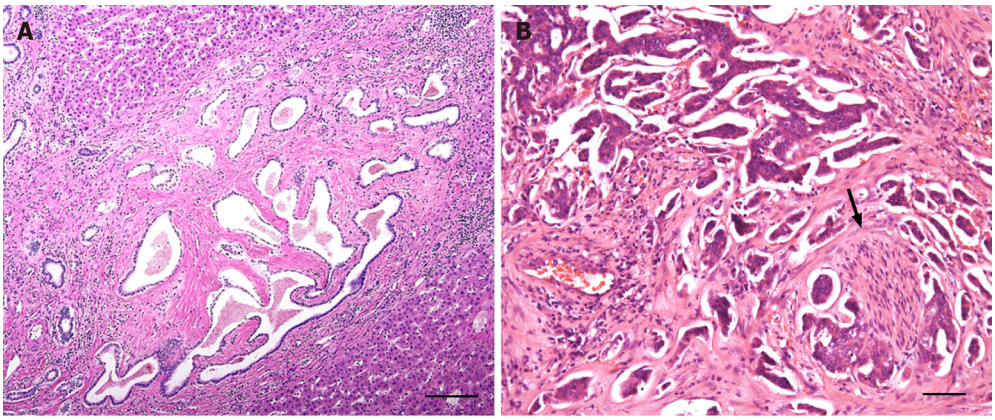
Neonatal sclerosing cholangitis (NSC) is a rare and severe genetic cholangiopathy, commonly progressing to liver failure. The disease usually manifests in the first weeks of life. Clinical features include jaundice, hepatosplenomegaly, pale stools, coagulopathy, signs of gastrointestinal bleeding, and high serum GGT levels. Percutaneous cholangiography typically reveals an intrahepatic sclerosing cholangiopathy[49,50].

Mutations in the doublecortin domain-containing 2 (*DCDC2*) gene have been recently associated with NSC induction. *DCDC2* encodes doublecortin domain-containing protein 2 (the DCDC2 protein), which binds tubulin and facilitates microtubule polymerisation. Histologically, DCDC2 defects are generally

Table 3 Ductal plate malformation – genes and encoded proteins

Disorder	Gene	Product
ARPKD	*PKHD*	Fibrocystin/polyductin
ADPKD	*PKD1*	Polycystin 1
	PKD2	Polycystin 2
	GANAB/PKD3	Glucosidase II alpha subunit
PCLD	*PRKCSH*	Glucosidase II beta subunit
	ALG8	Alpha-1,3-glucosyltransferase
	SEC61B	SEC61 translocon subunit beta
	SEC63	Translocon component in the endoplasmic reticulum
	LRP5	LDL receptor-related protein 5
CHF-SC	*ZFYVE19*	Zinc finger FYVE-type containing 19

ARPKD: Autosomal recessive polycystic kidney disease; ADPKD: Autosomal dominant polycystic kidney disease; PCLD: Polycystic liver disease (without kidney); CHF-SC: Congenital hepatic fibrosis and sclerosing cholangiopathy.



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Figure 7 Tumour-forming biliary processes. A: Hamartomatous proliferation of ductular structures in ductal plate malformation. B: Intrahepatic cholangiocarcinoma with perineural invasion (arrow). Haematoxylin and eosin; bar corresponds to 100 μm (A) and 50 μm (B).

expressed in the form of small-duct cholangiopathies, featuring focal concentric periductal lamellar fibrosis and progressive destruction of the small bile ducts[51,52]. Immunohistochemical examination typically confirms the absence of both the DCDC2 protein and the primary ciliary protein ACALT (acetylated alpha-tubulin), especially in septal and perihilar bile ducts, along with focal irregular expression in small interlobular bile ducts. Simple liver cysts have also been observed in some patients with DCDC2 deficiency[51,52].

DRUG- AND TOXIN-INDUCED CHOLANGIOPATHY

Drug-induced liver injury (DILI) encompasses a wide variety of acute and chronic forms of hepatobiliary injury that can mimic virtually any liver disease, including primary biliary conditions such as PBC or PSC. Bile duct loss, ductopenia, and biliary-type fibrosis/cirrhosis are well-documented consequences of exposure to various drugs, remedies, and toxins[53-55].

In general, drug-induced injury can be dose-dependent (intrinsic) or dose-independent (idiosyncratic). Some drugs and remedies selectively damage larger bile ducts, predominantly by dose-dependent mechanisms, and can induce biliary abnormalities with cholangiographic and histomorphological features of sclerosing cholangiopathy[49]. In most cases, however, drug-induced mechanisms are dose-independent, unpredictable, and affect cholangiocytes of small bile ducts[53].

Drug-induced cholangiopathy usually manifests in fatigue, upper abdominal pain, and features of clinical and biochemical cholestasis. Histopathological findings depend on the severity and stage of

disease progression. Mixed portal-based inflammation with eosinophils, inflammatory bile-duct injury, and centrilobular hepatocanalicular bilirubinostasis are the most common features reported in the acute phase. The chronic stage is characterised by degenerative changes of cholangiocytes, prominent bile duct injury and loss, as well as acute and chronic cholestasis (cholate stasis). Periportal ductular proliferation, associated with fibrous portal tract expansion and relatively mild portal inflammation, also occurs. Small bile duct injury can eventually progress to drug-induced ductopenia. In addition, drug-induced portal and periductal granulomata are not uncommon[53-55].

The most common drugs implicated in bile duct injury are neuroleptics, tricyclic antidepressants, anticonvulsants, antibiotics, and non-steroidal anti-inflammatory drugs. Recently, significant bile duct pathology was observed in patients treated with immune checkpoint inhibitors (CPI). Non-infective cholangitis with predominant neutrophilic or lymphocytic infiltrates, PBC-like changes, and fibrosing cholangitis mimicking sdPSC are the most common patterns of CPI-related cholangiopathy, which responds poorly to immunosuppression and can eventually lead to bile duct loss[56,57].

Diagnosis of DILI requires clinical suspicion, knowledge of the clinicopathological patterns of injury associated with the suspected agent, and exclusion of other possible causes. Underlying genetic defects and predispositions to adverse drug reactions should also be considered in certain cases.

ISCHAEMIC CHOLANGIOPATHY

Supplied by branches of the hepatic artery, the rich peribiliary vascular plexus is vulnerable to any disruption in arterial flow. Ischaemic cholangiopathy, a form of secondary sclerosing cholangiopathy (SSC), is a well-documented complication of transcatheter arterial chemoembolisation (TACE), intra-arterial infusion of chemotherapeutic agents, irradiation, radiofrequency ablation of hepatocellular carcinoma, arterial spasm after cocaine use, sickle cell crisis, HELLP syndrome, and hepatic artery thrombosis after liver transplantation[58,59]. Ischaemic cholangiopathy is characterised by injury to cholangiocytes, cytoplasmic vacuolisation, pyknotic nuclei, and subsequent desquamation of the biliary epithelial lining. Advanced lesions can lead to necrosis of the biliary tree resulting in bile leak, multiple fibrous strictures of the extrahepatic and intrahepatic bile ducts, cholangiectasis, and progressive bile duct loss. Bacterial and fungal infections are common complications[59].

Secondary sclerosing cholangitis in critically ill patients

Ischaemia of the intrahepatic bile ducts is likely involved in secondary sclerosing cholangitis in critically ill patients (SSC-CIP), a newly recognised form of SSC observed in patients after long-term treatment in intensive care units[60,61]. In addition to ischaemia, infections and toxic bile compounds are understood to contribute to the development of SSC-CIP. Interestingly, a cholangiopathy associated with some features of SSC-CIP was documented in patients with critical coronavirus disease 2019 (COVID-19) (see below).

The most frequent symptoms of SSC-CIP typically persist after recovery from the primary illness. These include jaundice, pruritus, and abdominal discomfort localised in the right upper quadrant of the abdomen. Recurrent biliary infections are also commonly observed. SSC-CIP is associated with rapid progression to liver cirrhosis[60,61,62].

Endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography (MRCP) are considered the gold standards for diagnosing SSC-CIP. Characteristic initial findings are multiple ribbon-like intraductal filling defects (biliary casts). In later stages, multiple irregular strictures, dilatations, thickening of the bile duct walls, and destruction of the intrahepatic bile ducts (except for the common bile duct, which results in a ‘pruned tree’ appearance) are routinely observed. Intrahepatic bile ducts are affected in all SSC-CIP patients, with abnormalities of the extrahepatic biliary tree occurring only in a minority of individuals[60,61].

Histopathological changes in SSC-CIP patients include degenerative epithelial alterations to the interlobular bile ducts, mild-to-moderate periductal inflammatory infiltrate, portal oedema, and periportal ductular reactions. Portal changes are accompanied by parenchymal hepatocanalicular bilirubinostasis, bile infarcts, and features of cholate stasis. Fibrous expansion of portal areas and rapid progression to advanced portoseptal fibrosis/cirrhosis have also been reported[61,62].

INFECTIOUS CHOLANGIOPATHY

A variety of microbial agents can directly impact the hepatobiliary system in both normal and immunocompromised hosts. Bacterial and viral infections are understood to play an important role in the progression of ischaemic and immune-mediated bile duct injury such as GVHD and hepatic allograft rejection. Cytomegalovirus (CMV) and hepatitis C virus (HCV) infections after interferon/ribavirin treatment have been shown to trigger ductopenic rejection after orthotopic liver transplantation[63]. Hepatitis-associated bile duct lesions featuring prominent swelling and vacuolisation of cholangiocytes

alongside periductal lymphocytic aggregates have been observed in association with HCV and hepatitis E virus infections. These lesions are mostly reversible and typically occupy individual segments along the circumferences of small bile ducts[64,65]. CMV infection of biliary epithelial cells in the presence of typical viral inclusions is not an uncommon finding in immunocompromised adults and neonates with CMV hepatitis[66].

Human immunodeficiency virus-associated cholangiopathy

Human immunodeficiency virus (HIV)-associated cholangiopathy is a biliary obstruction caused by a benign stricture of the biliary system in patients with advanced acquired immune deficiency syndrome (AIDS)[67,68]. Although the aetiology of this disease remains unclear, it is understood to occur in association with various opportunistic infections, such as CMV, *Cryptosporidium* spp., and *Giardia lamblia*. In most patients, MRCP usually results in an accurate diagnosis of HIV-associated cholangiopathy based on identification of characteristic ductal abnormalities (multiple intrahepatic and/or extrahepatic biliary strictures, papillary stenosis). Liver biopsy and histomorphological examination are reserved for inconclusive and complicated cases[68].

Post-COVID-19 cholangiopathy

Recently, a syndrome of progressive bile duct injury characterised by a marked elevation in serum ALP was reported in some patients recovering from severe COVID-19[69,70]. Although the pathogenesis of post-Covid-19 cholangiopathy has not been fully elucidated, it is likely that some of its pathogenic mechanisms resemble those observed in SSC-CIP. A combination of ischaemic cholangiopathy related to microvascular coagulopathy and an imbalance between the deleterious effects of bile components and biliary protective mechanisms likely contribute to bile duct injury. In addition, direct virus-mediated alteration of the biliary epithelium may also be involved in the pathogenesis of cholangiopathy[70,71].

The most common MRCP findings are intrahepatic and extrahepatic bile duct beading with multiple short segmental strictures and intervening dilatations together with bile duct wall thickening and hyper-enhancement, all of which are consistent with SSC. Histology typically reveals fibro-oedematous expansion of portal tracts, prominent ductular reaction, neutrophilic inflammation, and severe parenchymal cholestasis[70,71].

NEOPLASTIC CHOLANGIOPATHY

Biliary system is affected by a variety of tumour-forming and neoplastic processes, both benign and malignant.

Von Meyenburg complex is a common hamartomatous tumour-like lesion likely representing a form of ductal plate malformation (Figure 7A). Biliary hamartomas are typically found adjacent to a portal area and may be multiple[72,73].

Bile duct adenoma (BDA) is a benign lesion composed of proliferating small bile ducts. The origin of BDA is controversial and a reactive process, a hamartomatous origin and a neoplastic aetiology are considered[73].

Precursor neoplastic lesions of the biliary tract

Many cases of cholangiocarcinoma have been reported to develop through multistep carcinogenesis. The current World Health Organization classification of tumours proposes several precursor lesions of the biliary tract that may precede invasive malignancy[74,75].

Dysplastic epithelial changes can take the form of microscopic, non-invasive lesions occurring in the extrahepatic and intrahepatic bile ducts and gallbladder. This condition is known as biliary intraepithelial neoplasia (BilIN). Based on the extent of cytoarchitectural atypia, BilIN can be subdivided into low-grade (former BilIN-1 and BilIN-2) and high-grade dysplasia (former BilIN-3)[74,75].

Intraductal papillary neoplasm of the bile duct (IPNB) is a macroscopic premalignant neoplastic process with multifocal intraductal papillary or villous projections covered by a single- to multi-layered biliary-type epithelium. While the epithelial lining is usually columnar, it can also exhibit pancreaticobiliary, gastric, intestinal, or oncocytic differentiation along with varying degrees of dysplasia. Resection is the standard therapy for IPNB. However, the multifocal nature of these tumours makes complete resection difficult; therefore, tumour recurrence is a frequent complication. Tubular and mucinous forms of invasive adenocarcinoma are associated with IPNB[75,76,77].

Mucinous cystic neoplasm (MCN) is a rare lesion occurring almost exclusively in women. These tumours are typically multilocular, with cystic spaces lined by the columnar mucinous epithelium overlying ovarian-like stroma. Based on the degree of dysplasia, MCN can be subdivided into low-grade and high-grade categories. This type of lesion can also progress to invasive adenocarcinoma[75,76,78].

Intrahepatic cholangiocarcinoma

Cholangiocarcinoma (CC) is a malignant epithelial neoplasm with biliary differentiation. CC may originate from intrahepatic bile ducts (intrahepatic or peripheral CC), the confluence of the right and left hepatic ducts (hilar CC or Klatskin tumour), or distal extrahepatic bile ducts (extrahepatic distal CC). Although these subtypes differ in overall clinical outcome, they share the basic histomorphological features (Figure 7B)[75,79].

Intrahepatic CC is the second most common primary hepatic malignancy and accounts for 10%-15% primary liver cancers. Incidence is highest in south-east Asia (> 80 cases/100000 persons per year in Thailand) but lowest in Europe (0.2-1.8 cases/100000 per year)[80].

Intrahepatic CC has two main subtypes: large duct CC, originating from the larger intrahepatic bile ducts near the hepatic hilus, and small duct CC, which preferentially occurs in the periphery of the liver parenchyma[75,79].

Aetiology in most cases of CC is unknown. However, several risk factors with a high geographic prevalence have been established. Large duct CC typically originates against a background of liver fluke infection, PSC, hepaticolithiasis, biliary tract malformations, and other rare conditions, mostly associated with chronic inflammation of the biliary tract. On the other hand, the risk factors for small duct intrahepatic CC are similar to those for primary hepatocellular carcinomas, including chronic viral hepatitis and/or non-biliary fibrosis and cirrhosis[80,81].

Recent research on intrahepatic CC has identified many molecular alterations, including KRAS, TP53, ARID1A, IDH1/2, BAP1, BRAF, and other mutations[75].

Both types of intrahepatic CC are aggressive carcinomas with high mortality and poor survival rates. Resectability of the tumour indicates better prognosis, but most patients present with unresectable tumours. Adjuvant therapy, usually a combination of gemcitabine and cisplatin, is recommended for patients with node-positive disease and positive resection margins. Other therapies such as radiation, TACE and ablation have been successful to varying degrees in unresectable cases. Furthermore, a combination of targeted therapy and immunotherapy has shown promise in the treatment of patients with CC[82,83].

CONCLUSION

The small intrahepatic bile ducts are affected by a wide range of non-neoplastic and neoplastic conditions and vary considerably in clinical and morphological presentation. Given their progressive nature and limited curative options, biliary diseases account for significant morbidity and mortality in both the adult and paediatric populations. Cholangiopathies often result in end-stage liver disease requiring liver transplantation.

Although the majority of cholangiopathies are long established, recent entities such as SSC-CIP and CPI-induced cholangiopathy have complicated the application of new therapeutic agents and approaches[56,60,61]. Cholangiopathy has also developed in some critically ill patients infected by β-coronavirus severe acute respiratory syndrome coronavirus 2, isolated for the first time in Wuhan in December 2019[69,71].

Although the pathogenesis of most cholangiopathies is complex and poorly understood, environmental and genetic factors are likely to be involved. Moreover, given that each cholangiopathy has a heterogeneous pathogenesis and a variable natural history, individual responses to therapy will be different[61,71].

Early identification of the pathological mechanisms that compromise cholangiocytes and the small bile ducts is crucial in determining appropriate treatment. While large bile duct pathology is usually visualised by imaging methods, liver biopsy is still considered an effective tool in the diagnosis of small bile duct injury. However, discrepancies between histomorphological, clinical, and biochemical presentations of small bile duct disorders can hinder the diagnostic process. Clinically clear cholestatic conditions manifesting in pruritus and elevated serum ALP, GGT, cholesterol and bile salts can progress without significant bilirubinostasis on biopsy. In addition, pathognomonic morphological signs of small duct cholangiopathies are often focal and may be easily overlooked during percutaneous liver biopsy. On the other hand, early-stage cholangiopathy with a fully developed histomorphological pattern may be accompanied by only minimal and non-specific biochemical abnormalities.

Uneven fibrosis progression within the liver parenchyma is another factor that complicates the diagnostic process, notably when staging fibrosis in a chronic biliary disease. The application of standard semiquantitative scoring systems can serve to underestimate or overestimate the fibrosis stage, particularly in a limited tissue specimen. The clinician should consider not only the data obtained from the liver biopsy but also the results of imaging and other methods (FibroScan, elastography) relevant to the assessment of liver fibrosis.

The implementation of new, predominantly non-invasive diagnostic tools and methods that bridge the shortcomings of liver biopsy is needed. Moreover, further studies are required to elucidate the environmental, genetic, and epigenetic background of the processes affecting the biliary tree and to improve the clinical management of both hereditary and acquired small duct cholangiopathies.

Understanding the underlying pathogenetic mechanisms, familiarity with basic morphological patterns, and the ability to correlate microscopical findings with clinical and laboratory results are important elements when forming an overall clinical picture and selecting the optimal therapeutic approach in patients with biliary diseases. To that end, the close cooperation of all medical specialists participating in the diagnostic process is recommended.

FOOTNOTES

Author contributions: Sticova E wrote the manuscript and prepared the figures and tables; Fabian O provided input on the writing of the paper and prepared the figures; Both authors revised and edited the draft and are in agreement with the content of the manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Inferior outcomes of liver transplantation for hepatocellular carcinoma during early-COVID-19 pandemic in the United States

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Abstract

BACKGROUND

Early in the coronavirus disease 2019 (COVID-19) pandemic, there was a significant impact on routine medical care in the United States, including in fields of transplantation and oncology.

AIM

To analyze the impact and outcomes of early COVID-19 pandemic on liver transplantation (LT) for hepatocellular carcinoma (HCC) in the United States.

METHODS

WHO declared COVID-19 as a pandemic on March 11, 2020. We retrospectively analyzed data from the United Network for Organ Sharing (UNOS) database regarding adult LT with confirmed HCC on explant in 2019 and 2020. We defined pre-COVID period from March 11 to September 11, 2019, and early-COVID period as from March 11 to September 11, 2020.

RESULTS

Overall, 23.5% fewer LT for HCC were performed during the COVID period (518 *vs* 675, $P < 0.05$). This decrease was most pronounced in the months of March-April 2020 with a rebound in numbers seen from May-July 2020. Among LT recipients for HCC, concurrent diagnosis of non-alcoholic steatohepatitis significantly increased (23 *vs* 16%) and alcoholic liver disease (ALD) significantly decreased (18 *vs* 22%) during the COVID period. Recipient age, gender, BMI, and MELD score were statistically similar between two groups, while waiting list time decreased during the COVID period (279 days *vs* 300 days, $P = 0.041$). Among

pathological characteristics of HCC, vascular invasion was more prominent during COVID period (P < 0.01), while other features were the same. While the donor age and other characteristics remained same, the distance between donor and recipient hospitals was significantly increased (P < 0.01) and donor risk index was significantly higher (1.68 *vs* 1.59, P < 0.01) during COVID period. Among outcomes, 90-day overall and graft survival were the same, but 180-day overall and graft were significantly inferior during COVID period (94.7 *vs* 97.0%, P = 0.048). On multivariable Cox-hazard regression analysis, COVID period emerged as a significant risk factor of post-transplant mortality (Hazard ratio 1.85; 95%CI: 1.28-2.68, P = 0.001).

CONCLUSION
During COVID period, there was a significant decrease in LTs performed for HCC. While early postoperative outcomes of LT for HCC were same, the overall and graft survival of LTs for HCC after 180 days were significantly inferior.

Key Words: Liver transplantation; Hepatocellular carcinoma; COVID-19; Mortality; Graft failure; United Network for Organ Sharing database

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Core Tip: Overall, 23.5% fewer liver transplants for hepatocellular carcinoma were performed during the coronavirus disease 2019 (COVID-19) early pandemic. Among liver transplant recipients for hepatocellular carcinoma, concurrent diagnosis of non-alcoholic steatohepatitis significantly increased. Liver transplant outcomes for hepatocellular carcinoma was worse during the early COVID-19 pandemic.

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INTRODUCTION

Since December 2019, after an initial cluster of cases of pneumonia was reported in Wuhan, China, the global spread of coronavirus disease 2019 (COVID-19) was swift, and the World Health Organization (WHO) declared it as a pandemic on March 11, 2020. This pandemic significantly impacted healthcare in the United States including transplantation[1,2] and cancer care[3], especially early in the pandemic when there were challenges in access to routine healthcare[4]. While the number of alcoholic liver disease (ALD) including alcoholic hepatitis increased during the pandemic, liver transplantation (LT) for hepatocellular carcinoma (HCC) were postponed due to the lower severity of the underlying liver disease[5]. The aim of this study was to analyze the impact of COVID-19 pandemic on LT for HCC in the United States.

MATERIALS AND METHODS

Patients and selection criteria

We evaluated all patient 18 years of age and older undergoing LT who were confirmed as HCC on pathology in the United States. Since WHO declared COVID-19 as a pandemic on March 11, 2020, we defined pre-COVID period as March 11 to September 11, 2019, and COVID period as March 11 to September 11, 2020. Patients who received re-transplant during the study were excluded. All study methods were approved by New York Medical College Institutional Review Board.

Patient characteristics and outcome variables

All data were collected from the United Network for Organ Sharing (UNOS) registry. Demographic data included diagnosis, age, gender and race. Evaluable recipient factors included body mass index (BMI), underlying etiology for liver disease, pre-transplant diabetes mellitus (DM) status, alpha-fetoprotein (AFP) level, presence of portal vein thrombosis (PVT), and model for end-stage liver disease (MELD) score at transplant. Milan criteria and UCSF criteria were created based on the pathological

findings[6,7]. HCC related factors included tumor size, number, presence of vascular invasion, and histological grade. High risk features of HCC were defined if one or more of the followings were present: More than 3 tumors, largest tumor > 5.0 cm, presence of vascular invasion, presence of metastases, and poorly differentiated[8]. Donor related factors included donor causes of death, BMI, hepatitis C virus (HCV) sero-status, cold ischemia time, distance between donor and recipient hospitals, and donor risk index[9].

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 26.0 (IBM Corp., Armonk, NY, United States) and R studio version 4.1.1 (R Studio, Inc., Boston, MA, United States). Non-parametric analysis was used to compare continuous variables between groups (Mann-Whitney *U* test 2 groups and for categorical data with the χ^2 test or Fisher's exact test for categorical data). The overall and graft survival were calculated from the date of transplant to the date of event using the Kaplan-Meier Method. The log-rank test was used to compare survival curves. Cox regression analysis was applied to assess the association of multiple covariate factors with survival between two groups. Results were presented as hazard ratios (HR) and reported with 95% confidence intervals (CI) and two-sided *P* values. For all statistical analyses, $P < 0.05$ was taken as statistically significant.

RESULTS

Recipient characteristics

During the study period, a total of 8384 individuals received LT, of which 1193 were confirmed as HCC on explant pathology. Of these patients, 675 underwent transplantation during the pre-COVID period and 518 underwent transplantation during the COVID period (Table 1). While there was a 4% reduction in all-cause LT during the COVID period, the reduction of LT for HCC was 23.5%. This decrease was most pronounced in months of March-April 2020 with a rebound in numbers seen from May-July 2020. Compared to pre-COVID period, the concurrent underlying primary etiology of liver disease among LT recipients for HCC showed a significant increase in non-alcoholic steatohepatitis (NASH) (23% *vs* 16%) and significant decrease in ALD (18% *vs* 22%) during the COVID period. The median waiting list time among patients who underwent LT, decreased during the COVID period (279 days *vs* 300 days, $P = 0.041$). There were no significant differences in the rates of pre-transplant diabetes, AFP levels, and MELD score at transplant between the two periods (Table 1).

Donor characteristics

The donor age (COVID 45 years-old *vs* pre-COVID 44 years-old, $P = 0.027$) and BMI (27.8 kg/m^2 *vs* 27.0 kg/m^2, $P < 0.001$) were significantly higher during the COVID period (Table 1). Although the distance between donor and recipient hospitals significantly increased during COVID period (88 miles *vs* 50 miles, $P < 0.001$), cold ischemia time was not significantly affected (5.87 hours *vs* 5.78 h, $P = 0.84$). The donor risk index was significantly higher during the COVID period (1.68 *vs* 1.59, $P < 0.01$) (Table 1).

Tumor characteristics

During the COVID period, the overall number of tumors with equal or greater than one high-risk features was the same (Table 2). However, there was a significantly higher rate of vascular invasion during the COVID period compared to pre-COVID period (16% *vs* 11%, $P = 0.016$). There were no significant differences in histological grade, outside of Milan criteria, and outside of UCSF criteria during the both periods.

Outcomes

Median follow-up period was 705 days in COVID period and 1059 days in pre-COVID period. Two-year overall survival (COVID period 86.5% *vs* pre-COVID 91.7%, $P = 0.0063$) and graft survival (COVID period 84.5% *vs* pre-COVID 89.6%, $P = 0.014$) were significantly inferior during the COVID period (Figure 1) (Table 3). The 180-day overall survival (COVID period 94.7% *vs* pre-COVID 97.0%, $P = 0.048$) and graft survival (COVID period 92.8% *vs* pre-COVID 95.7%, $P = 0.035$) were inferior during the COVID period. The 90-day overall survival (COVID period 97.7% *vs* pre-COVID 97.9%, $P = 0.95$) and graft survival (COVID period 96.1% *vs* pre-COVID 96.7%, $P = 0.71$) were comparable between the two periods. The risk of acute rejection after transplant before discharge was the same between pre-COVID and COVID period (17 *vs* 11, $P = 0.061$) and incidence of treatment of rejection within 6 months and 1 year were same.

Cox regression analysis was performed for overall survival and graft survival (Supplementary-Table 1 and 2). On multivariable analysis (Table 4), COVID period (HR, 1.85; 95%CI: 1.28-2.68, $P = 0.001$), recipient diabetes (HR 1.47; 95%CI: 1.04-2.06, $P = 0.027$), MELD score at transplant (HR, 1.04; 95%CI: 1.01-1.07, $P = 0.016$) significantly impacted recipient overall survival. On pathology, intrahepatic metastasis (HR 2.03; 95%CI: 1.20-3.42, $P = 0.008$) and poorly differentiated cancer (HR 2.76; 95%CI: 1.48-

Table 1 Characteristics of the study population, *n* (%)

Variable	COVID, *n* = 518	Pre-COVID, *n* = 675	*P* value
Recipient			
Age, yr, median (IQR)	64.0 (60.0, 67.8)	63.0 (59.0, 67.0)	0.2
Male	405 (78)	529 (78)	0.98
Race, White	328 (63)	388 (57)	0.12
African American	36 (6.9)	47 (7.0)	
Hispanic	108 (21)	153 (23)	
Asian	39 (7.5)	69 (10)	
Others	7 (1.4)	19 (2.8)	
BMI median (IQR) kg/m^2	29.3 (25.7, 33.2)	28.9 (25.3, 33.0)	0.26
Etiology, HCV	180 (35)	258 (38)	0.034
HBV	33 (6.4)	43 (6.4)	
ALD	95 (18)	149 (22)	
NASH	118 (23)	107 (16)	
Others	92 (18)	118 (17)	
Blood Type, A	203 (39)	235 (35)	0.13
AB	18 (3.5)	27 (4.0)	
B	76 (15)	82 (12)	
O	221 (43)	331 (49)	
Diabetes	208 (40)	255 (38)	0.4
HCV positive serostatus	229 (46)	319 (49)	0.28
Previous abdominal surgery	229 (45)	330 (49)	0.19
Portal vein thrombosis	66 (13)	106 (16)	0.19
Hemodialysis at transplant	8 (1.6)	8 (1.2)	0.59
TIPSS	30 (5.8)	32 (4.7)	0.42
AFP ng/mL, median (IQR)	6.0 (3.0, 15.0)	7.0 (4.0, 16.0)	0.21
MELD score, median (IQR)	10 (8, 14)	10 (8, 14)	0.33
MELD exception	510 (98)	658 (97)	0.24
Mechanical ventilation	0 (0)	2 (0.3)	0.51
Waiting days, median (IQR)	279 (221, 392)	300 (228, 424)	0.041
Patient location at transplant			0.62
ICU at time of transplant	2 (0.4)	5 (0.7)	
Non-ICU inpatient at time of transplant	19 (3.7)	31 (4.6)	
Outpatient	487 (96)	639 (95)	
Donor			
Age, yr, median (IQR)	45.0 (32.0, 59.0)	44.0 (30.0, 56.0)	0.027
Male	326 (63)	421 (62)	0.84
Race, White	322 (62)	428 (63)	0.058
African American	104 (20)	117 (17)	
Hispanic	73 (14)	92 (14)	
Asian	15 (2.9)	17 (2.5)	
Others	4 (0.8)	21 (3.1)	

BMI median (IQR) kg/m^2	27.8 (24.4, 33.2)	27.0 (23.8, 30.9)	< 0.001
Blood Type, A	204 (39)	237 (35)	0.12
AB	14 (2.7)	16 (2.4)	
B	80 (15)	88 (13)	
O	220 (42)	334 (49)	
HCV NAT positive	37 (7.1)	39 (5.8)	0.34
HCV antibody positive	59 (11)	76 (11)	0.94
Donor causes of death: Anoxia	234 (45)	265 (39)	0.026
Cerebrovascular accident	158 (31)	221 (33)	
Head trauma	104 (20)	172 (25)	
Central nervous system tumor	2 (0.4)	4 (0.6)	
Others	20 (3.9)	13 (1.9)	
Donor after cardiac death	62 (12)	80 (12)	0.95
Organ Share, Local	241 (47)	508 (75)	< 0.001
Regional	174 (34)	145 (21)	
National	103 (20)	22 (3.3)	
Donor risk index, median (IQR)	1.68 (1.45, 2.06)	1.59 (1.35, 1.91)	< 0.001

COVID: Coronavirus disease; IQR: Interquartile range; BMI: Body mass index; HCV: Hepatitis C virus; HBV: Hepatitis B virus; ALD: Alcoholic liver disease; NASH: Non-alcoholic steatohepatitis; TIPSS: Transjugular intrahepatic portosystemic shunt; AFP: Alpha-fetoprotein; MELD: Model for end-stage liver disease; ICU: Intensive care units; NAT: Nucleic acid amplification test.

5.15, P = 0.001) significantly impacted recipient overall survival.

DISCUSSION

This study was undertaken to examine the effect of COVID-19 on LT for HCC in the United States using the UNOS database. During COVID period, there was a significant reduction in the number of LTs performed for HCC compared to pre-COVID period. Even with overall reduction in the number LTs performed during the same period, the decrease was much pronounced in patients with underlying HCC (4% decrease in all-cause LT *vs* 23.5% decrease in LT for HCC). In addition, both graft and patient survival after 180 days during COVID period were significantly inferior compared to pre-COVID period.

During early pandemic, the unprecedented burden of COVID-19 on healthcare system disrupted both transplantation and oncological care. Due to resource reallocation, cancer patients experienced delays in diagnosis and treatment, as well as treatment interruptions including surgery and chemotherapy[3,4]. HCC is an aggressive form of liver cancer which requires a prompt, multimodal approach for diagnosis and management. Similar to outpatient management of other cancers, challenges in care of HCC were prompted by overall disruption of healthcare during COVID-19[10,11]. Despite efforts to optimize care, there was an overall reduction in HCC surveillance and subsequent treatment during the early COVID pandemic[12-14]. Because of the unknown risk-benefit of proceeding with liver transplantation and introducing iatrogenic immune-suppression, liver transplantation was limited to patients who had higher risk of imminent death while on the waitlist[1,2]. Since LT candidates for HCC often had lower MELD scores, this likely contributed to the disproportional decrease in LT for HCC during early COVID period.

The current leading indication for liver transplant in the United States is ALD[15]. During the COVID pandemic, there was a significant increase in alcohol misuse, which resulted higher rates of hospitalization from ALD, progression to fulminant liver disease and LT for acute and chronic ALD[16,17]. However, when looking at the underlying primary etiology of HCC for patients who received LT for HCC, the number and ratio of ALD significantly decreased during the COVID, while the number and ratio of NASH significantly increased. Though further studies are needed to investigate the cause of this seemingly contradictory finding, one possible explanation is increased alcohol recidivism in HCC patients with ALD as the primary underlying etiology, which could disqualify them as LT candidates in most transplant centers.

Table 2 Pathological characteristics of hepatocellular carcinoma in liver transplant, *n* (%)

Variable	COVID, *n* = 518	Pre-COVID, *n* = 675	*P* value
Number of tumors			
1	229 (44)	333 (49)	
2	116 (22)	146 (22)	
3	80 (15)	77 (11)	
4	36 (6.9)	47 (7.0)	
5	22 (4.2)	33 (4.9)	
6	31 (6.0)	38 (5.6)	
Tumor numbers > 3	89 (17)	118 (17)	0.89
Outside UCSF criteria	131 (25)	178 (26)	0.71
Outside Milan criteria	203 (39)	262 (39)	0.84
Tumor max size, cm median (IQR)	2.80 (1.80, 4.00)	2.90 (1.90, 4.00)	0.92
Worst tumor histology			
Complete tumor necrosis	132 (25)	185 (27)	0.5
Moderate differentiated	265 (51)	344 (51)	
Poorly differentiated	26 (5.0)	41 (6.1)	
Well differentiated	95 (18)	105 (16)	
Vascular invasion	6 (1.2)	11 (1.6)	0.019
Macro			
Micro	77 (15)	65 (9.6)	
None	435 (84)	599 (89)	
Lymph node involvement	8 (1.5)	4 (0.6)	0.1
Extra hepatic spread	7 (1.4)	6 (0.9)	0.76
Intrahepatic metastasis	42 (8.1)	36 (5.3)	0.066
Previous treatment for HCC	511 (99)	657 (97)	0.21
High risk features	174 (34)	198 (29)	0.12

COVID: Coronavirus disease; IQR: Interquartile range; UCSF: University of California San Francisco; HCC: Hepatocellular carcinoma.

Table 3 Outcomes of liver transplant for hepatocellular carcinoma, *n* (%)

Variable		COVID, *n* = 518	Pre COVID, *n* = 675	*P* value
Month	March	68 (13)	132 (20)	0.008
	April	86 (17)	123 (18)	
	May	108 (21)	97 (14)	
	June	78 (15)	95 (14)	
	July	84 (16)	99 (15)	
	August	94 (18)	129 (19)	
Distance from donor hospital, miles, median (IQR)		88 (23, 188)	50 (8, 164)	< 0.001
Cold ischemia time, hours median (IQR)		5.87 (4.71, 7.07)	5.78 (4.65, 7.12)	0.84
Acute rejection before discharge		17 (3.3)	11(1.6)	0.061
Treatment rejection within 6 month		27 (6.4)	38 (6.6)	0.88

Treatment rejection within 1 year	35 (8.4)	45 (8.4)	0.99
Length of stay, days median (IQR)	8 (6, 11)	8 (6, 12)	0.60
Survival rate			
90-day overall survival	97.7	97.9	0.95
180-day overall survival	94.7	97.0	0.048
Two-year overall survival	86.5	91.7	0.0063
90 day graft survival	96.1	96.7	0.71
180 day graft survival	92.8	95.7	0.035
Two-year graft survival	84.5	89.6	0.014

IQR: Interquartile range; COVID: Coronavirus disease.

Table 4 Multivariable Cox regression analysis of factors affecting overall mortality

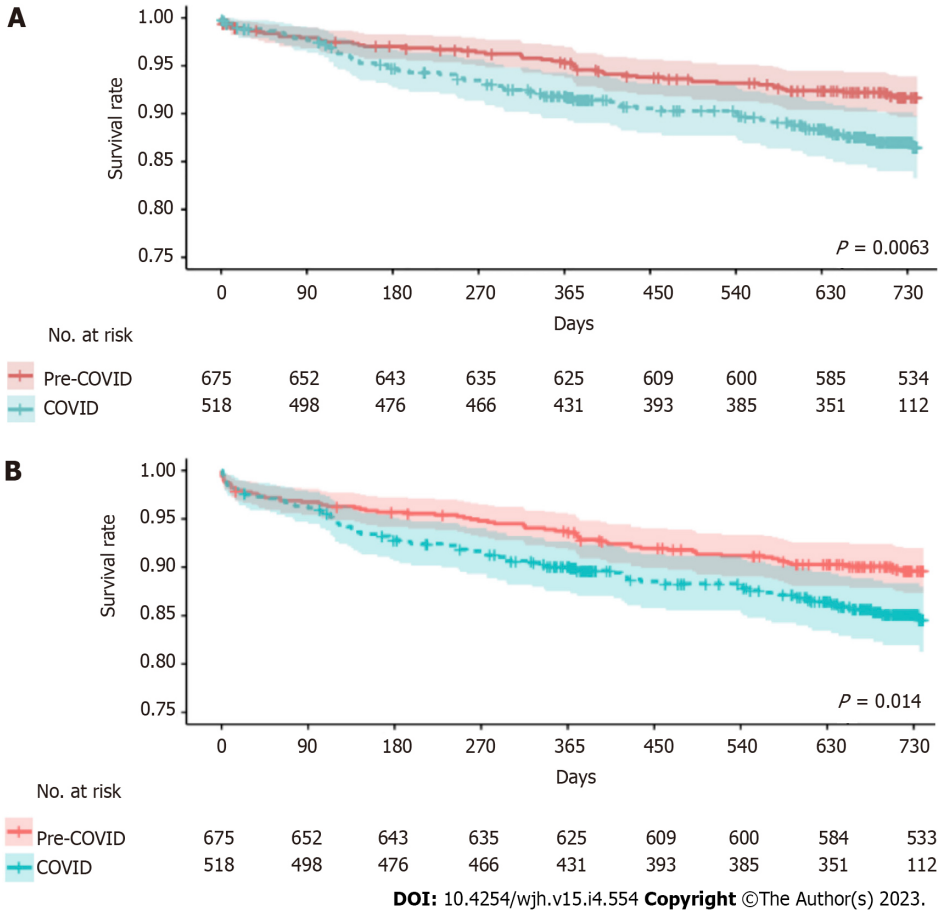
Variable	HR	95%CI	*P* value
COVID period	1.85	1.28, 2.68	0.001
Recipient age	1.02	0.99, 1.05	0.15
Recipient diabetes	1.47	1.04, 2.06	0.027
MELD score	1.04	1.01, 1.07	0.016
Donor age	1.01	1.00, 1.02	0.10
Donation after cardiac death	0.59	0.31, 1.14	0.12
Pathology			
Intrahepatic metastasis	2.03	1.20, 3.42	0.008
Lymph node invasion	2.49	0.90, 6.86	0.078
Worst tumor histology			
Complete tumor necrosis	reference	-	
Well differentiated	1.11	0.63, 1.97	0.70
Moderate differentiated	1.27	0.81, 1.99	0.30
Poorly differentiated	2.76	1.48, 5.15	0.001

HR: Hazard ratio; CI: Confidence intervals; MELD: Model for end-stage liver disease; COVID: Coronavirus disease.

Similar to the decrease in graft and patient survival seen in all-cause LT during the early COVID period[5], the cause for significant decrease in graft and patient survival after 180 days in LT for HCC is likely multifactorial. During early COVID, many centers decreased the use of immunosuppression to mitigate the risk of infection[18]. Such conservative approaches may have contributed to increased rates of acute rejection prior to discharge[5]. In our cohort, although it is not statistically significant, we observed a similar trend in increased rates of rejection prior to discharge in those who were transplanted during COVID period. During the follow up, the incidences of acute rejection were comparable between pre-COVID and COVID period.

Another potential contributing factor towards inferior graft and patient survival in LT for HCC during the COVID period is the progression of HCC at the time of LT. Although there were no significant differences in histological grade and the number of patients outside Milan/USCF criteria, there was a significantly higher rates of vascular invasion during the COVID period compared to pre-COVID period. Vascular invasion is one of the known risk factors for recurrent HCC and detection of metastasis post-LT, which is associated with high morbidity and mortality[19]. As delays in oncological care and radiological testing were prevalent during this period, any such delay may have resulted in progression of HCC which was not grossly evident prior to LT.

In February 2020, the new liver allocation policy was also implemented which allowed for broader sharing of the organs across different organ procurement organizations[20]. This likely contributed to increase in donor risk index and farther distances between donor and recipient hospitals. Long term



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Figure 1 Post-transplant survival curves between pre-coronavirus disease and coronavirus disease period. A: Comparison of overall survival between pre-coronavirus disease (COVID) and COVID period; B: Comparison of graft survival between pre-COVID and COVID period.

impact of these factors on the graft and overall outcomes still needs to be determined.

Limitations

This study was performed retrospectively using UNOS database. Other indirect effects of COVID-19, such as psychosocial impact, medication non-compliance, rates of recidivism of alcohol use, rates of community/household spread of COVID-19 are not available in the database and may impact the outcomes.

CONCLUSION

During the early-COVID period (from March 11, 2020 to September 11, 2020), the overall number of LT for HCC decreased and post-transplant graft and patient survival were inferior compared to pre-COVID period.

ARTICLE HIGHLIGHTS

Research background

Coronavirus disease 2019 (COVID-19) pandemic significantly impacted healthcare in the United States including transplantation and cancer care, especially early in the pandemic when there were challenges in access to routine healthcare.

Research motivation

To analyze the impact of COVID-19 pandemic on Liver transplantation (LT) for Hepatocellular carcinoma (HCC) in the United States.

Research objectives

All patient 18 years of age and older undergoing LT who were confirmed as HCC on pathology in the United States.

Research methods

Since WHO declared COVID-19 as a pandemic on March 11, 2020, we defined pre-COVID period as March 11 to September 11, 2019, and COVID period as March 11 to September 11, 2020.

Research results

Overall, 23.5% fewer LT for HCC were performed during the COVID period (518 *vs* 675, $P < 0.05$). Among pathological characteristics of HCC, vascular invasion was more prominent during COVID period ($P < 0.01$), while other features were the same. Among outcomes, 90-day overall and graft survival were the same, but 180-day overall and graft were significantly inferior during COVID period (94.7 *vs* 97.0%, $P = 0.048$). On multivariable Cox-hazard regression analysis, COVID period emerged as a significant risk factor of post-transplant mortality (Hazard ratio 1.85; 95%CI: 1.28-2.68, $P = 0.001$).

Research conclusions

During COVID period, there was a significant decrease in LTs performed for HCC. While early postoperative outcomes of LT for HCC were same, the overall and graft survival of LTs for HCC after 180 days were significantly inferior.

Research perspectives

To analyze the effects of COVID-19 pandemic in the long-term effects for LT for HCC.

FOOTNOTES

Author contributions: Lee IS and Okumura K contributed equally to this work; Lee IS, Okumura K, and Dhand A contributed to study design; Lee IS, Okumura K, Misawa R, Nishida S and Dhand A analyzed data and writing of the manuscript; Sogawa H, Veillette G, John D and Diflo T contributed to critical revision; all authors approved the final manuscript.

Institutional review board statement: All study methods were approved by New York Medical College Institutional Review Board.

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.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Data sharing statement: The data that support the findings of this study are available from the corresponding author upon reasonable request. The data reported here have been supplied by the UNOS as the contractor for the OPTN. The interpretation and reporting of these data are the responsibility of the authors and in no way should be seen as an official policy of or interpretation by the OPTN or the United States Government.

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ORIGINAL ARTICLE

Retrospective Study

Peptic ulcer disease in non-alcoholic fatty liver disease hospitalizations: A new challenge on the horizon in the United States

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Abstract

BACKGROUND

Peptic ulcer disease (PUD) is frequently seen in patients with liver cirrhosis. However, current literature lacks data on PUD in non-alcoholic fatty liver disease (NAFLD) hospitalizations.

AIM

To identify trends and clinical outcomes of PUD in NAFLD hospitalizations in the United States.

METHODS

The National Inpatient Sample was utilized to identify all adult (≥ 18 years old) NAFLD hospitalizations with PUD in the United States from 2009-2019. Hospitalization trends and outcomes were highlighted. Furthermore, a control group of adult PUD hospitalizations without NAFLD was also identified for a comparative analysis to assess the influence of NAFLD on PUD.

RESULTS

The total number of NAFLD hospitalizations with PUD increased from 3745 in 2009 to 3805 in 2019. We noted an increase in the mean age for the study population from 56 years in 2009 to 63 years in 2019 ($P < 0.001$). Racial differences were also prevalent as NAFLD hospitalizations with PUD increased for Whites and Hispanics, while a decline was observed for Blacks and Asians. The all-cause inpatient mortality for NAFLD hospitalizations with PUD increased from 2% in 2009 to 5% in 2019 ($P < 0.001$). However, rates of *Helicobacter pylori* (*H. pylori*) infection and upper endoscopy decreased from 5% in 2009 to 1% in 2019 ($P < 0.001$) and from 60% in 2009 to 19% in 2019 ($P < 0.001$), respectively. Interestingly, despite a significantly higher comorbidity burden, we observed lower inpatient mortality (2% *vs* 3%, $P = 0.0004$), mean length of stay (LOS) (11.6 *vs* 12.1 d, $P < 0.001$), and mean total healthcare cost (THC) ($178598 *vs* $184727, $P < 0.001$) for NAFLD hospitalizations with PUD compared to non-NAFLD PUD hospitalizations. Perforation of the gastrointestinal tract, coagulopathy, alcohol abuse, malnutrition, and fluid and electrolyte disorders were identified to be independent predictors of inpatient mortality for NAFLD hospitalizations with PUD.

CONCLUSION

Inpatient mortality for NAFLD hospitalizations with PUD increased for the study period. However, there was a significant decline in the rates of *H. pylori* infection and upper endoscopy for NAFLD hospitalizations with PUD. After a comparative analysis, NAFLD hospitalizations with PUD had lower inpatient mortality, mean LOS, and mean THC compared to the non-NAFLD cohort.

Key Words: Non-alcoholic fatty liver disease; Peptic ulcer disease; Trends; Outcomes; Mortality

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Core Tip: Due to dietary and lifestyle changes, the prevalence of non-alcoholic fatty liver disease (NAFLD) is on the rise worldwide. Peptic ulcer disease (PUD) is commonly seen in patients with liver cirrhosis. However, data on PUD in NAFLD hospitalizations is currently lacking. In this study, we noted an increase in inpatient mortality for NAFLD hospitalizations with PUD in the United States. The rates of *Helicobacter pylori* infection and upper endoscopy for NAFLD hospitalizations with PUD were on the decline. Despite a higher comorbidity burden, inpatient mortality, mean length of stay, and mean total healthcare cost were lower for NAFLD hospitalizations with PUD compared to the non-NAFLD cohort.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a wide range of conditions primarily charac-

terized by the presence of hepatic steatosis which is identified on radiological imaging or histology after other secondary causes of fat deposition have been excluded[1]. Based on histological findings, it is further subdivided into NAFL, which is hepatic steatosis without hepatocellular injury, and non-alcoholic steatohepatitis (NASH) characterized by hepatic steatosis and inflammation with hepatocyte injury. NAFLD usually progresses linearly from steatosis and hepatitis to fibrosis, cirrhosis, and ultimately hepatocellular carcinoma. Risk factors commonly implicated in the development of NAFLD include obesity, diabetes mellitus, hypertriglyceridemia, and metabolic syndrome[2]. The global prevalence of NAFLD is estimated to be 25% and is expected to rise further due to the rising incidence and prevalence of obesity worldwide[3]. In the United States, approximately 80 million people have NAFLD, with NASH being the second leading cause of liver transplant[4].

Peptic ulcer disease (PUD) involves acid-induced mucosal disruption in the upper gastrointestinal (GI) tract, usually in the stomach or proximal duodenum. *Helicobacter pylori* (*H. pylori*) infection and the excessive use of non-steroidal anti-inflammatory drugs are the leading causes of PUD[5]. Additionally, increasing age, smoking, alcohol use, and obesity have a strong association with PUD[6]. Although the prevalence of self-reported physician-diagnosed ulcer disease in the United States was as high as 10% at the end of the 20th century, there has been a decrease in prevalence and hospitalizations for PUD in the last few decades, primarily due to advancements in *H. pylori* eradication and increasing utilization of proton pump inhibitors for acid suppression[7,8].

The association between alcoholic liver disease and PUD has been well established with studies reporting a higher prevalence of PUD in patients with alcoholic liver cirrhosis[9]. Additionally, a study by Nojkov and Cappell noted that PUD was the most common cause of non-variceal hemorrhage in cirrhotics and carried a higher rate of re-bleeding, delayed ulcer healing, and recurrence when compared to the general population[10]. However, there continue to be significant knowledge gaps on PUD in NAFLD hospitalizations. Hence, we addressed the knowledge gaps in current literature as we identified hospitalization trends, outcomes, and predictors of mortality for NAFLD hospitalizations with PUD. Furthermore, we also performed a comparative analysis between NAFLD hospitalizations with PUD and non-NAFLD PUD hospitalizations to determine the influence of NALFD on PUD.

MATERIALS AND METHODS

Design and data source

The study cohort was derived from the National Inpatient Sample (NIS) database which is one of the largest, publicly available, multi-ethnic databases in the United States. The NIS, part of the Healthcare Cost and Utilization Project group of databases, consists of data on inpatient admissions submitted by hospitals across the United States to state-wide data organizations, covering 97% of the United States population[11]. It approximates a 20-percent stratified sample, and the dataset is weighted to obtain national estimates[12]. For the 2009-2019 study period, the NIS database was coded using the International Classification of Diseases (ICD)-9/10 coding systems.

Study population

We utilized the NIS to obtain all adult (≥ 18 years) NAFLD hospitalizations with PUD in the United States from 2009-2019. Furthermore, a control group of all adult PUD hospitalizations without NAFLD were identified for comparative analysis.

Statistical analysis and outcome measures

Statistical analysis was conducted using SAS 9.4 (SAS Institute Inc, Cary, NC) while accounting for the weights in the stratified survey design. The weights were considered during statistical estimation by incorporating variables for strata, clusters, and weight to discharges in the NIS. Descriptive statistics were provided, including mean (standard deviation) for continuous variables and count (percentage) for categorical variables. To test for the trend for proportions of binary variables in years, the Cochran-Armitage trend test was implemented. The trend for the averages of continuous variables in years was examined using linear regression. The Rao-Scott design-adjusted chi-square test, which takes the stratified survey design into account, examined the association between two categorical variables. All analytical results were statistically significant when the *P*-values ≤ 0.05.

Ethical considerations

The NIS database lacks any patient and hospital-specific identifiers. Hence, this study was exempt from Institutional Review Board (IRB) review as per guidelines put forth by our institutional IRB for research on database studies.

RESULTS

Hospitalization characteristics for NAFLD hospitalizations with PUD

Overall, there was no decline in the total number of NAFLD hospitalizations with PUD (3745 in 2009 to 3805 in 2019, with a peak of 6885 in 2014) (Table 1). The mean age increased from 56.0 years in 2009 to 63.0 years in 2019 ($P < 0.001$), with a significant increase noted for the 65-79 age group (26% in 2009 to 46% in 2019). A majority of NAFLD hospitalizations with PUD were for females and Whites. Racial differences were also noted as there was a declining trend of NAFLD hospitalizations with PUD for Blacks from 10% in 2009 to 5% in 2019 ($P = 0.01$) and Asians from 3% in 2009 to 2% in 2019 ($P = 0.01$), while Whites and Hispanics had a rising trend (Table 1 and Figure 1). Furthermore, rates of *H. pylori* infection and upper endoscopy decreased from 2009 to 2019 (Table 1). Most NAFLD hospitalizations with PUD were at large hospitals, and admissions in urban teaching hospitals increased from 40% in 2009 to 80% in 2019 ($P < 0.001$). Overall, Medicare was the largest insurer for NAFLD hospitalizations with PUD.

Outcomes for NAFLD hospitalizations with PUD

We noted a rising trend of all-cause inpatient mortality for NAFLD hospitalizations with PUD from 2% in 2009 to 5% in 2019 ($P < 0.001$) (Table 2). However, inpatient mortality for Whites declined from 81% in 2009 to 64% in 2019 ($P = 0.04$) within the race analysis. We did not find a statistically significant trend for mean length of stay (LOS) and mean total healthcare cost (THC). Furthermore, the rates of GI tract perforation decreased [33% (2009) to 8% (2019), $P = 0.02$] but the proportion of patients with GI bleeding increased [0% (2009) to 11% (2019), $P = 0.04$].

Comparative analysis of NAFLD and non-NAFLD hospitalizations with PUD

NAFLD hospitalizations with PUD were younger (58.6 *vs* 65.3 years, $P < 0.001$), and had more Whites and Hispanics compared to the non-NAFLD subgroup (Table 3). A Charlson Comorbidity Index (CCI) ≥ 3 was noted in a higher proportion of NAFLD hospitalizations with PUD (55%) compared to non-NAFLD PUD hospitalizations (49%) ($P < 0.001$). Although NAFLD hospitalizations with PUD had a higher proportion of patients that underwent upper endoscopy (49% *vs* 41%, $P < 0.001$), the rates of *H. pylori* infection was not statistically different between the cohorts (Table 3).

Despite a higher CCI score, the all-cause inpatient mortality was lower (2% *vs* 3%, $P = 0.0004$), for NAFLD-PUD hospitalizations compared to the non-NAFLD-PUD hospitalizations (Table 4). Furthermore, the mean LOS was shorter (11.6 *vs* 12.1 d, $P < 0.001$), the and mean THC was lower ($178598 and $184727, $P < 0.001$) for NAFLD-PUD hospitalizations compared to non-NAFLD-PUD hospitalizations (Table 4). There was no statistical difference in the proportion of patients with complications such as GI bleeding and perforation between the 2 groups.

Predictors of mortality for NAFLD hospitalizations with PUD

Significant predictors of all-cause inpatient mortality for NAFLD hospitalizations with PUD included GI tract perforation (aHR = 2.71, 95%CI: 1.37-5.35, $P = 0.004$), coagulopathy (aHR = 2.24, 95%CI: 0.74-0.84, $P < 0.001$), fluid and electrolyte disorders (aHR = 1.92, 95%CI: 1.82-2.01, $P < 0.001$), alcohol abuse (aHR = 1.51, 95%CI: 1.42-1.61, $P < 0.001$), and protein-calorie malnutrition (aHR = 1.16, 95%CI: 1.11-1.22, $P < 0.001$) (Table 5).

DISCUSSION

PUD is believed to be the most common cause of non-variceal GI bleeding in liver cirrhosis patients. However, data on PUD in NAFLD hospitalizations is lacking. Ours is the only study in current literature that evaluates the trends of hospitalization characteristics and outcomes for NAFLD hospitalizations with PUD and further compares NAFLD hospitalizations with PUD to non-NAFLD PUD hospitalizations using the NIS database. In this study, we did not find a decline in the total number of NAFLD hospitalizations with PUD. The all-cause inpatient mortality increased from 2% in 2009 to 5% in 2019 in the United States. However, the rates of upper endoscopy and *H. pylori* infection declined for NAFLD hospitalizations with PUD. Furthermore, NAFLD hospitalizations with PUD had lower mortality, LOS, and THC compared to the non-NAFLD group, despite a higher comorbidity burden. An understanding of the trends, outcomes, and influence of NAFLD on PUD is crucial as it may help gastroenterologists identify individuals at the greatest risk of adverse outcomes and complications, thereby preventing morbidity and mortality.

Recent studies have demonstrated a dramatic increase in the prevalence and hospitalization rates for patients with NAFLD, which could have led to the slightly increased number of hospitalizations noted in our study[13,14]. This is despite the fact that there has been an overall decline in hospitalization rates for PUD in the United States[15]. Furthermore, racial differences have also been noted in NAFLD hospitalizations for ethnic minorities. Per current literature, the highest and lowest rates of NAFLD

Table 1 Hospitalization trends for non-alcoholic fatty liver disease hospitalizations with peptic ulcer disease in the United States from 2009-2019

Variable	Years									
	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Total hospitalizations	3745	4563	5230	5655	5759	6885	5745	2880	3070	3430
Mean age (yr)	56.01 ± 0.8	57.07 ± 0.75	56.68 ± 0.81	57.1 ± 0.14	57.1 ± 0.41	57.4 ± 0.59	57.5 ± 0.97	62.4 ± 0.41	62.8 ± 0.8	63.7 ± 0.74
Age groups, yr (%)										
18-34	9	8	8	8	7	8	8	3	4	2
35-49	25	24	25	25	23	22	21	12	11	15
50-64	39	42	41	39	42	42	39	44	40	36
65-79	26	25	26	27	28	28	32	42	44	47
≥ 80	< 1	1	1	1	< 1	< 1	< 1	1	1	1
Gender (%)										
Male	45	42	43	43	44	44	45	36	40	40
Female	55	48	57	58	56	56	55	64	60	50
Race (%)										0.01
White	72	73	70	71	72	71	70	74	76	76
Black	10	8	9	10	9	11	10	6	5	5
Hispanic	11	13	14	13	12	11	13	14	15	15
Asian	3	3	3	3	2	3	3	2	2	2
Native American	1	< 1	1	1	1	1	1	2	1	1
Other	3	3	3	3	3	4	3	3	2	4
CCI (%)										
CCI = 0	0	0	0	0	0	0	0	0	0	0
CCI = 1	34	32	34	32	32	31	31	6	6	6
CCI = 2	27	24	23	22	22	22	25	13	8	10
CCI ≥ 3	39	44	43	47	46	47	44	86	86	86
Hospital region (%)										
Northeast	13	12	14	16	15	14	14	13	13	16
Midwest	24	24	22	22	22	21	21	28	29	29
South	39	41	43	21	42	43	44	38	37	35
West	24	23	21	21	21	21	21	20	21	19
Hospital bed-size (%)										
Small	8	11	11	13	11	16	16	15	16	16
Medium	25	25	22	29	26	32	29	24	25	28
Large	67	64	67	59	63	52	55	61	59	56
Hospital location and teaching status (%)										
Rural	8	10	11	10	10	7	7	6	5	5
Urban non-teaching	47	50	45	41	37	26	27	21	20	14
Urban teaching	46	40	44	49	53	67	66	73	75	81
Primary payer										
Medicare	41	42	41	44	43	44	44	53	60	60

Medicaid	13	15	14	15	15	16	16	14	11	11
Private	36	35	35	33	34	33	33	30	25	26
Other	10	8	10	8	8	7	7	2	3	3
Median household income (%)										
1st (0-25th)	26	27	30	28	31	28	31	31	27	29
2nd (26th-50th)	25	24	25	24	26	29	24	27	32	32
3rd (51st-75th)	30	24	24	26	24	23	26	24	25	21
4th (76th-100th)	19	24	21	22	19	20	19	17	15	18
Upper endoscopy (%)	60	59	62	58	59	59	62	20	22	18

CCI: Charlson Comorbidity Index.

Table 2 Trends of outcomes for non-alcoholic fatty liver disease hospitalizations with peptic ulcer disease in the United States from 2009-2019

Outcome	Years											*P* value
	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	
Inpatient mortality (%)	2	1	2	2	2	2	1	4	4	5	5	< 0.001
Gender-specific inpatient mortality (%)												0.2
Male	25	21	28	57	53	38	40	37	52	32	31	
Female	75	79	72	43	47	62	60	63	48	48	48	
Race-specific inpatient mortality (%)												0.4
White	81	53	89	48	79	73	73	71	81	61	64	
Black	7	0	0	14	0	4	0	4	4	6	11	
Hispanic	12	47	11	19	16	19	7	17	8	19	14	
Asian	0	0	0	10	5	0	0	4	0	3	6	
Native American	0	0	0	5	0	0	7	0	4	3	6	
Others	0	0	0	5	0	4	13	4	4	6	0	
Age-group specific inpatient mortality (%)												0.1
18-34	7	0	0	0	0	3	0	0	0	0	0	
35-49	13	26	16	13	0	17	14	9	22	14	6	
50-64	51	36	33	31	29	34	36	41	52	31	48	
65-79	28	24	36	56	71	41	50	50	26	48	45	
≥ 80	0	13	14	0	0	3	0	0	0	3	0	
Length of stay (d)	17.1 ± 0.59	11.8 ± 0.76	16.3 ± 0.58	9.2 ± 0.85	9.0 ± 0.01	8.2 ± 0.68	10.1 ± 0.33	9.6 ± 0.49	17.3 ± 0.33	9.2 ± 0.90	11.2 ± 0.22	0.06
Total hospital charge ($)	182296 ± 760	104265 ± 620	215085 ± 130	172837 ± 170	157079 ± 890	105031 ± 760	136876 ± 800	134573 ± 730	373045 ± 450	143284 ± 940	174044 ± 550	0.8
Complications (%)												
Bleeding	0	21	0	0	5	10	7	17	7	10	11	0.4
Perforation	33	12	35	29	16	**3**	13	17	11	3	8	0.02

Table 3 Comparative analysis of hospitalization characteristics for non-alcoholic fatty liver disease hospitalizations with peptic ulcer disease and non-NALFD hospitalizations with peptic ulcer disease in the United States from 2009-2019

	NAFLD hospitalizations with PUD	Non-NAFLD hospitalizations with PUD	*P* value
Total hospitalizations	50769 (1%)	4624628 (99%)	
Mean age (yr)	58.6 ± 0.27	65.3 ± 0. 80	< 0.001
Age group, yr (%)			< 0.001
18-34	6	6	
35-49	21	15	
50-64	40	33	
65-79	32	40	
≥ 80	1	6	
Gender (%)			< 0.001
Male	42	49	
Female	48	51	
Race (%)			< 0.001
White	72	70	
Black	9	14	
Hispanic	13	9	
Asian	3	3	
Native American	1	1	
Other	3	3	
CCI (%)			< 0.001
CCI = 0	0	0	
CCI = 1	25	30	
CCI = 2	20	22	
CCI ≥ 3	55	49	
Upper endoscopy (%)	49	41	< 0.001
H. pylori (%)	3	3	0.5
Hospital region (%)			< 0.001
Northeast	14	18	
Midwest	24	23	
South	41	39	
West	21	20	
Hospital bed size (%)			< 0.001
Small	14	16	
Medium	27	28	
Large	59	56	
Hospital location and teaching status (%)			< 0.001
Rural	8	10	
Urban nonteaching	32	34	
Urban teaching	60	56	
Expected primary payer (%)			< 0.001
Medicare	47	61	

Medicaid	14	12	
Private	32	21	
Other	7	5	
Median household income (quartile) (%)			< 0.001
1st (0-25th)	29	31	
2nd (26th-50th)	27	26	
3rd (51st-75th)	25	24	
4th (76th-100th)	20	19	

CCI: Charlson Comorbidity Index; NAFLD: Non-alcoholic fatty liver disease; PUD: Peptic ulcer disease.

Table 4 Comparative analysis of outcomes for non-alcoholic fatty liver disease hospitalizations with peptic ulcer disease and non-NAFLD hospitalizations with peptic ulcer disease in the United States from 2009-2019

Outcomes	NAFLD hospitalizations with PUD	Non-NAFLD hospitalizations with PUD	*P* value
Inpatient mortality (%)	2	3	0.0004
Gender-specific inpatient mortality (%)			< 0.001
Male	38	54	
Female	62	46	
Race-specific inpatient mortality (%)			< 0.001
White	70	72	
Black	6	12	
Hispanic	16	9	
Asian	3	4	
Native American	3	1	
Others	3	3	
Age-group specific inpatient mortality (%)			< 0.001
18-34	1	2	
35-49	13	7	
50-64	39	30	
65-79	44	49	
≥ 80	2	12	
Length of stay (d)	11.6	12.1	< 0.001
Total healthcare cost ($)	178598	184727	< 0.001
Complications (%)			
Bleeding	8	6	0.2
Perforations	14	18	0.1

NAFLD: Non-alcoholic fatty liver disease; PUD: Peptic ulcer disease.

hospitalizations are for Hispanics and African Americans, respectively[13,16]. This has been attributed to dietary habits, lifestyle, and genetics as Hispanics have an allele of the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) that favors hepatic fat storage, while African Americans possess a different allele of the same gene associated with lower hepatic fat content[17,18]. Our study echoed similar findings as we noted a rising trend of NAFLD hospitalizations with PUD for Hispanics from 11% in 2009 to 15% in 2019 (P = 0.01) and a declining trend for Blacks from 10% in 2009 to 5% in 2019 (P = 0.01). These racial differences are important as we advocate for the need for aggressive public health

Table 5 Predictors of all-cause inpatient mortality for non-alcoholic fatty liver disease hospitalizations with peptic ulcer disease in the United States from 2009-2019

Variable	Adjusted hazard ratio	95% confidence interval	*P* value
Coagulopathy	2.24	2.14-2.34	< 0.001
Obesity	0.79	0.74-0.84	< 0.001
Protein calorie malnutrition	1.16	1.11-1.22	< 0.001
Fluid and electrolyte disorder	1.92	1.82-2.01	< 0.001
Alcohol abuse	1.51	1.42-1.61	< 0.001
Perforation	2.71	1.37-5.35	0.004

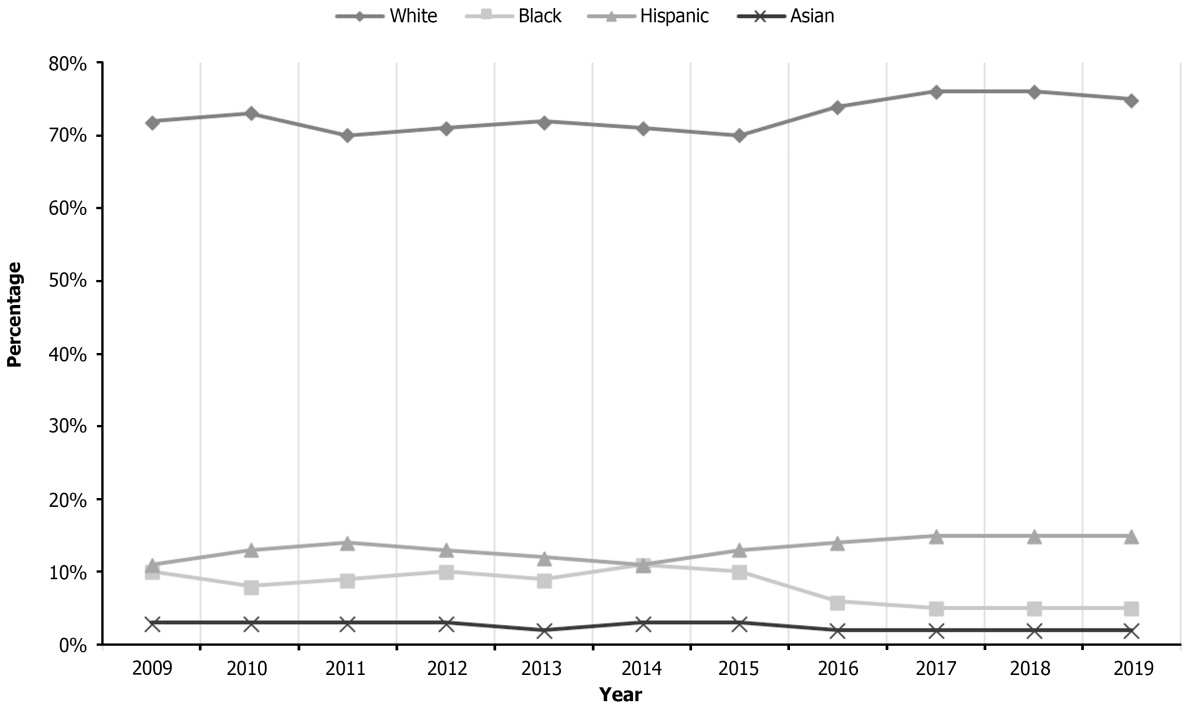


Figure 1 Racial trends for non-alcoholic fatty liver disease hospitalizations hospitalizations with peptic ulcer disease in the United States from 2009-2019.

measures for Hispanic populations to increase awareness about the burden of PUD in those who have NAFLD. Moreover, hospitalists and gastroenterologists who take care of NAFLD hospitalizations should have a high degree of suspicion of PUD in these patients.

In the United States, there has been a significant increase in inpatient mortality for NAFLD-cirrhosis by 32% between 2005-2015, despite a decrease in the inpatient mortality rates for patients with all other causes of liver cirrhosis[19]. However, there has been a significant decline in inpatient PUD mortality due to the increasing use of therapeutic endoscopic procedures for bleeding ulcers[20]. In our study, there was a rising trend of all-cause inpatient mortality for NAFLD hospitalizations with PUD (Table 2). From a race perspective, we noted an increasing trend of all-cause inpatient mortality for Blacks and Hispanics, while a decline was observed for Whites. The exact reasons for these findings are unknown, but they may, in part, be attributed to a higher comorbidity burden and the increasing mean age for NAFLD hospitalizations with PUD, particularly for ethnic minorities *i.e.,* Blacks and Hispanics (Table 1), leading to greater severity of disease and adverse outcomes. Interestingly, we noted lower all-cause inpatient mortality, mean LOS, and mean THC for NAFLD hospitalizations with PUD compared to non-NAFLD PUD hospitalizations, despite a higher comorbidity burden. But there was no statistical difference in the proportion of patients with complications such as GI bleeding and perforation between the two groups. The exact reason for this protective effect of NAFLD is unclear and needs further investigation by multi-center prospective studies.

There has been a rapid decline in *H. pylori* infection rates in the western world secondary to higher standards of living and improved hygiene[21]. An analysis of outpatient endoscopy centers in the United States noted a significant fall in *H. pylori* infections from 11% in 2009 to 9% in 2018[22]. However,

a recent cross-sectional study identified a positive relationship between *H. pylori* infection and NAFLD in females after adjusting for metabolic variables, gastrin factors, and liver enzymes[23]. This implies that rates of *H. pylori* infection may rise with an increasing prevalence of NAFLD. Our study contradicts these findings as we observed a declining trend of *H. pylori* infection in NAFLD hospitalizations with PUD (Table 1). Furthermore, we also noted a decline in the trends of utilization of upper endoscopy from 60% in 2009 to 19% in 2019. This may be due to increased adherence to guideline-directed management which advocates for non-endoscopic testing for uninvestigated dyspepsia without alarm features in individuals < 60 years of age[24]. However, due to an increasing trend of all-cause inpatient mortality in this subset population, it may be justified to perform upper endoscopy at an early stage to prevent adverse clinical outcomes. Interestingly, NAFLD hospitalizations with PUD had a higher proportion of patients that underwent upper endoscopy compared to non-NAFLD PUD hospitalizations. The exact reason for this is currently unknown and needs further investigation.

Our study has numerous strengths and some limitations. A key strength of this study is the study population, which is derived from one of the largest, publicly available, all-payer, multi-ethnic databases in the United States. An analysis over the 11-year study period allowed us to obtain meaningful information on the trends of hospitalization characteristics and outcomes for NAFLD hospitalizations with PUD, adding to scarce literature. Moreover, through our unique and multifaceted comparative analysis, we were able to determine the influence of NAFLD on PUD. Furthermore, as the NIS database covers 97% of the United States population, the results of our study are applicable to almost all NAFLD hospitalizations with PUD, offering a national perspective. However, we do acknowledge the limitations associated with our study. This was a retrospective study design which is subject to all biases associated with retrospective studies. The NIS database does not contain information on the severity, time from hospitalization to discharge, hospital course, and other treatment aspects of the disease. Lastly, the NIS is an administrative database maintained through data collection organizations that use the ICD coding system to store inpatient data. Hence, the possibility of human coding errors cannot be excluded. However, despite these limitations, we believe that the large sample size and a comprehensive analysis technique help us better understand the trends, characteristics, and outcomes of NAFLD hospitalizations with PUD, and promote future research on the topic.

CONCLUSION

NAFLD is a public health concern and places a significant burden on the United States healthcare system. There is a significant knowledge gap on PUD in patients with NAFLD who are admitted to the hospital. We noted an increase the all-cause inpatient mortality for NAFLD hospitalizations with PUD in the United States between 2009-2019. There was a rising trend of NAFLD hospitalizations with PUD for Hispanics, reflecting the need for urgent public health measures to increase awareness in this subset population. Compared to non-NAFLD PUD hospitalizations, NAFLD hospitalizations with PUD had lower inpatient mortality, mean LOS, and mean THC despite a higher comorbidity burden. However, there was no statistical difference in GI bleeding and perforation between the two groups. Perforation of the GI tract, coagulopathy, alcohol abuse, malnutrition, and fluid and electrolyte disorders were identified to be independent predictors of all-cause inpatient mortality for NAFLD hospitalizations with PUD. Additional multi-center prospective studies are needed to further confirm these findings.

ARTICLE HIGHLIGHTS

Research background

The association between peptic ulcer disease (PUD) and liver cirrhosis has been thoroughly investigated. However, there are knowledge gaps on PUD in non-alcoholic fatty liver disease (NAFLD) hospitalizations. As the prevalence of NAFLD continues to rise across the globe and in the United States, it is vital to identify individuals with NAFLD at high risk of adverse clinical outcomes from PUD.

Research motivation

In current literature, there is a knowledge gap on PUD in NAFLD hospitalizations. Hence, this study was designed to help fill the knowledge gaps that currently exist in this subset population.

Research objectives

Our main objective was to identify national trends in hospitalization characteristics, clinical outcomes, and complications for NAFLD hospitalizations with PUD. We also performed a comparative analysis between NAFLD and non-NAFLD hospitalizations with PUD to assess the influence of NAFLD on PUD.

Research methods

The National Inpatient Sample was used from 2009-2019 to identify all adult (≥ 18 years) NAFLD hospitalizations with PUD in the United States. Furthermore, a control group of all adult PUD hospitalizations without NAFLD were identified for comparative analysis. Statistical analysis was conducted using SAS 9.4. To test for the trend for proportions of binary variables, the Cochran-Armitage trend test was implemented. The trend for the averages of continuous variables in years was examined using linear regression. The Rao-Scott design-adjusted chi-square test examined the association between two categorical variables.

Research results

NAFLD-PUD hospitalizations increased from 3745 (2009) to 3805 (2019). Racial differences were noted as NAFLD hospitalizations with PUD increased for Whites and Hispanics, while a decline was observed for Blacks and Asians. There was an increase in all-cause inpatient mortality for NAFLD hospitalizations with PUD from 2% in 2009 to 5% in 2019 ($P < 0.001$). However, the rates of *Helicobacter pylori* (*H. pylori*) infection and upper endoscopy decreased during the study period. Despite a high comorbidity burden, we observed lower inpatient mortality, mean length of hospital stay, and mean total healthcare cost (THC) for NAFLD-PUD hospitalizations *vs* the non-NAFLD-PUD cohort. Perforation of the gastrointestinal tract, coagulopathy, alcohol abuse, malnutrition, and fluid and electrolyte disorders were identified as independent predictors of inpatient mortality for NAFLD hospitalizations with PUD.

Research conclusions

Between 2009-2019, inpatient mortality for NAFLD hospitalizations with PUD increased. However, there was a significant decline in *H. pylori* infections and esophagogastroduodenoscopy for NAFLD-PUD hospitalizations. After a comparative analysis, NAFLD-PUD hospitalizations had lower rates of mortality, mean length of hospital stay, and mean THC *vs* the non-NAFLD-PUD cohort despite a higher comorbidity burden.

Research perspectives

This is one of the few national studies which investigates trends, clinical outcomes, and complications of NAFLD hospitalizations with PUD, using one of the largest, multi-ethnic databases in the United States. Future research should be directed toward identifying the underlying cause of racial disparities for this subset population.

FOOTNOTES

Author contributions: Dahiya DS, Ali H, Inamdar S, Sanaka MR, and Al-Haddad M were responsible for the conception and design; Dahiya DS, Inamdar S, Sanaka MR, and Al-Haddad M provided administrative support; Dahiya DS and Ali H were responsible for provision, collection, and assembly of data; all authors were responsible for review of literature, drafting the manuscript, and revision of key components of the manuscript; and all authors have read and approve the final version of the manuscript.

Institutional review board statement: The National Inpatient Sample database contains numerous safeguards to protect patient privacy. It also lacks patient- and hospital-specific identifiers. Therefore, our study was exempt from Institutional Review Board (IRB) evaluation as per guidelines put forth by our institutional IRB for analysis of HCUP databases.

Informed consent statement: The data for this study was collected from the National Inpatient Sample (NIS) database. As the NIS database lacks patient-specific and hospital-specific identifiers, this study did not require informed consent.

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

Data sharing statement: The National Inpatient Sample is a publicly available database that can be accessed at https://www.hcup-us.ahrq.gov. Due to the availability of a large sample size within the NIS database, it is an ideal choice for the estimation of national trends and clinical outcomes.

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ORIGINAL ARTICLE

Observational Study

Clinicopathological features of 11 cases of chronic hepatitis B infection complicated with primary biliary cholangitis

Yun Ye, Qian Zhang, Zhong-Hua Lu, You-Wen Tan

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Abstract

BACKGROUND

Only a few cases of chronic hepatitis B (CHB) with primary biliary cholangitis (PBC) have been reported based on histological evidence from liver biopsies.

AIM

To observe the clinicopathological features and outcomes of 11 patients with CHB infection complicated by PBC.

METHODS

Eleven patients with CHB and PBC who underwent liver biopsy at the Zhenjiang Third Hospital, affiliated with Jiangsu University, and Wuxi Fifth People's Hospital, from January 2005 to September 2020, were selected. All patients initially visited our hospital with CHB and were pathologically diagnosed with CHB and PBC.

RESULTS

Only five had elevated alkaline phosphatase levels, nine were positive for anti-mitochondrial antibody (AMA)-M2, and two were negative for AMA-M2. Two had jaundice and pruritus symptoms, 10 had mildly abnormal liver function, and one had severely elevated bilirubin and liver enzyme levels. The pathological characteristics of CHB complicated by PBC overlapped with those of PBC-autoimmune hepatitis (AIH). When necroinflammation of the portal area is not obvious, the pathological features of PBC are predominant, similar to the features of PBC alone. When the interface is severe, biliangitis will occur, with a large number of ductular reactions in zone 3. Unlike the PBC-AIH overlap pathology, this pathology is characterized by a small amount of plasma cell infiltration. Unlike PBC, lobulitis is often observed.

CONCLUSION
This is the first large case series to show that the rare pathological features of CHB with PBC are similar to those of PBC-AIH and small duct injury was observed.

Key Words: Chronic hepatitis B; Primary biliary cholangitis; Clinicopathological features; Anti-mitochondrial antibody

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Core Tip: We retrospectively observed the clinicopathological features and outcomes of 11 patients with chronic hepatitis B (CHB) infection complicated by primary biliary cholangitis (PBC). We found that CHB complicated with PBC had pathological characteristics overlapping with PBC-autoimmune hepatitis. When necroinflammation of the portal area is not obvious, the pathological features of PBC are superior, similar to the features of PBC alone, this pathology is characterized by a small amount of plasma cell infiltration. Unlike PBC alone, lobulitis is often present. All patients improved after antiviral and ursodeoxycholic acid treatment and stabilized after 1 year.

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INTRODUCTION

Chronic hepatitis B (CHB) remains the largest public health burden in China, with nearly 60 million people infected with the CHB virus and nearly 300000 deaths related to liver disease every year[1,2]. Primary biliary cholangitis (PBC) is a slowly progressing autoimmune disease that is prevalent in Northern Europe and North America. PBC remains uncommon compared with CHB in Asian populations. Large cohort studies have reported that, in Japan and Hong Kong, only 2.4% and 1.3% of PBC cases occur in patients with chronic liver disease, respectively[3]. A recent study from southern China reported that PBC occurred in 49.2 cases out of 100000 adults who underwent routine annual examinations[4]. Anti-mitochondrial antibody (AMA)-M2 was reported in 22 of the 325 patients (6.8%) with CHB[5]. A positive AMA result did not confirm the presence of PBC. Only a few cases of CHB with PBC based on histological evidence from liver biopsies have been reported. We retrospectively examined the clinicopathological features and outcomes of 11 patients with CHB complicated by PBC.

MATERIALS AND METHODS

Methods

Eleven cases of CHB patients with PBC who underwent liver biopsy were all at the Third People's Hospital of Zhenjiang City and the Fifth People's Hospital of Wuxi City from January 2005 to September 2020. CHB infected persons were defined as hepatitis B surface antigen (HBsAg) lasting for more than half a year. PBC was diagnosed if two of the following three criteria were met: (1) Biochemical abnormalities reflecting cholestasis, such as elevated alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) levels given that extra- or intrahepatic bold tube obstruction is excluded by imaging examination; (2) positive serum AMA/AMA-M2 or other PBC-specific autoantibodies such as anti-GP210 and anti-SP100; and (3) histological evidence of chronic non-suppurative destructive cholangitis (CNSDC) and interlobular bile duct destruction in liver biopsy[6]. The exclusion criteria were a history of excessive alcohol consumption (defined as ≥ 20 g/d for men and ≥ 10 g/d for women)[7], drug-induced hepatitis, schistosomiasis liver disease, autoimmune hepatitis (AIH), or hepatitis A, C, or D. This study was approved by the Ethics Committee of The Third People's Hospital of Zhenjiang City.

Data collection

The following demographic data (sex and age) were collected: clinical history (medical history, drinking history, family history); antiviral treatment status; results of routine blood tests; biochemical indicators; hepatitis B pathogenic serological examination; and tumor indicators during liver biopsy, including total bilirubin, albumin, prealbumin, alanine aminotransferase, aspartate aminotransferase, GGT, ALP, platelet count, HBsAg, hepatitis B e antigen, hepatitis B virus (HBV) DNA level, alpha-fetoprotein,

indicators of other types of viral hepatitis, human immunodeficiency virus antibody, and autoantibody examination results; antibodies (AMA) and subtypes (AMA-M2), antibodies against gp210, sp100, and immunoglobulins G (IgG) and M (IgM).

Pathological assessment

Liver biopsies were performed using a 16G puncture needle (Bard Peripheral Vascular Inc., United States) under ultrasound guidance by an experienced hepatologist. The length of the puncture tissue was greater than 1.5 cm and the tissue was fixed in formalin. The tissues were evaluated by two liver pathologists using the METAVIR and Ludwig staging systems[8,9].

RESULTS

Clinical features

There were 11 cases of CHB diagnosed with PBC, of which four were male and seven were female, with an average age of 42 years (range, 28-60 years); nine cases were positive for AMA-M2. Two AMA-M2-negative patients with elevated ALP and GGT levels but without other etiological explanations were diagnosed with PBC by liver biopsy. ALP levels were elevated in five of the 11 cases. Six patients had normal ALP levels, while four had elevated GGT levels and positive AMA-M2 results. Two patients attained a complete HBV response (HBV DNA < 10 U/L) before liver biopsy, and the remaining patients with CHB underwent primary antiviral treatment, with five receiving entecavir, three receiving tenofovir, and three receiving propofol tenofovir fumarate. Patient 7 was hospitalized for severe liver injury for approximately 2 mo. After control was achieved, patient 7 received outpatient treatment similar to that of the other patients (Table 1).

Pathological characteristics

The lesions were primarily caused by necroinflammation of the portal tract. All cases showed bile duct injury; five showed a typical florid duct lesion (FDL) and three cases with severe interfacial necroinflammation showed PBC-AIH-like injury with fewer plasma cells. A ductular reaction was evident that extended deep into the hepatocyte plate, and bile duct interfacial necroinflammation was apparent. Six patients with normal ALP and AMA-M2 expression had bile duct injuries. Overall, the METAVIR staging was consistent with the Ludwig staging (Table 2, Figure 1).

Treatments and outcomes

Three patients with CHB had previously received antiviral therapy, and eight patients with HBV DNA-positive liver biopsies received antiviral therapy. The antiviral drugs used were entecavir in five cases, tenofovir in three cases, and propofol tenofovir in three cases. Ten patients experienced viral remission (< 10 U/L) after six months, and one experienced viral remission after one year. ALP levels returned to normal in four of the five patients with elevated ALP levels, and one patient returned to normal levels. Of the eight patients with elevated GGT levels, seven returned to normal after six months and one returned to normal after one year (Table 3).

DISCUSSION

Hepatitis B is a lifelong immune-related disease. Raynaud's phenomenon occurs in 2% of patients with CHB, arthralgia or arthritis in 3%, myalgia in 3%, Sjögren's syndrome in 3%, glomerulonephritis in 3%, uveitis in 2%, and cryoglobulin in 2%[10]. In a Chinese study on the detection rate of autoantibodies against CHB, 58.2% of patients with CHB were found to have AMA-M2 (approximately 7%)[5]. The 11 patients in our study were selected from approximately 1500 CHB liver histology examiners at two centers. The AMA-M2 positivity rate was < 1%, which may be related to the fact that many patients did not undergo the AMA-M2 examination.

Logically, preclinical PBC refers to patients with positive AMA but normal enzymatic indicators (*i.e.*, ALP and GGT) that reflect cholestasis and no PBC manifestation on histology who nonetheless progress to PBC during follow-up. A prospective multicenter cohort study in France[11] found a 5-year incidence of PBC of 16% in a population with positive AMA and normal ALP levels. A recent single-center study in Austria[12] reported that, after an average follow-up of 5.8 years, only 10.2% of 122 AMA-positive patients developed PBC. An earlier study also found that only one (4%) of 26 AMA-positive first-degree relatives with normal ALP levels developed PBC after an 8-9-year follow-up[13].

In most reported cases, the diagnosis of PBC in patients with CHB is delayed for many years[14]. All 11 patients initially visited the hospital because of CHB, and eight patients were found to be AMA-M2 positive during the course of treatment. Of these, two with CHB showed ALP elevation after antiviral treatment, and a bile duct injury was detected by liver puncture. Only two patients had jaundice and pruritus symptoms, ten had mild abnormal liver function, and one patient had very serious increases in

Table 1 Clinical characteristics of 11 cases of chronic hepatitis B complicated with primary biliary cholangitis

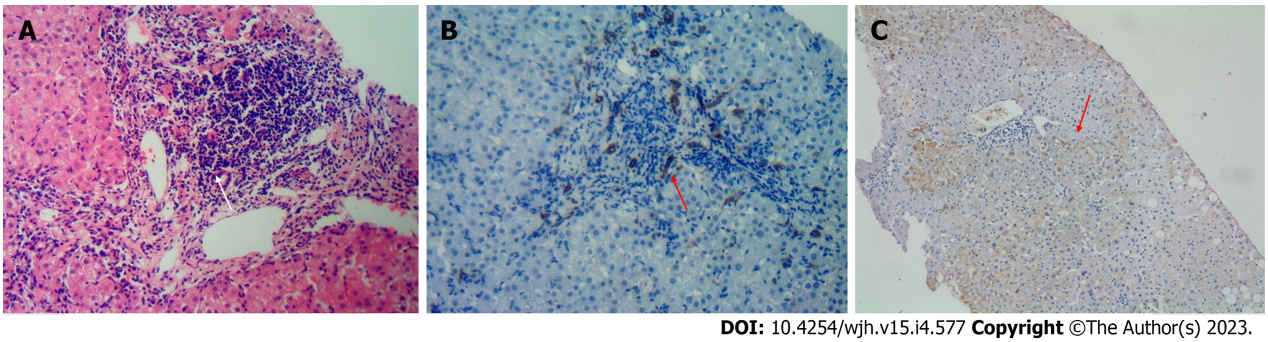
No.	Sex	Age	TBIL	ALT	AST	ALP	GGT	TBA	Albumin (g/L)	AMA-M2	gp120	sp100	ANA	IgM	IgG	HBV DNA (Log)	Platelet (1 × 10^9 /L)	Fatigue	Pruritus
1	Woman	42	22.2	87	56	47	67	17	32.2	++	+	-	+	4.25	21.15	7.21	9.21	+	+
2	Man	60	45.2	224	175	154	116	30	36.5	+	-	-	-	1.25	11.23	4.25	12.66	-	-
3	Woman	52	13.5	35	24	57	57	12	41.2	+	-	-	-	1.65	9.32	4.68	8.35	-	-
4	Man	37	15.5	22	16	46	47	8	38.2	+	-	-	-	1.42	8.65	5.32	16.35	-	-
5	Man	39	16.0	35	24	65	37	12	37.9	+	-	-	-	1.87	10.32	-	15.32	-	-
6	Woman	37	16.3	27	18	74	58	14	36.4	++	-	-	-	2.01	11.56	6.14	11.25	-	-
7	Man	57	422.4	243	215	767	837	147	32.2	+	-	-	-	4.36	24.32	4.35	13.65	+	+
8	Woman	52	20.4	156	87	167	224	25	40.2	++	+	-	+	0.98	10.48	3.57	14.25	+	-
9	Woman	32	11.3	25	11	37	26	37	41.6	+	-	-	-	1.14	9.65	4.25	24.22	-	-
10	Woman	28	32.7	18	15	166	89	18	39.2	-	-	-	-	1.58	13.64	-	21.03	-	-
11	Woman	51	25.6	87	26	185	98	9	38.1	-	-	-	-	1.67	10.35	-	18.21	-	-

TBIL: Total bilirubin; TBA: Total bile acid; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; AMA-M2: Antimitochondrial antibody and subtypes-M2; ANA: Antibodies against gp210, antibodies against sp100, antinuclear antibodies; Ig: Immunoglobulin.

Table 2 Changes of biochemical indexes before and after treatment

	TBIL	ALT	AST	ALP	GGT	TBA	IgM	IgG
Before treatment	20.4 (11.3-422.4)	35 (18-243)	24 (11-215)	66 (37-767)	58 (17-837)	17 (8-147)	1.65 (0.98-4.37)	12.7 (8.6-24.3)
After treatment	17.7 (6.7-21.2)	27 (12-63)	21 (15-43)	47 (38-107)	25 (16-42)	9 (6-20)	1.4 (1.1-2.5)	11.3 (6.8-15.3)
Statistical value	1.223	2.351	1.656	1.326	2.369	1.632	2.435	0.931
P value	0.248	0.041	0.129	0.214	0.039	0.134	0.031	0.374

TBIL: Total bilirubin; TBA: Total bile acid; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; Ig: Immunoglobulin.



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Figure 1 Histological characteristics of liver in patient 3. A: Severe necroinflammation of the portal tract, chronic non-suppurative cholangitis and florid duct injury (arrow); B: CK7 staining showed interfacial ductular reaction (arrow); C: Hepatitis B surface antigen staining showed hepatitis B infection (arrow).

bilirubin and liver enzyme levels that rapidly improved after antiviral and ursodeoxycholic acid (UDCA) treatment; liver histology showed severe necrosis and fibrosis.

In a recent single-center study in China, up to 80% of patients positive for AMA and with normal ALP were pathologically confirmed to have PBC[15], which is similar to the findings of a multicenter study in Switzerland[16]. Both studies[15,16] suggested that a high AMA titer and elevated IgM and ALP levels close to the upper limit of normal are predictive factors for PBC expression on histology.

Table 3 Pathological characteristics of 11 cases of chronic hepatitis B complicated with primary biliary cholangitis

No.	II	BN	LI	DI	FDL	BIH	DU	HBsAg staining	HBcAg staining	METAVIR activity score	METAVIR stage	Ludwig stage
1	1	-	2	+	+	-	-	+	+	2	F1	I
2	1	-	1	+	-	-	-	-	-	1	F1	II
3	0	-	1	+	+	-	-	+	-	1	F1	I
4	2	+	2	+	-	+	-	-	+	2	F1	II
5	0	-	1	+	+	-	-	+	-	1	F2	I
6	1	-	1	+	-	+	-	+	-	1	F1	I
7	3	+	2	+	+	+	-	+	+	3	F3	II
8	2	+	1	+	-	+	-	-	-	2	F1	I
9	1	-	1	+	-	+	+	+	-	1	F4	III
10	0	-	0	+	+	-	-	-	-	0	F0	I
11	1	-	1	+	-	-	-	+	-	1	F0	I

II: Interfacial inflammation; BN: Bridging necrosis; LI: Lobular inflammation; DI: Ductal injure; FDL: Florid duct lesion; BIH: Biliary interface hepatitis; DU: Ductopenia; HBsAg: Hepatitis B surface antigen; HBcAg: Hepatitis B core antigen.

Notably, despite normal ALP levels, GGT levels were elevated in most patients in our study. In our 11 cases, only five had elevated ALP, and only three were AMA-M2 positive; nine cases were AMA-M2 positive, seven cases had elevated GGT, and only three cases had elevated ALP. For cases of CHB that are AMA-positive, even though ALP and GGT levels are normal, PBC is likely to be combined; therefore, it cannot be considered that CHB has autoantibodies at present. Changes in liver biochemical indicators in this population should be monitored every year, and for patients with elevated IgM and GGT levels, liver biopsy should be considered to determine the presence of PBC.

The pathological characteristics of CHB is primarily characterized by necroinflammation of the portal tract and lobular inflammation. Bridge-like multilobular necrosis can also occur in severe cases. Fibrosis usually occurs in the portal tract and gradually bridges the adjacent portal areas to form a package. The pathological characteristics of CHB are nonspecific and are difficult to differentiate from those of other chronic liver injuries. The main pathological feature of PBC is CNSDC, involving the interlobular bile duct (small bile duct). The characteristic lesion is the infiltration of lymphocytes around the bile duct and the formation of epithelioid granulomas, which is called incandescent cholangiopathy (FDL). When no small bile ducts were accompanied by a small artery in > 50% of the portal area, it was defined as a reduction or disappearance of the bile duct. Ludwig *et al*[8] divided PBC into four phases. We found that CHB combined with PBC had overlapping pathological characteristics similar to those of PBC-AIH. When necroinflammation in the portal area is not evident, the pathological characteristics of PBC are predominant over those of simple PBC. In contrast, when the interface inflammation is serious, cholangio interface inflammation occurs, and a large number of ductular reactions occur in zone 3. The overlapping pathological feature that differs from that of PBC-AIH is the small amount of plasma cell infiltration. Compared with simple PBC[8], lobular inflammation often occurs; 10 cases had lobular inflammation, and three cases were more serious. FDL are characteristic PBC lesions that do not always occur in patients with CHB or PBC. FDL was observed in five cases in Scheuer's original report[17]. CNSDC has also been observed in the livers of patients with nodular cirrhosis. Additionally, the pathology of PBC is not always evenly distributed in the liver; therefore, sampling errors may occur when determining the stage of these systems.

Currently, there are no cohort observations of CHB overlapping with PBC. A Chinese study surveyed 379 HBsAg-negative patients with PBC, 52 of whom underwent a liver biopsy. The enrolled patients were divided into the anti-HBC-positive and anti-HBC-negative groups. Histological examination revealed that patients in the anti-HBC-positive group had more advanced PBC than those in the anti-HBC-negative group ($P < 0.05$)[18]. In a single-center retrospective review of all follow-up HBV (n = 1493) and hepatitis C virus (HCV; n = 526) patients[14], 17 were identified as having concurrent viral hepatitis and PBC, and most were found to have cirrhosis (10/17, 58.8%). The authors speculated that chronic viral hepatitis combined with PBC was a risk factor for cirrhosis; however, the diagnosis of PBC was mainly based on the presence of anti-mitochondrial antibodies and elevated cholesterol levels. Only one of our 11 patients was diagnosed with cirrhosis. After treatment, the disease was quickly controlled, and the liver transaminase levels stabilized and were normal. All patients achieved biochemical normality within one year. However, the aforementioned pathological characteristics of HBV infection combined with PBC suggest that, as observed in these cohort studies, HBV infection combined with

PBC is more prone to disease progression.

CONCLUSION

The shortcomings of this cohort study are its small sample size and descriptive nature. Nevertheless, this is the first large case series to show that the rare pathological features of CHB with PBC are similar to those of PBC-AIH. Although ALP was normal in nearly 50% of patients, small duct injury was observed, and all patients responded effectively after antiviral and UDCA treatment. The lack of overlap between these two diseases is an aggravating phenomenon.

ARTICLE HIGHLIGHTS

Research background

Chronic hepatitis B (CHB) and primary biliary cholangitis (PBC) are chronic liver diseases; however, CHB combined with PBC is uncommon.

Research motivation

There are few studies on the clinical and pathological characteristics of CHB combined with PBC, and current research is limited to case reports.

Research objectives

To explore the clinicopathological characteristics, diagnoses, and treatments of patients with CHB and PBC.

Research methods

Eleven patients with chronic hepatitis B virus (HBV) infection and PBC who underwent liver biopsy at our hospital between January 2005 and September 2020 were selected. Demographic data, clinical biochemical indicators, autoantibodies, and virological indicators were also collected. The liver pathology was evaluated using the METAVIR and Ludwig staging systems.

Research results

Eleven patients with CHB were diagnosed with PBC, and nine were anti-mitochondrial antibody-M2 positive. Alkaline phosphatase (ALP) increased in five of the 11 cases. ALP levels were normal in six patients, but γ-glutamyl transferase levels were elevated in four patients. Pathological changes were primarily caused by inflammation of the portal area. All cases showed bile duct injury, five cases showed a typical florid duct region, and three cases with severe interfacial inflammation showed similar autoimmune hepatitis-PBC-like injury with few plasma cells. All the patients received antiviral and ursodeoxycholic acid treatment.

Research conclusions

For the first time, the pathological characteristics of rare CHB complicated with PBC were observed in a large sample size. A normal ALP level cannot exclude the diagnosis of PBC. The pathological characteristics were similar to those of PBC-autoimmune hepatitis, but with fewer plasma cells.

Research perspectives

Attention should be paid to the possibility of HBV combined with PBC. Pathology can provide important information, even if biochemical changes or specific antibodies are negative.

FOOTNOTES

Author contributions: Ye Y, Zhang Q, and Lu ZH contributed equally to the research; Tan YW and Ye Y designed the research; Zhang Q and Lu ZH collected and analyzed the data and drafted the manuscript; Tan YW performed the liver pathological evaluations; Tan YW and Ye Y wrote and revised the manuscript; all authors read and approved the final version for publication.

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REVIEW

Current and novel modalities for management of chronic hepatitis B infection

Iman Ibrahim Salama, Samia M Sami, Somaia I Salama, Ghada A Abdel-Latif, Fatma A Shaaban, Walaa A Fouad, Aida M Abdelmohsen, Hala M Raslan

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Abstract

Over 296 million people are estimated to have chronic hepatitis B viral infection (CHB), and it poses unique challenges for elimination. CHB is the result of hepatitis B virus (HBV)-specific immune tolerance and the presence of covalently closed circular DNA as mini chromosome inside the nucleus and the integrated HBV. Serum hepatitis B core-related antigen is the best surrogate marker for intrahepatic covalently closed circular DNA. Functional HBV "cure" is the durable loss of hepatitis B surface antigen (HBsAg), with or without HBsAg seroconversion and undetectable serum HBV DNA after completing a course of treatment. The currently approved therapies are nucleos(t)ide analogues, interferon-alpha, and pegylated-interferon. With these therapies, functional cure can be achieved in < 10% of CHB patients. Any variation to HBV or the host immune system that disrupts the interaction between them can lead to reactivation of HBV. Novel therapies may allow efficient control of CHB. They include direct acting antivirals and immunomodulators. Reduction of the viral antigen load is a crucial factor for success of immune-based therapies. Immunomodulatory therapy may lead to modulation of the host immune system. It may enhance/restore innate immunity against HBV (as toll-like-receptors and cytosolic retinoic acid inducible gene I agonist). Others may induce adaptive immunity as checkpoint inhibitors, therapeutic HBV vaccines including protein (HBsAg/preS and hepatitis B core antigen), monoclonal or bispecific antibodies and genetically engineered T cells to generate chimeric antigen receptor-T or T-cell receptor-T cells and HBV-specific T cells to restore T cell function to efficiently

clear HBV. Combined therapy may successfully overcome immune tolerance and lead to HBV control and cure. Immunotherapeutic approaches carry the risk of overshooting immune responses causing uncontrolled liver damage. The safety of any new curative therapies should be measured in relation to the excellent safety of currently approved nucleos(t)ide analogues. Development of novel antiviral and immune modulatory therapies should be associated with new diagnostic assays used to evaluate the effectiveness or to predict response.

Key Words: Current modalities; Novel modalities; Management; Chronic hepatitis B infection; Direct acting antiviral therapy; Immunotherapy; Therapeutic vaccination

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Core Tip: Chronic hepatitis B virus (HBV) infection is a result of immune tolerance and the presence of covalently closed circular DNA and the integrated HBV. The currently approved therapies are nucleos(t)ide analogues, interferon-alpha, and pegylated-interferon-alpha, with functional cure achieved in < 10% of patients. Disruption of the interaction between HBV or the host immune system can lead to HBV reactivation. Novel therapies include direct acting antivirals and immunomodulators. Immunomodulators may enhance/restore innate and adaptive immunity against HBV (as toll-like-receptors and retinoic acid inducible gene-1 agonist), checkpoint inhibitors, therapeutic HBV vaccines, and genetically engineered T cells to restore T cell function.

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INTRODUCTION

Hepatitis B is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV). It is a major global health problem. It can cause chronic infection and puts people at high risk of death from cirrhosis and liver cancer[1]. In 2019, the World Health Organization (WHO) reported that there were three million people living with new HBV infection and 296 million people living with chronic HBV (CHB) infection. The target of the Sustainable Development Goals and Global Health Sector Strategy in 2020 to decrease the incidence of hepatitis B has been met, as the global prevalence of CHB infection among children aged < 5 years decreased to less than 1% (0.94%) in 2019 compared to 5% in the pre-vaccine era[2]. The prevalence of CHB ranges from < 2% in the United States and Western Europe to ≥ 8% in western Africa[3]. The endemicity of HBV in Asia is heterogeneous, and most of the region has a moderate to high prevalence of HBV infection, except for a few low endemic areas.

The WHO[4] reported that in 2019 approximately 820000 deaths were related to HBV infection, mainly from cirrhosis and hepatocellular carcinoma (HCC). A National Egyptian study reported that among vaccinated children aged 1-16 years, 1535 (42.8%) were identified to have non-seroprotective levels of anti HBs (< 10 IU/L), and CHB prevalence was 0.39%[5,6]. Nearly, 95% of infections occurring during infancy and early childhood progress to CHB[7]. Over 90% of infections occurring during adulthood resolve as a result of developing robust immune responses[8]. In naïve CHB patients, the cumulative incidence of hepatic cirrhosis within 5 years of infection is about 10%-20% among patients with active CHB, and about 2%-5% develop liver cancer annually[9]. Infections during infancy usually carry a greater risk of developing cirrhosis and liver cancer[10].

HBV can be transmitted by sexual exposure, perinatal (mother-to-child transmission), percutaneous, and direct contact with the blood and other body fluids of HBV-infected persons[11]. Acute HBV infection manifestations range from subclinical to icteric to fulminant hepatitis in few cases. Patients who resolved the acute infection develop an efficient B cell immune response displayed by high levels of antibodies (anti-HBs) to surface antigen (HBsAg). They also develop a vigorous T cell response including CD4 and CD8 T cells to produce antiviral cytokines that kill infected hepatocytes. The difference between patients who resolved the acute HBV infection and those with CHB infection is primarily dependent on the magnitude and effectiveness of their immune response. CD8+ T cells are considered responsible for viral clearance during acute recovery but are impaired by continuous exposure to high viral HBsAg and immunosuppression in CHB[12]. The patient immune response directed towards the virus-infected hepatocytes usually leads to hepatocytes damage with an increasing

risk of liver cirrhosis and HCC due to long-term liver inflammation associated with lack of viral clearance[13]. The present review summarized the current and novel diagnostic and therapeutic modalities for HBV infection.

HBV LIFE CYCLE

HBV is a DNA hepatotropic virus[14]. HBV is a double-stranded DNA virus that belongs to the Hepadnaviridae family. It consists of an outer envelope containing HBsAg and a capsid core. The capsid core bears the viral genome and DNA polymerase[15]. Inside the host nucleus, the relaxed circular DNA genome is transformed to a covalently closed circular DNA (cccDNA), which is the stable form of the virus acting as a mini-chromosome. The cccDNA produces a template for generating subgenomic mRNAs and pre-genomic RNA (pgRNA) and uses the host transcription factors for viral synthesis[16]. HBV polymerase catalyzes the pgRNA to synthesize the viral genomic DNA, while the mRNA is translated to various viral proteins as a part of the HBV life cycle[17]. The HBV viral genome encodes RNA transcripts and seven proteins. The DNA polymerase gene is the main part of the virus genome sequence. The S gene includes three parts: pre-s1, pre-s2, and s encoding envelope HBsAg. The C gene encodes both hepatitis B core (HBcAg) and "e" antigens (HBeAg). The HBx gene is encoded in both the polymerase gene and the X region[18,19].

There are three different sized proteins derived from the envelope gene: Small from s (SHB), middle from pre-s2 + s (MHB) , and large from pre-s1 + pre-s2 + s (LHB)[20]. The virus secretes many defective particles as enveloped nucleocapsids that are empty or contain defective immature genomes and subviral lipid particles (SVPs) containing the viral surface antigens. These SVPs are noninfectious smaller particles containing the most plentiful form of 17-25 nm spherical particles of SHB. They also contain filamentous (or tubular) particles of variable length and comprised of SHB, MHB, and LHB proteins[21]. HBsAg proteins can gather to noninfectious SVPs approximately 22 nm in diameter containing the HBV envelope proteins (LHB, MHB, and SHB) and are secreted at a level 1000-fold to 100000-fold higher than the infectious particles[22].

During the chronic phase, HBsAg may lead to dysregulation of innate and adaptive host immunity through interacting with either the immune or non-immune cells causing impairment of the immune system and liver damage. SVPs inhibit antibody responses to the virus[23]. HBsAg could activate the unfolded protein response leading to cellular premalignant changes, *i.e.* it is associated with both hepatic inflammation and HCC[24]. LHB plays a crucial role in the virus envelope and initiates infectivity[25], and the form of the HBV particles appears to be determined by the ratio of the various HBsAg proteins[26]. SHB may have a role in HCC metastasis and progression. Among CHB patients, SHB can be a target for prevention or intervention of HCC progression. A continuous low level of HBsAg in CHB patients is due to failure of the host to eliminate it due to several effects related to the virus, host, and other factors[27]. Moreover, HBeAg and HBx are also major carcinogenesis-related proteins as they are essential to initiate and maintain virus replication. Various cellular events are related to HBx, such as ubiquitination, autophagy, epigenetic modifications, and non-coding RNA regulation. Subsequently, they might contribute to hepatic inflammation, fibrosis, and HCC[28].

After resolution of an acute infection, there is disappearance of serum HBV DNA, appearance of anti-HBc, HBeAg seroconversion to anti-HBe, and finally HBsAg seroconversion to anti-HBs during recovery[11]. Occult HBV infection is defined by the presence of isolated anti-HBc with the absence of HBsAg and anti-HBs[29,30]. HBV DNA is a direct measurement of the viral load. It indicates viral replication activity and disappears at the recovery from HBV infection or gradually diminishes in CHB. High titers of HBV DNA may lead to more quickly to liver cirrhosis and HCC[31]. HBV DNA is immunostimulatory and is recognized by cyclic GMP-AMP synthase and stimulator of interferon genes. Lauterbach-Rivière *et al*[32] found that in infected hepatocytes HBV passively escapes recognition by cellular sensors of nucleic acids by producing non-immunostimulatory RNAs and avoiding sensing of its DNAs by cyclic GMP-AMP synthase/stimulator of interferon genes without active inhibition of the pathway.

NEW HBV BIOMARKERS

Novel biomarkers such as hepatitis B core-related antigen (HBcrAg) and serum HBV RNA are recognized as important markers to monitor the antiviral effects of the emergent therapies. HBcrAg is made up of three related viral proteins sharing an identical 149 amino acid sequence. These include HBcAg, HBeAg, and a truncated 22 kDa pre-core protein[33]. Several Asian and European studies reported a significant positive correlation between serum HBcrAg levels and the amount of intrahepatic cccDNA[34]. The presence of HBcrAg among patients with undetectable HBV DNA indicates continued transcription of cccDNA and can predict clinical relapse and be an aid for clinicians in identifying patients with a higher risk of HCC development[35,36]. HBcrAg can monitor the response to novel therapy targeting cccDNA[37]. The combination of both HBsAg and HBcrAg was found to be an

outstanding biomarker for assessing the risk of HCC[38]. Treated patients with persistently high HBcrAg levels had a higher risk of HCC[39]. In addition, HBcrAg is a good virologic marker differentiating active HBV from inactive HBV in the presence of negative HBeAg[40].

The level of serum HBV RNA, presented as a pgRNA-containing virion, is correlated with intrahepatic pgRNA and cccDNA content, and it signifies high viral replication activities[39,41]. pgRNA is present in serum at lower levels than HBV DNA in treatment-naïve patients. It is enriched during nucleos(t)ide analogue (NA) therapy, which inhibits reverse transcriptase activities by blocking the transcription of pgRNA into HBV DNA. This could explain the presence of HBV RNA in serum after NA therapy, despite the undetectable serum HBV DNA[39,42]. The HBV RNA level was found to be associated with a higher risk of HCC and recurrence in patients treated with NAs[43,44]. However, the way for serum HBV RNA detection and assessment should be standardized before its clinical application.

Yang *et al*[45] found that HBV can encode HBV-related microRNA and named it HBV microRNA 3 (HBV-miR-3). It is used in the monitoring of HBV infection, and it is positively correlated to HBV DNA, HBsAg, and pgRNA. HBV-miR-3 was secreted by HBV-infected hepatocytes and existed in the peripheral blood exosomes of CHB patients. It is positioned at site 373–393 in the HBV genome and can be encoded by three mRNAs, (except for HBx mRNA). HBV-miR-3 and pgRNA are synthesized using cccDNA. Little effect on HBsAg, pgRNA, and HBV-miR-3 was found after antiviral drug treatment[46].

MECHANISMS OF HBV REACTIVATION

The integrated HBV DNA in the host genome can produce viral RNAs and proteins, although it does not produce progeny virus[47]. In the early HBV life cycle, HBV DNA integration occurs at the double stranded breaks throughout the whole host genome. Mutants of HBV genes may be the products of HBV DNA integration, which can stably express mutant HBV proteins. This may lead to HCC and might be a useful biomarker to monitor disease progression from CHB to HCC[48]. HBV reactivation may occur among HBV patients with resolved infection or inactive carrier state and spontaneously or as a complication of immunosuppressive therapy[7]. The basic initial step is the loss of HBV immune control. The Asian Pacific Association for the Study of the Liver (APASL) recommends the need to screen all patients for hepatitis B preceding the initiation of immunosuppressive therapy and to administer protective NAs to those patients with a considerable risk of hepatitis and acute on chronic liver failure due to hepatitis B reactivation[49].

CURRENT THERAPIES FOR CHB

The current available treatments can control viral replication and decrease HCC progression, but these regimens are not curative and may require lifelong therapy[50].

Interferon-alpha and pegylated-interferon-alpha

Interferon-alpha (IFN-α) and pegylated-IFN-α (PEG-IFN-α) act as immune modulators of finite duration. They work through inducing immune-mediated control of HBV infection with long-lasting viral replication suppression after therapy[10,50]. They inhibit viral cell entry, increase host immune response, induce cccDNA degradation, inhibit pgRNA encapsidation, and exert epigenetic modification of cccDNA transcription. Epigenetically they suppress cccDNA transcription and viral protein secretion [51], with a higher success rate shown in PEG-IFN compared to IFN[52]. IFN-α and PEG-IFN-α are the only licensed anti-HBV therapies capable of eliminating cccDNA[53]. PEG-IFN-α-2a and PEG-IFN-α-2b improve the expression of innate antiviral genes and proteins, stimulate natural killer (NK) T and CD8 immune cells, and enhance a non-cytolytic viral clearance through cytokines or cytolysis of the infected cells[54].

They have high HBsAg clearance rates, especially with genotypes A and B. However, the rate of success of IFN therapy is still low with major side effects. The long-term 5-year HBsAg loss post treatment is less than 20%[55]. A study by Wu *et al*[27] demonstrated that after a short-course of PEG-IFN-α-2b re-treatment in patients with HBsAg recurrence a high rate of functional cure could be achieved after therapy withdrawal, which was relatively safe.

Nucleo(t)ide therapy

NAs are nucleos(t)ide reverse transcriptase inhibitors. As an oral therapy targeting viral reverse transcriptase activity, it inhibits viral replication and prevents new HBV DNA formation from pgRNA [56]. However, it has minimal effects on the existing or newly formed cccDNA because cccDNA formation does not depend on the viral reverse transcriptase activity. This allows viral relapse after NA withdrawal[57]. Trials with finite treatment duration have been conducted[58]. There are currently seven approved NAs, mainly tenofovir disoproxil fumarate (TDF), tenofovir alafenamide, and entecavir.

They have high effectiveness in decreasing the risk of cirrhosis and HCC with good tolerability and minimal side effects[10,11]. However, HBV disease progression can still occur even with sustained viral suppression[39]. The probability of HBsAg loss after NA cessation varies according to patient ethnicity, HBV genotype, and viral antigen levels at the end of treatment. Non-Asian patients are more likely to achieve favorable outcomes[59]. Irrespective of ethnicity, lower serum levels of HBcrAg and HBsAg and HBV genotype C are associated with higher rates of viral response, HBsAg loss, and lower rates of alanine aminotransferase (ALT) flares[60].

NAs inhibit viral replication but not viral transcription or translation[61]. The newly developed generation of NAs should be safer and more efficient through novel prodrug methods. ATI-2173 is a noncompetitive non-chain terminating clevudine derivative that can inhibit HBV polymerase[62]. After dosing for 28 d, the mean HBV DNA reduced to 2.8 Log10 IU/mL without any serious adverse events in phase I studies[63]. But during the short dosing interval, no changes were seen in HBsAg levels. Pradefovir, HS-10234, and NCO-48 fumarate are other new NA prodrugs derived from adefovir and tenofovir and increase antiviral potency and reduce metabolite toxicity[64,65]. Similar effectiveness is derived from TDF. Agents targeting the RNase H function of the HBV polymerase are in preclinical development[66].

When HBV DNA was undetectable, NAs had little effect on the HBV-miR-3 levels. PEG-IFN following NA therapy had a positive impact on the decrease of HBV-miR-3, pgRNA, and HBsAg[46].

Patient selection and therapy withdrawal

The three major liver societies [the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver, and APASL] strongly agree that patients with active disease (defined as those with elevated HBV DNA and ALT levels), those with decompensated liver disease, and those with cirrhosis should receive treatment[67]. Table 1 summarized the current therapy regimens for CHB as recommended by AASLD 2023[68].

After seroconversion and consolidation therapy, drug withdrawal may be considered as recommended by guidelines in HBeAg-positive patients[69]. The European Association for the Study of the Liver guidelines in 2017[10] recommended a minimum of 3 years of viral inhibition, while the AASLD guidelines in 2018[70] recommended at least 2 years of combined therapy and viral inhibition. In 2021, APASL guidelines recommended NA withdrawal in HBeAg-positive patients who experienced HBeAg seroconversion and a 3-year consecutive treatment in HBeAg-negative patients who have had undetectable HBV DNA with normalization of ALT[71]. The disagreement of when antiviral therapy should be discontinued in CHB patients is due to the lack of effective methods for assessing cccDNA in the hepatocyte nucleus[72,73]. Moreover, stopping NAs carries a high incidence of virological relapse and surge of ALT levels, with high risk of fibrosis progression, decompensation, and HCC. Over 40% of CHB patients who stopped NAs eventually received re-treatment[71].

NEW TREATMENT MODALITIES

Complete cure is defined as an undetectable HBsAg and HBV DNA in the serum and complete eradication of both the intrahepatic cccDNA and integrated HBV DNA. Functional cure is defined as sustained undetectable levels of both HBsAg and HBV DNA in the serum with or without anti-HBs seroconversion and the persistence of low amounts of intrahepatic cccDNA and HBV DNA integration [74]. Since it is currently not possible to eradicate the cccDNA mini-chromosome or the integrated HBV DNA from the infected cell, functional cure is more attainable with the approved therapies. In addition, to reach high rates of HBsAg loss, there is a crucial need for short duration regimens because current treatments do not restore the immune dysfunction occurring in CHB.

Ongoing efforts are directed towards developing novel anti-HBV drugs that achieve a complete cure. Two main classes of novel therapies are recognized: (1) Agents that directly interfere with the HBV life cycle; and (2) Agents that reinforce the host immune response against HBV infection (immune modulators). The combination of these two novel agents is expected to be more effective. Antiviral immune responses should be restored to allow elimination of both cccDNA and integrated HBV. Figure 1 shows the HBV life cycle and the effects of current and novel therapies. Table 2 shows the new HBV treatment modalities and its clinical trial phases.

Agents that directly target the virus

The direct-acting antiviral (DAA) therapy should be non-toxic; however, off-target effects may inevitably occur. Clinical trials have been carried out on novel NAs targeted to overcome the drawbacks of the currently approved NAs. Besifovir, a prodrug of tenofovir comprised of tenofovir exalidex and tenofovir disoproxilorotate, is currently under investigation[75-78]. The following are the novel DAAs that are currently in experimental or clinical trials.

Table 1 Current therapy regimens for chronic hepatitis B viral infection as recommended by the American Association for the Study of Liver Diseases, 2023[68]

For adult patients	Treatment
Immune-active CHB (HBeAg negative or positive)	Antiviral therapy as PEG-IFN, tenofovir, or entecavir to decrease the risk of liver complications
Immune-tolerant CHB	Against antiviral therapy. Continuous monitoring of ALT levels at least every 6 mo for potential transition to immune-active or inactive CHB
Immune-active CHB HBeAg-negative	Indefinite antiviral therapy, unless there is a strong indication for treatment withdrawal
Compensated cirrhosis with low levels of viremia (< 2000 IU/mL)	Antiviral therapy to reduce the risk of decompensation, regardless of ALT level
Decompensated cirrhosis with positive HBsAg	Indefinite antiviral therapy, irrespective of the level of HBV DNA, ALT, or HBeAg status to decrease risk of worsening the condition
HBeAg-positive CHB without cirrhosis seroconvert to anti-HBe on NA therapy	Discontinue NAs after a period of treatment consolidation
HBeAg-positive CHB with cirrhosis seroconvert to anti-HBe on therapy	Indefinite antiviral therapy unless there is a strong indication for treatment withdrawal
For children (2 to < than 18 years)	**Treatment**
HBeAg positive with elevated ALT and HBV DNA levels	Antiviral therapy (IFN-α, PEG-IFN, and NAs) aiming for achieving sustained HBeAg seroconversion. PEG-IFN-α is recommended for use compared to NAs due to absence of viral resistance and finite duration of treatment
HBeAg-positive with persistently normal ALT, regardless of HBV DNA level	Against antiviral therapy

ALT: Alanine aminotransferase; anti-HBe: Antibody to hepatitis B e antigen; CHB: Chronic hepatitis B viral infection; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; IFN-α: Interferon-alpha; NAs: Nucleos(t)ide analogues; PEG-IFN: Pegylated interferon; PEG-IFN-α: Pegylated-interferon-alpha.

HBV entry inhibitors

HBV enters the hepatocyte through binding irreversibly to the Na^+-taurocholate co-transporting polypeptide (NTCP), which is a receptor exclusively expressed on hepatocytes with high-affinity to HBV and hepatitis D virus (HDV). The myristoylated preS1 domain (myr- $preS1_{2-48}$ lipopeptide) of the LHB is important for this binding complex, triggering viral endocytosis[79]. It is believed that de novo infection through the NTCP receptor is required to maintain the cccDNA pool. Hepcludex (bulevirtid, formerly known as myrcludex B) is a synthetic analogue of the myr-$preS1_{2-48}$ lipopeptide and specifically blocks NTCP HBV/HDV binding. It is in a clinical phase II trial[80].

They found that bulevirtide monotherapy or with PEG-IFN-α resulted in a significant, undetectable level of HDV RNA within 24 wk of treatment. For these results, it was approved for HDV treatment by the European Medicine Agency[81,82]. On the other hand, it was not expected that bulevirtide would provide a sustained decrease of hepatocyte HBV DNA levels, especially when used as monotherapy [83]. The reported adverse effect for this drug was interfering with the physiological function of NTCP (reuptake of circulating bile acids from the portal blood into the liver), leading to the elevation of plasma bile acids[84]. Further studies are required before bulevirtide can be applied for the treatment of HBV infection[85].

Betulin derivatives and cyclosporin derivatives were found to have a promising potent and selective inhibition of NTCP to HBV without affecting its role as a bile acid transporter[86]. Neutralizing antibodies are another promising entry inhibitor directed toward the antigenic S, preS1-domain, cellular heparan sulphate proteoglycans (*e.g.*, poly-Lysin), or attachment inhibitors that bind the virus (*e.g.*, heparin)[80].

Rather than targeting the receptor, several monoclonal/polyclonal antibody preparations are being developed that bind to the N-terminal region of pre-S1, the site of viral interaction with NTCP[87]. In addition to blocking viral entry, monoclonal antibodies can lower viremia and the level of subviral particles and may cross-present viral antigens with stimulation of T cells leading to HBsAg loss[67].

Agents targeting covalently closed circular DNA

In the hepatocyte nucleus, the relaxed circular DNA genome is converted into cccDNA, the mini-chromosome for HBV transcription and replication, resulting in persistence of HBV infection. IFN-α decreases cccDNA transcription *via* epigenetic modifications[88]. Gene therapy including editing, silencing, or epigenetics are currently assessed to modify HBV, most of them aiming to permanently disable cccDNA and lead to HBV infection cure.

Table 2 New hepatitis B virus treatment modalities and clinical trial phases

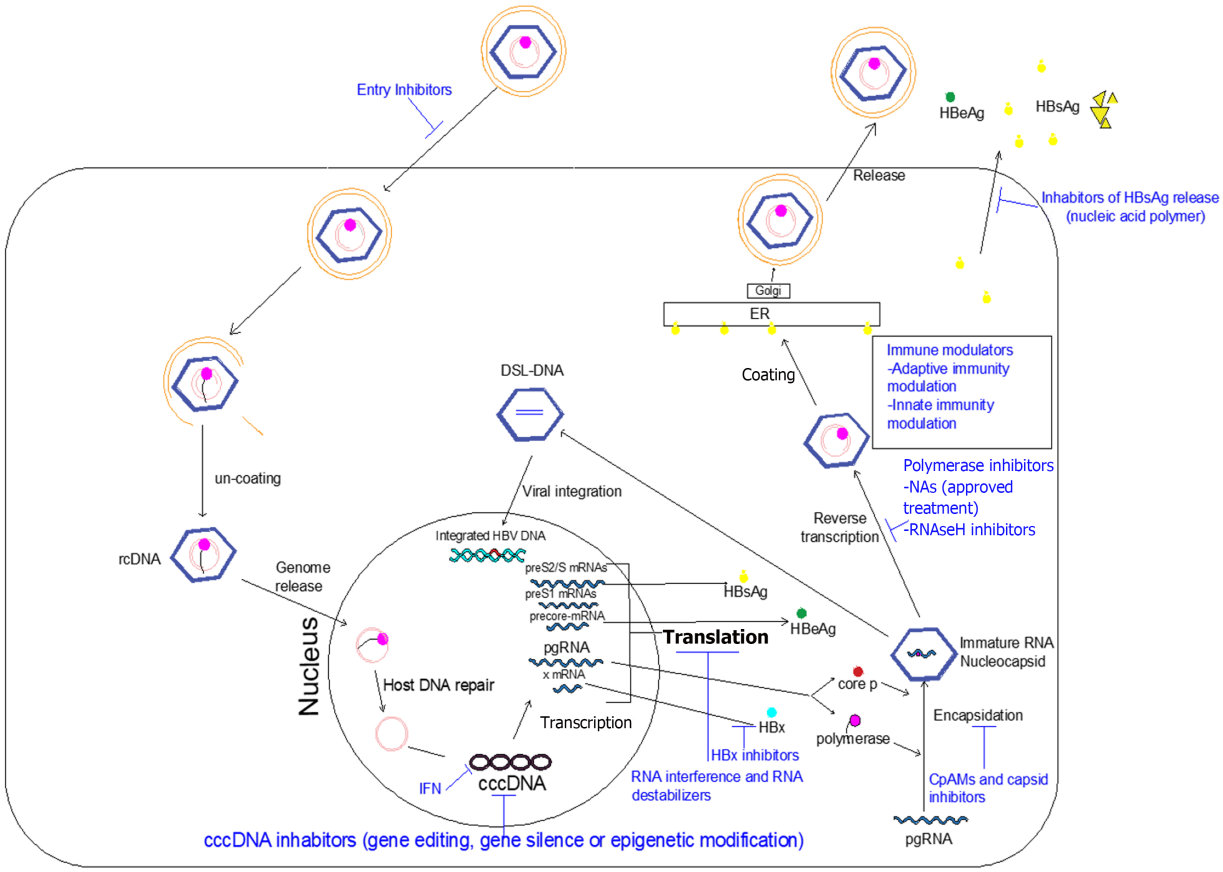
Class	Drug name	Mechanism
HBV entry inhibitors	Hepcludex (Bulevirtid, formerly known as Myrcludex B)	Block HBV binding to NTCP receptor; clinical trial phase II
	Betulin derivatives and cyclosporin derivatives	
	IFN-α, γ	Decreases cccDNA transcription *via* epigenetic modifications
	ZFNs	Site-specific cleavage of DNA creates DSBs to target the viral cccDNA; Pre-clinical phase
Agents targeting cccDNA	TALENs	
	CRISPR and CRISPR-Cas9 as EBT106 HBV CRISPR Cas9 lipid nanoparticle	Target and reduce the viral cccDNA reservoir; destroy the intrahepatic HBV genome and reduce HBsAg levels; reduction of intracellular pgRNA; preclinical phase
	Arginine methyltransferase 5 (PRMT5)	Restriction of HBV transcription and replication through cccDNA transcription suppression and pgRNA encapsidation inhibition
Targeting HBx	SMC5/6 complex	Block all HBV mRNA transcription except HBx mRNA transcription
	Nitazoxanide	Restore SMC5/6 protein levels and block HBV transcription by inhibiting the HBx-DDB1 binding; clinical trial phase II
	CRV-431	Pan-cyclophilin inhibitor that inhibits liver HBV DNA and HBsAg; clinical trial phase I
Agents targeting viral transcripts	ASO: IONIS-HBVRx (GSK3228836) IONIS-HBVLRx (GSK33389404) RG6004 RO7062931	HBV RNA degradation, inhibit the expression of the corresponding gene, and bind viral mRNA to prevent viral protein production; clinical trial phase II
	siRNAs: AB-729, ARB-1467, ARB-1740, Lunar-HB Vir-2218 (also known as ALN HBV02), JNJ-3989 (ARO-HBV), RG6346 (DCR-HBVS), GSK3228836 (IONIS-HBVRx), and Hepbarna (BB-HB-331)	HBV RNA degradation and reduce viremia, antigenemia, core, and HBx protein inside the hepatocyte; clinical trial phase I and II
	asRNA agent	Block HBV translation; clinical trial phase II
Capsid assembly modulators (core protein assembly modulators)	HAPS as Morphothiadin (GLS4)	Core protein binding that inhibits encapsidation of pgRNA or nucleocapsid assembly, to an extent that the HBV pgRNA could not enter inside the capsid resulting in morphologically normal capsids with no nucleic acid content and therefore the virus will be noninfectious; clinical trial phase I and II
	Phenylpropenamides derivatives as 3711, AT-61, and AT-130	
	Sulfamoylbenzamide derivatives as AB-423, AB 506, JNJ-6379, JNJ-0440, NVR 3-778	
	Morphothiadin, JNJ 56136379, and ABI-H0731	Core protein binding led to a significant decrease in HBV DNA, but with smaller reductions of HBV RNA and HBsAg; clinical trial phase II
	RO7049389, ABI-H2158, GLS4JHS, ABI-H0731, ABI-H3733, RG7907, QL-007, EDP-514, CB-HBV-001	Core protein binding; clinical trial phase I and II
Targeting HBsAg	DNA based NAPs (REP-2055 and REP- 2031) or RNA-based NAPs (REP-2139 and REP-2165)	Block HBsAg release and improve the HBV-specific immune response; clinical trial phase II
	NAPs: REP 301, REP 301-LFT and REP 401	Functional cure in the form of undetectable HBV DNA and HBV RNA, decrease HBsAg and HBcrAg, normalize ALT levels, and detect anti-HBsAg; clinical trial phase II
	STOPS	Disrupt HBsAg secretion, ALG-010133; further development of this compound has been discontinued
	mAb against HBsAg, mAb E6F6A, and VIR-3434	Overcome persistent HBV replication; clinical trial phase I and II
	E6F6 immunotherapy	Restore anti-HBV T cell response; clinical trial phase II
	GC1102 mAb and HBIg	Anti-HBs (against HBsAg) and boost humoral immunity; clinical trial phase II
	Apoptosis inducer as APG-1387 and Cyclophilin Inhibitor as CRV 431 (CPI 431-32)	Target host pathways; clinical trial phase II

Immune modulators		
-Therapeutic vaccination	GS-4774	Enhance antiviral cytokine production by HBV-specific T cells as CD8+ cells; clinical trial phase III
	DNA vaccines as INO-1800, HB-110, and JNJ-64300535	Encode HBsAg, HBcAg, and polymerase proteins; clinical trial phase I
	NASVAC, Sci-B-Vac derivative BRII-179, and HepTcell	T cell vaccines; clinical trial phase I and II
	TG1050/T101 and TomegaVax HBV	Non-replicative adenovirus serotype 5 encode three HBV proteins; clinical trial phase II
	ChAdOx1 HBV	Adjuvanted ChAd and MVA vector; clinical trial phase I
	AIC 649	iPPVO nonspecific vaccine; clinical trial phase I
	ABX-203	Contain both HBsAg and HBcAg; clinical trial phase I
-Checkpoint inhibitors	Tim-3, CTLA-4 (anti-CTLA-4), Nivolumab, ASC22 (KN035), and Cemiplimab (REGN2810)	Anti-PD-1/anti-PD-L1 antibodies that restore virus-specific CD8+ T cell responses that boost adaptive immunity; clinical trial phase I and II
-Genetically engineered T cells	cTCR with anti-HBs antibody or HBV specific T cell receptor gene	Recognize HBV-infected cells carrying HBV proteins on their surfaces resulting in disappearance of HBV-infected cells and decreasing cccDNA; Pre-clinical trial
	LTCR-H2-1 and CAR-T cells	LTCR-H2-1 targets TCR gene transfer, which boosts adaptive immunity; Preclinical trial
-Pathogen recognition receptors	Vesatolimod (GS-9620), RO7020531 (RG7854), RO6864018 (RG7795), ANA773, and JNJ-4964 (AL-034/TQ-A3334)	TLR-7 agonists activate intrahepatic dendritic cells, NK cells, and mucosal-associated invariant T cells and trigger the production of type I and II IFNs causing inhibition of HBV replication and boost innate immunity; clinical trial phase I and II
	Selgantolimod (GS-9688)	TLR-8 agonists activate intrahepatic dendritic cells, NK cells, and mucosal-associated invariant T cells and enhance the production of antiviral cytokines (IL-12/IL-18) and boost innate immunity; clinical trial phase II
	STING agonist and RIG-I and NOD2 agonist as Inarigivir (SB9200)	Induce production of IFN-stimulated genes and proinflammatory cytokines that can cytopathically or noncytopathically clear virus, inhibit HBV replication, and boost innate immunity; clinical trial phase II
-Other immune approaches	IMC-I109V	Immune mobilizing monoclonal T cell receptors against the virus; clinical trial phase I

ALT: Alanine aminotransferase; ASO: Antisense oligonucleotides; asRNA: Antisense RNA; CAR: Chimeric antigen receptor; cccDNA: Covalently closed circular DNA; CRISPR: Clustered regularly interspaced short palindromic repeats; CRISPR-Cas9: CRISPR-associated protein nine nuclease; cTCR: Chimeric T-cell receptor; CTLA-4: Cytotoxic T-lymphocyte associated protein 4; PDSBs: Double stranded breaks; HAPS: Heteroaryldihydropyrimidines; HBcAg: Hepatitis B core antigen; HBcrAg: Hepatitis B core-related antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HBx: Hepatitis B virus X protein; IFN-α: Interferon-alpha; IL: Interleukin; iPPVO: Inactivated parapoxvirus; mAb: Monoclonal antibody; NAPs: Nucleic acid polymers; NK: Natural killer; NOD2: Nucleotide-binding oligomerization domain-containing protein 2; NTCP: Sodium taurocholate cotransporting polypeptide; PD-1: Programmed cell death protein 1; PD-L1: Programmed death ligand 1; pgRNA: Pre-genomic RNA; PRMT5: Protein arginine methyltransferase 5; RIG-I: Cytosolic retinoic acid inducible gene I; siRNAs: Small interfering RNAs; SMC5/6: structural maintenance of chromosomes protein 5 and 6; STING: Stimulator of interferon genes; STOPS: S-antigen transport-inhibiting oligonucleotide polymers; TALENs: Transcription activator-like effector nucleases; Tim3: T cell immunoglobulin and mucin containing 3; TLR: Toll-like receptor; ZFNs: Zinc finger nucleases.

Gene editing introduces genome targeted modifications using engineered nucleases for cutting a specific genomic target sequence[89]. They induce double strand breaks (DSBs) at a particular site in the HBV genome resulting in activation of the repair mechanisms of cellular DNA, aiding in producing targeted genome modifications at specific areas[90]. Three classes of nucleases have been developed: zinc finger nucleases (ZFN); transcription activator-like effector nucleases (TALENs); and the clustered regularly interspaced short palindromic repeats (CRISPR) system[91]. They have the ability to make DSBs and target cccDNA. The DSBs are repaired by nonhomologous end-joining pathways, producing insertions and deletions and disrupting the genes open reading frames. Moreover, they lead to degradation of cccDNA[92-94].

ZFNs are designed with zinc finger domains and Fok1 nuclease domain. The zinc finger domains bind to specific sites on DNA, which facilitate the introduction of a DSB into a specific targeted locus. It stimulates gene targeting 100-fold to 10000-fold *via* the activation of DNA repair mechanisms[95,96]. However, ZFN production needs extensive protein engineering, and for every new genome target a new ZFN must be created. Moreover, its production is time-consuming and costly. Therefore, it is not an attractive therapeutic agent[97].



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Figure 1 Hepatitis B virus life cycle and the effects of current and novel therapies. CpAMs: Core protein assembly modulators; cccDNA: Covalently closed circular DNA; DSL-DNA: Double stranded linear DNA; ER: Endoplasmic reticulum; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; IFN: Interferon; NAs: Nucleos(t)ides analogues; pgRNA: Pre-genomic RNA; rcDNA: Relaxed circular DNA.

TALENs are superior to ZFNs, regarding simplicity. Instead of binding to three nucleotides, they only bind to one nucleotide, executing an increased specificity with a decline in the off-target effects. TALENs use a catalytic or functional domain of a nonspecific FokI endonuclease, which can effectively induce the formation of DSBs to the target[98,99]. The DNA binding domains of TALENs are the repeat region of transcriptional activator-like effector protein[100].

CRISPR and CRISPR-associated protein nine nuclease (Cas9) is produced from *Streptococcus pyogenes* [101]. It directly targets and reduces the viral cccDNA reservoir. CRISPR/Cas9 is simply formed of Cas, and it precisely allows gene sequence editing with specific cleavage of cccDNA[75]. Unfortunately, it bears the risk of host genomic rearrangement and damage when targeting the integrated HBV DNA causing DSBs of the host genome[102,103]. The CRISPR/Cas9 approach revealed a potent effect in destroying the HBV genome and reducing HBsAg levels both *in vitro* and in animal models[104]. It is more efficient and more cost-effective than ZFNs and TALENs[62]. However, this strategy has several unwanted effects such as accurate targeting of the DNA site, off-target results on the host genome, mutation introduction, and resistance development towards the Cas9/single guide RNA system[105, 106].

Targeting HBV DNA using HBV-infected HepG2-NTCP cells with CRISPR-Cas9 resulted in cleavage and the subsequent appearance of episomal HBV DNA variants. The effects of Cas9 were sustainable, signifying permanent changes in the HBV genome (not just transient effects owing to transcriptional interference)[107].

A novel technology of the CRISPR system has been developed to allow permanent damage to the HBV genome and finally reach a complete cure. This was accomplished by producing premature stop codons, without causing a DSB of the host genome[108]. Persistent high levels of HBsAg are considered a critical risk factor for the development of HCC. *In vitro* experiments of the CRISPR/Cas9 system showed significant functional inactivation of cccDNA by mutagenesis, inducing suppression of viral replication and potent synergy with NAs[109,110]. Results suggest that additive modulation of host DNA repair pathways enhances the antiviral activity of CRISPR/Cas9 and causes profound reduction of intracellular pgRNA[111]. However, fragments of viral DNA appear to be repaired in transcriptionally active "small cccDNA" formations[110]. The HBV DNA variants were generated after dual pgRNA targeting, highlighting the importance of understanding the fate of cccDNA after gene editing.

It is expected that cccDNA transcription is controlled by epigenetic machinery, mainly through cccDNA-bound histone 3 and histone 4 acetylation. DNA epigenetic modification could silence gene expression. Epigenetic modifications include cccDNA methylation and acetylation. HBV transcription and replication can be effectively restricted by protein arginine methyltransferase 5. HBV replication is limited by protein arginine methyltransferase 5 through cccDNA transcription suppression and pgRNA encapsidation inhibition[111-113]. Epigenetic editor studies are still preliminary for assessing its effect on HBV replication and sustainability[53].

A recent study revealed that non-histone host DNA-binding protein high mobility group box 1 targets the HBV cccDNA minichromosome and induces epigenetic silencing of viral transcription, which could be antagonized by the viral HBx protein[114]. Base editors are alternative gene editing tools that were engineered by fusing partially inactivated Cas nucleases to deaminases. They induce accurate mutations in the targeted sequences *via* deamination reactions, without promoting DSBs. This technique does not require donor DNA templates for gene correction[107]. The key to curing CHB infection is to eliminate or silence cccDNA within the hepatocyte nucleus. Therefore, there are several approaches in preclinical development.

Agents targeting HBx

The structural maintenance of chromosomes protein 5 and 6 (SMC5/6) complex binds to the episomal cccDNA templates. Through this binding, the SMC5/6 can block all HBV mRNA transcription except HBx mRNA transcription. In the cytoplasm, the HBx protein binds to the cullin 4 RING E3 ubiquitin ligase complex *via* its interaction with the cellular damaged DNA binding protein 1 (DDB1) forming the DDB1-CUL4 complex[115,116]. This complex is then transported to the nucleus targets to destroy the cccDNA-bound Smc5/6 complex, allowing cccDNA transcription[117]. In addition, it promotes development of HCC[118]. Accordingly, antiviral agents that interfere with HBx action can also lead to silencing of cccDNA transcription[119]. In cell culture models, nitazoxanide was found to restore SMC5/6 protein levels and block HBV transcription by inhibiting the HBx-DDB1 binding[120]. In a pilot study, nitazoxanide was given to nine treatment-naïve CHB patients for 48 wk. Results revealed undetectable HBV DNA in 89% of patients, and HBsAg disappeared in 33% of patients. HBeAg seroconversion occurred in the 2 HBeAg-positive patients. The drug was well tolerated with mild to moderate transient side effects that resolved during treatment. However, there was no follow-up for the responders[121].

Pevonedistat, a neuronal precursor cell expressed developmentally, downregulated protein 8 activating enzyme inhibitor and dicoumarol, an inhibitor of NAD(P)H quinone oxidoreductase 1, which was shown to reduce HBx expression[122,123], restore SMC5/6 levels, and suppress viral transcription in cultured hepatocytes[124] in a humanized mouse model. However, the major limitation to this approach may be the reactivation of cccDNA as soon as HBx becomes available again[122].

Agents targeting viral transcripts

Viral RNA is the basis of the viral antigens and proteins. Prevention of viral replication and HBsAg production can be achieved through RNA inhibition of viral transcripts translation. This might restore the HBV-specific immune response and potentially lead to a functional cure. RNA interference (RNAi) through small-interfering RNA (siRNA) and anti-sense oligonucleotide (ASO) are new strategies for CHB treatment. RNAi targets post-transcriptional mRNAs and pgRNAs to decrease both HBV antigen production and viral replication. Reducing viral antigens may promote host immune reconstruction against HBV[125]. siRNA and ASO have different mechanisms of gene silencing. The double-stranded siRNA enters the cell cytoplasm, the duplex opens, and the antisense strand binds to the RNA-induced silencing complex (RISC). The antisense-RISC complex recognizes the mRNA. After binding, RISC induces gene silencing and mRNA degradation[126]. RISC is defined as a multiple turnover enzyme; a single siRNA could silence multiple mRNA transcripts after being induced by RISC[127]. However, single-stranded DNA oligomers (ASO) enter the cell and the nucleus, and they can bind to target mRNA alone. It binds to its corresponding site on the target viral RNA forming a DNA-RNA duplex. This allows HBV RNA degradation by ribonuclease H (a family of endonucleases), resulting in inhibition of the corresponding gene expression[128].

Small interfering RNAs (siRNAs) are designed to lower serum HBsAg through targeting specific viral mRNA sequences for all HBV RNA levels, even HBx mRNA[129]. Thus, siRNAs permit lowering viremia, antigenemia, and reducing the core and HBx proteins inside the hepatocyte. Several agents have been developed and reached phase II clinical trials, including the siRNAs VIR-2218 (also known as ALN HBV02), JNJ-3989 (ARO-HBV), RG6346 (DCR-HBVS), and JNJ-3989 (ARO-HBV) and the ASO GSK3228836 (IONIS-HBVRx)[130]. Studies are currently assessing if restoration of SMC5/6 complex induced by siRNA can lead to cccDNA silencing[119,131]. In a phase II study, a single dose siRNA ARC-520 in combination with entecavir resulted in a profound and durable decrease in serum HBV DNA and HBsAg levels[42]. In phase I/II clinical trials, re-designing siRNA to be able to target both integrated and cccDNA viral sequences resulted in an effective decrease in serum HBsAg levels in both HBeAg-positive and HBeAg-negative patients[132].

Several siRNA drugs are in phase II clinical trials. In 98% of the participants, NA co-administration with JNJ-3989 induced a decrease in HBsAg more than 1.0 Log10 IU/mL and a decrease of 1.93 Log10 IU/mL[133]. AB-729, Vir-2218, and RG6346 showed similar reductions in HBsAg titer[134]. An ASO phase II clinical study was conducted for GSK3228836. Each group of treatment-naïve and previously NA-treated received 300 mg six times. The serum HBsAg titer mean was reduced by more than 1.5 Log10 IU/mL[135]. The risk of post-treatment reactivation by the remaining cccDNA, the potential toxicity of the delivery vehicle, and the off-target toxicity are the main topics of interest for these agents.

Capsid assembly modulator inhibitors

The HBV core protein or HBcAg is essential for genome packaging, reverse transcription, and potentially for modulation of cccDNA. The HBc has recently emerged as a promising direct antiviral target that would affect multiple aspects of the viral life cycle. Several small molecules (chemical classes) are named core protein assembly modulators (CpAMs). There are two main classes of CpAMs. Class I are known as heteroaryldihydropyrimidines. They target the core protein and alter the kinetics of nucleocapsid formation resulting in deformed virus particles that would be noninfectious. Class II includes phenylpropenamides and sulfamoylbenzamide derivatives. They speed up capsid assembly to an extent that the HBV pgRNA could not enter inside the capsid resulting in morphologically normal capsids with no nucleic acid content and are therefore noninfectious[136,137]. Several CpAMs are in preclinical and clinical studies including morphothiadin, JNJ 56136379, and ABI-H0731[50]. They led to a significant decrease in HBV DNA but with smaller reductions of HBV RNA and HBsAg[138,139]. Thus, CpAM compounds directly revoke viral replication and post-infection spread[140].

In a preclinical study, adding GS-SBA-1 (a CpAM-E class, which is a potent inhibitor of extracellular HBV DNA *in vitro*) at the time of infection inhibited cccDNA establishment and the downstream viral products (HBsAg, HBeAg, and viral RNA). *In vitro*, GS-SBA-1 avoids creation of extracellular HBV RNA-containing viral particles. It is a potent CpAM that improved viral suppression when combined with an NA[141]. In clinical trials, the combination of NAs and CpAMs might lead to more potent suppression of viral replication, which could decrease the renewal of the cccDNA and inhibit its formation in newly infected cells[142]. However, withdrawal of the CpAM and NAs after short-term administration resulted in minimal changes in HBeAg and HBsAg levels coupled with a high rate of virologic relapse[143].

HBsAg release inhibitor

HBsAg enhances viral entry through binding of the preS1 region to the NTCP receptor. HBsAg is the most abundant HBV antigen, representing about 99.99% of circulating SVPs. This high circulating HBsAg contributes to the suppressive immune environment through interference with the signaling pathways of innate and adaptive immunity, contributing to chronicity of HBV[144]. Inhibition of HBsAg release is a potential step in preventing the release of enveloped HBV and the spread of infection and allowing improvement of the HBV-specific immune response. In addition, it clears circulating HBsAg in patients causing persistence control of HBV infection even after therapy withdrawal[124].

Nucleic acid polymers (NAPs) reduce the release of HBsAg through interrupting the assembly and secretion of HBV subviral particles. They are single-stranded nucleotides. They are either DNA-based NAPs (REP-2031 and REP- 2055) or RNA-based NAPs (REP-2165 and REP-2139). In the REP 102 study by Al-Mahtab *et al*[145], REP-2139-Ca monotherapy was given to 12 Bangladeshi patients; 9/12 patients showed 2.79–7.10 Log reduction of HBsAg. After a combination with PEG-INF-α or thymosin alpha 1 immunotherapy, there was a disappearance of HBsAg. Adverse effects occurred primarily during combination therapy. They were in the form of loss of appetite, dyspepsia, fever, and hair loss.

A phase II clinical trial of REP 2165 and REP 2139 revealed that the combination of either NAP with tenofovir TDF and PEG-IFN-α significantly decreased HBsAg levels in 15/20 patients, with seroconversion occurring in 11/20 patients with CHB in the first 24 wk. However, hepatitis flares were more common among patients receiving NAPs, especially those with undetectable HBsAg, suggesting that host-induced flares are the result of immune control of infection[146]. In clinical trials, a functional cure was developed after receiving REP 301, REP 301-LFT, and REP 401[147]. These effects of NAPs need to be confirmed in larger studies with monitoring the long-term safety and efficacy.

A monoclonal antibody (mAb) against HBsAg, mAb E6F6A, revealed a significant effect in a mouse model to overcome persistent HBV replication[148]. Another study reported restoration of anti-HBV T cell response with E6F6 immunotherapy in mice[149]. Recently, GC1102 mAb against HBsAg was investigated in a phase II clinical trial[80].

Like NAPs, S-antigen transport-inhibiting oligonucleotide polymers (STOPS) are single-stranded oligonucleotides that sequester cellular proteins necessary for HBsAg production and disrupting HBsAg secretion. STOPS were shown to be more potent than NAPs *in vitro*. STOPS could contribute to the functional cure of CHB as it inhibits the production of HBsAg and HBeAg and accelerates degradation of HBsAg. Antiviral compounds that act on cellular proteins will decrease the chance of the appearance of viral resistant mutations[71]. However, these molecules acting on cellular targets may exhibit toxicity. A phase I study evaluating the STOPS agent ALG-010133 demonstrated no reduction of HBsAg. Therefore, its further development has been discontinued[150].

Immune modulators

To overcome acute HBV infection, the adaptive immune responses are the cornerstone for viral resolution through B and T viral specific cell function activation. Failure of the host immune response results in liver damage and CHB[151]. In CHB, the CD8+ T cells show functional impairment in both antigen-specific cytolytic activity and non-cytolytic functions, as the secretion of IFNγ and tumor necrosis factor-alpha are needed for intracellular viral elimination through killing the infected host cell [152]. High HBsAg load also leads to T cell functional exhaustion and apoptosis[153]. NK cells in the blood and liver have additional killing and depletion effects on HBV-specific CD8+ T cells[154]. In CHB, surviving HBV-specific CD8+ T cells are markedly dysfunctional compared to that in acute HBV-resolved patients[155].

Endosomal toll-like receptors (TLR) and cytosolic retinoic acid inducible gene I (RIG-I), melanoma differentiation antigen 5, or cyclic GMP-AMP synthase recognizes intracellular pathogenic nucleic acids leading to cytokine production and transcription of IFN-stimulated genes. HBV is able to evade and suppress the recognition of innate immunity[156]. An innovative immunotherapy approach is targeting functional restoration of T cell[151]. TLR agonists, RIG-1 agonists, checkpoint inhibitors, monoclonal antibodies, genetically engineered T cells, and therapeutic vaccines are being discovered.

Therapeutic vaccination

The concept behind the therapeutic vaccines is the introduction of modified HBV antigens that will interact with antigen-presenting cells. The antigen-presenting cells stimulate HBV-specific T cells to produce antiviral cytokines such as IFN-γ[157]. Preclinical data in HBV mice models revealed good efficacy with therapeutic vaccines. However, in humans the results were unsatisfactory. This may be attributed to the high level of HBsAg in CHB patients, which interferes with the ability of the vaccine to induce HBV-specific T cells. GS-4774, a yeast-based T cell vaccine, was the first HBV therapeutic vaccine studied in HBV-infected humans. It was safe, well-tolerated, and increased cytokine production. However, there was no significant decline in HBsAg by week 48[158,159].

The therapeutic vaccine BRII-179 was evaluated in a phase I/IIb trial. It is a virus-like particle encoding for the three HBsAg proteins. BRII-179 produced a good humoral response in all vaccinated patients, and it increased the frequency of IFN-γ-producing T cells. However, there was no significant decrease in the level of HBsAg, HBV RNA, or HBcrAg[160]. To enhance the effectiveness of therapeutic vaccines, a combination with antiviral approaches that induce a satisfactory immune response are ongoing. This includes therapeutic vaccines with checkpoint inhibitors, TLR or RIG-I agonists, and/or other novel therapy to allow viral antigen clearance[80].

Checkpoint inhibitors

T cells of patients with CHB infection overexpress inhibitory receptors such as cytotoxic T lymphocyte associated antigen 4, programmed cell death protein 1 (PD-1), and T cell immunoglobulin and mucin containing 3. This may affect the T cell function resulting in decreasing the T cell immune response[153, 161-163]. Blocking these checkpoint inhibitors may recover the exhausted T cells and restore their activity. T cell immunoglobulin and mucin containing 3 and cytotoxic T lymphocyte associated protein 4 checkpoint blockade were able to restore virus-specific CD8+ T cell responses in CHB patients[164,165]. An *in vitro* study was carried out on HBV-specific CD4+ T cells from CHB with HBeAg-negative infected persons. There was a functional boost of HBV-specific CD4+ T cells combined with programmed death ligand 1 blockade, leading to a marked increase in interleukin (IL)-21 and IFN-γ production. After blocking IL-10 receptors there was a reverse immune dysfunction in CHB infection [166]. Another *in vitro* study using anti-PD-1/anti-programmed death ligand 1 antibodies revealed only mild restoration of both peripheral and intrahepatic HBV-specific T cells[167]. However, in a preliminary clinical trial only 1 out of 12 patients achieved HBV cure after receiving minimal doses of anti-PD-1 antibodies[168].

Among the non-responder CHB patients, IL-2 therapy was able to significantly increase the frequency and function of HBV-specific CD8+ T cells, which was associated with an improvement in clinical outcomes and HBeAg seroconversion. It was concluded that the sequential IL-2 therapy has an effect on liberating the immune function in refractory CHB patients[169]. Another study revealed that HBV-specific T cells from immune-tolerant HBV-infected patients were liberated by IL-2 treatment, with high levels of IFN-γ, which was similar to immune-active patients. They concluded that a modified IL-2 molecule has the ability to reduce the toxic effects on T cells and are only directed towards CD8+ T cells [170].

However, the use of checkpoint inhibitors in the clinic has been constrained due to the death of hepatocytes causing acute liver failure and risk of autoimmunity[67]. The use of checkpoint blockade in CHB infection may be limited, despite their potential usefulness due to their safety and unpredictable response. Therefore, future safety studies are expected.

Toll-like receptor agonists

Several receptors, defined as pathogen recognition receptors, are required for effective function of the innate immune cells. The pathogen recognition receptors include TLRs, RIG-I-like receptors, formyl-

methionyl-leucyl-phenylalanine, and others[171]. These receptors are the primary sensors of infection, and they represent the essential component for innate immune responses[12]. TLRs are the first line of defense against invading pathogens. They are accountable for detecting self and non-self-antigens, stimulating the maturation of dendritic cells, and initiating antigen-specific adaptive immune responses [172,173].

TLR agonists (TLR-7 and TLR-8) initiate the manufacture of endogenous IFNs, the induction of IFN-stimulated genes, and the activation of other signaling cascades such as the Janus kinase/signal transducer and activator of transcription signaling pathway, leading to HBV replication inhibition[174]. GS-9620, a TLR-7 agonist, increases the T cell and NK cell responses, while it reduces the capability of NK to suppress T cells[175]. GS-9620 achieved a durable suppression of HBV replication in preclinical studies. It allowed the induction of type I IFN[176]. In HBV-infected chimpanzees, oral GS-9620 induced a decrease in circulating levels of HBsAg and HBV DNA in the serum and hepatic cells[177] as well as in woodchucks[176]. In a phase II study, GS-9620 was given once weekly and was safe and well-tolerated in CHB patients who were virally suppressed by oral antiviral treatment. The study revealed a regular dose-dependent pharmacodynamic induction of IFN-stimulated gene mRNA expression. However, HBsAg levels did not significantly decline[178]. It does not produce antiviral effects despite the stimulation of host NK and HBV-specific T cell responses[175].

Adding to its ability for immunomodulation, TLR stimulation was found to directly stimulate T cell function through metabolic regulation[179]. TLRs play an important role in sensing the initial invasion and activation of the innate immune response. In a phase Ia and phase II trial, selgantolimod (GS-9688), an oral TLR-8 agonist, enhanced the production of antiviral cytokines in preclinical studies and was used with no significant decline of HBsAg in virally suppressed patients with CHB and viremic CHB [180,181].

Genetically engineered T cells

In order to strengthen HBV-specific T cell responses (overcome HBV-specific T cell exhaustion), an *in vitro* genetically engineered T cell carrying a chimeric T cell receptor containing anti-HBs-specific antibody or an HBV-specific T cell receptor gene transferred through a vector have been developed. In animal models and *in vitro* studies these engineered T cells were able to recognize HBV-infected cells, carrying HBV proteins on their surfaces resulting in disappearance of HBV-infected cells and decreasing cccDNA[182,183]. They induced HBV infection clearance through cytokine secretion and cytotoxicity [184,185]. It has the advantage that the T-cell receptor (TCR)-T cells are not suppressed by the high levels of soluble antigens in the serum of CHB patients, as they are not recognized by them[186].

However, there are some concerns about the safety of this technology, as it carries the risk of going beyond immune control resulting in liver damage, and this limited its clinical use. Therefore, the addition of potent antiviral treatments to selective immune modulation may be a good strategy inducing functional cure for HBV, without causing severe liver damage progression[187]. TCR-reprogrammed non-lytic T cells have been developed to avoid the risk of severe liver damage. It can produce low amounts of perforin and granzyme B. However, in HBV-infected hepatocytes it is able to decrease viral infection by activation of the anti-viral cytidine deaminases APOBEC3[187,188]. Despite several studies currently addressing engineered T cell therapy challenges, its clinical application is still limited as it is technically difficult.

iC9 and HSV-TK were assessed as safety systems in the context of adoptive T cell transfer for the treatment of persistent CHB[189]. *In vitro* T cell cytotoxicity and cytokine production were abruptly stopped after HBV-specific TCR and S-chimeric antigen receptor T cells were infected with iC9 or HSV-TK. *In vivo* S-chimeric antigen receptor T cell induction of iC9 resulted in a rapid and significant decrease of transplanted T cells and prevented cytokine release and liver damage. However, it resulted in a decrease of antiviral effectiveness.

Preliminary evidence in an animal model demonstrated the reduction of HBsAg and HBV DNA levels without inducing significant liver damage. To prove the safety and efficacy of using genetically engineered T cells, an HBsAg-specific TCR or adoptive transfer of autologous T cells expressing HBV-specific TCR were demonstrated in patients with HCC caused by HBV[190,191]. To better understand the safety and efficacy of these novel approaches, results from clinical studies in non-HCC patients are necessary.

COMBINATION THERAPY

In theory, triple combinations of the three categories of therapy (reduction of viral antigen load, inhibition of viral replication, and immune stimulation) might be considered the ideal combination. To inhibit transcription and replication and boost immunity, vonafexor is a new agent. It is a farnesoid X receptor agonist that reduces HBV transcription. In a phase II open label study, HBeAg-negative patients with CHB were treated with TDF for 24 wk and randomly allocated to receive 24 wk of a NAP (REP 2139 or REP 2165) plus TDF and PEG-IFN or PEG-IFN plus TDF to reduce antigen burden, inhibit replication, and boost immunity[146].

The data available recommend combination therapy to achieve functional cure in CHB[192]. The best clinical evidence of the benefits of the alternative therapy is the combination of NAs and PEG-IFN, which were examined in a meta-analysis. Initial combination therapy *vs* initial NA monotherapy showed a nonsignificant increased relative risk of HBsAg loss of 1.44. While PEG-IFN add-on *vs* NA monotherapy showed a significantly improved relative risk of 4.52[193].

A recent study evaluated the combination of siRNA (JNJ-3989) with or without a CpAM (JNJ-6379) plus NA. It revealed that the triple regimen (siRNA plus CpAM plus NA) had the lowest rate of response compared with siRNA plus NA only[194]. This raises the possibility of an interaction between the CpAM and siRNA. Therefore, not all combinations will result in synergy.

Kuipery *et al*[195] treated infected HepG2-NTCP with TDF, TLR 7/8 agonists, or RNAi using siRNAs. They found that TDF and TLR7/8 agonists had no impact on T cell recognition. The administration of siHBVs, which inhibit viral replication and antigen expression, strongly suppressed HBV-specific CD8 T cell recognition. Similarly, we demonstrated that siHBV-mediated antigen reduction could negate the ability of TLR conditioned media to enhance antigen presentation to HBV-specific CD8 T cells. Understanding how CD8 T cell recognition is altered by these drug classes will help inform logical combination strategies for hepatitis B treatments. Immunomodulation and RNAi, but not NAs, alter the recognition of infected HepG2-NTCP by HBV-specific CD8 T cells. This could present a significant obstacle for combining immunotherapies and require careful immunological follow-up post-RNAi treatment.

ASSESSMENT OF SAFETY AND INDICATIONS FOR STOPPING TRIALS OF NEW HBV THERAPIES

The safety of new HBV therapies should always be compared with the well-proven safety of NAs. Increased additional risk should be weighed *vs* the predictable clinical outcomes. ALT flares are a major concern in HBV drug application, which may be induced either in relation to host immune responses or antiviral or drug-induced flares. Those related to the host immune response are associated with a decrease in serum HBV DNA and viral antigen levels. Virus-induced flares may be related to increased viral activity, lack of drug efficacy or resistance, or the side effects of antiviral agents[196]. Development of ALT flares necessitate close patient monitoring to identify the type of flare, whether to stop the antiviral, and whether it can be resumed. Even after ceasing treatment, close monitoring is mandatory, as flares may also occur due to viral reactivation, immune recovery, or delayed liver toxicity.

WORLDWIDE ELIMINATION OF HBV

Hepatitis B elimination is achievable but needs greater financial support. It will not be attained without finding and treating all HBV-infected people. In developing countries, many people are unaware of their infection, and few are receiving antiviral therapy[197,198]. The goals are to reduce new infections by 90% by 2030, with a prevalence in children of no more than 0.1%, by improving the delivery of a birth dose of vaccine, which will increase from 39% in 2015 to 90% globally. In addition, all pregnant women will be tested for HBV, and new antiviral treatment-based interventions will be developed[199]. The vaccine is highly cost–effective, safe, and available as a combination with other vaccines in the Expanded Program on Immunization. Universal hepatitis B vaccination should be undertaken in all countries regardless of HBV endemicity. The hepatitis B vaccine is the first "anti-cancer" vaccine, as it prevents hepatitis B, the leading cause of HCC[200]. The introduction of HBV immunization in 189 countries by the end of 2018 resulted in an estimated 84% of the world's population receiving three doses of the HBV vaccine. Nonetheless, the estimates are still insufficient (76%) in Africa, and just 11% of newborn babies in Africa received the HBV birth dosage, which is still a very low coverage rate (38%) internationally[199].

In Egypt, birth dose has been nationally added to the HBV vaccination program at the beginning of 2017[6]. After vaccination, anti-HBs levels decline over time[201]. Immunocompetent people who achieve an anti-HBs level ≥ 10 mIU/mL after receiving three doses of the hepatitis B vaccine remain protected, even if anti-HBs levels decline to < 10 mIU/mL due to persistent memory cells. Booster doses are not recommended for people with normal immune status who have been vaccinated[202-204]. Revaccination may be recommended for non-responders and certain high-risk populations[5,205].

Intense viral deactivation alone does not attain HBsAg reduction or loss. Combining NAs and immunotherapy reduces HBsAg levels and induces HBsAg loss in some patients, particularly those with low baseline HBsAg levels. Agents that are specifically prepared to decrease viral antigen load could not achieve constant HBsAg loss when used alone. Thus, the use of combinations of all three therapy types is recommended[142]. Moreover, combination therapy with the current anti-HBV therapy would be more effective in causing a drop in HBsAg levels and the eventual curing of HBV. To evaluate the synergistic effect of anti-HBV drugs and eradicate this global health issue, combination therapy with

CpAM and entry inhibitors, siRNA, immunomodulators, or therapeutic vaccines to rebuild the immune system may be used[140].

CONCLUSION

HBV persistence can occur in an overt state with the presence of serum HBsAg due to induction and maintenance of a defective immune response, especially an adaptive immune response resulting from the interaction between viral and host factors. It can also persist in a long-term occult state with the absence of serum HBsAg, owing to the long life of the HBV genome, including cccDNA and integrated DNA within the nucleus of hepatocytes. Nine medications are now licensed for the treatment of CHB, including two IFN conventional and PEG-IFN formulations as well as seven NAs (lamivudine, telbivudine, adefovir, entecavir, TDF, tenofovir alafenamide +, and besifovir dipivoxil) (only in Korea). The therapeutic vaccines that have been studied to date are largely ineffective at significantly and permanently suppressing HBV in animal models or CHB patients, but they are quite successful at priming particular T and B cell responses to HBV antigens.

Although a functioning T cell response is necessary for the management of HBV, it is unquestionably insufficient for an effective immunotherapeutic strategy. Gene therapy including editing, silencing, and epigenetic alterations have been assessed to modify HBV. Most of them act through direct deactivation of cccDNA and HBV infection cure. Monotherapy of entry inhibitors is not expected to induce complete elimination of cccDNA due to a slow rate of hepatocyte turnover and cccDNA decay. In order to effectively treat CHB, a combination of NA-based antiviral therapy, intrahepatic innate immunity activation, stimulation of particular T cell responses, and activation of non-immune mechanisms for sustained HBV control without unfavorable side effects may be required. The combined use of potent antiviral treatments and selective immune modulation may be the best strategy to accomplish a functional cure for HBV, without inducing severe tissue damage and disease progression. To reach the goal of global elimination of HBV by 2030, the WHO published certain measures that include full childhood vaccination, preventing perinatal transmission, harm reduction, blood safety, and testing and treatment. Unfortunately, most countries are not on track to meet these targets by 2030.

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REVIEW

Noninvasive biomarkers in pediatric nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease worldwide among children and adolescents. It encompasses a spectrum of disease, from its mildest form of isolated steatosis, to nonalcoholic steatohepatitis (NASH) to liver fibrosis and cirrhosis, or end-stage liver disease. The early diagnosis of pediatric NAFLD is crucial in preventing disease progression and in improving outcomes. Currently, liver biopsy is the gold standard for diagnosing NAFLD. However, given its invasive nature, there has been significant interest in developing noninvasive methods that can be used as accurate alternatives. Here, we review noninvasive biomarkers in pediatric NAFLD, focusing primarily on the diagnostic accuracy of various biomarkers as measured by their area under the receiver operating characteristic, sensitivity, and specificity. We examine two major approaches to noninvasive biomarkers in children with NAFLD. First, the biological approach that quantifies serological biomarkers. This includes the study of individual circulating molecules as biomarkers as well as the use of composite algorithms derived from combinations of biomarkers. The second is a more physical approach that examines data measured through imaging techniques as noninvasive biomarkers for pediatric NAFLD. Each of these approaches was applied to children with NAFLD, NASH, and NAFLD with fibrosis. Finally, we suggest possible areas for future research based on current gaps in knowledge.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Steatosis; Fibrosis; Serological; Imaging

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Core Tip: Nonalcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease in children and adolescents worldwide. Early diagnosis is essential and currently, liver biopsy is the gold standard for diagnosis and staging. However, noninvasive serological biomarkers, composite scores, and imaging biomarkers are being extensively studied for the diagnosis of NAFLD, nonalcoholic steatohepatitis, and liver fibrosis in children. This work reviews recent research on noninvasive biomarkers in pediatric NAFLD, identifies circulating biomarkers and imaging techniques that show the most promise, and suggests topics for future research.

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INTRODUCTION

Pediatric nonalcoholic fatty liver disease (NAFLD) is a spectrum of disease that ranges from isolated steatosis, or nonalcoholic fatty liver (NAFL), to its more severe form nonalcoholic steatohepatitis (NASH) (characterized by ≥ 5% hepatic fat infiltration with inflammation and/or hepatocellular ballooning), to fibrosis and even cirrhosis, or end-stage liver disease. It is the most common cause of chronic liver disease in children[1] and has an estimated global prevalence of 5%–10%[2]. In the United States, NAFLD was found to have a prevalence of 9.6% in children[3]. This percentage increases markedly in pediatric patients with other metabolic conditions including overweight, obesity, type 2 diabetes mellitus and/or dyslipidemia, with a prevalence of up to 50%-80%[4,5].

The early identification and management of pediatric NAFLD is crucial in the prevention of disease progression. Alanine aminotransferase (ALT) levels (with upper limit of normal 26 U/L for boys and 22 U/L for girls) are typically used to screen for NAFLD in patients with risk factors (including overweight/obesity, prediabetes/diabetes, features of metabolic syndrome, positive family history of NAFLD) starting at 9-11 years of age[6]. One study found that using an ALT threshold of twice the upper limit of normal (≥ 50 U/L for boys and ≥ 44 U/L for girls) had a sensitivity of 88% and specificity of 26% for the diagnosis of NAFLD in overweight and obese children[7]. In adults, a two-step approach is often used for screening before considering a liver biopsy. This typically involves using a predictive scoring algorithm (*e.g.* Fibrosis-4 (FIB-4) index), which – if elevated – is followed by subsequent imaging (*e.g.* elastography) to screen for NASH or fibrosis[8,9].

Currently, liver biopsy is considered the most accurate method for diagnosing pediatric NAFLD[6]. The Pathology Committee of the NASH Clinical Research Network (CRN) has developed and validated a scoring system that is widely used to assess the severity of NAFLD. This system includes 14 histological features, four of which are evaluated semi-quantitatively: steatosis (0-3), lobular inflammation (0-3), hepatocellular ballooning (0-2) and fibrosis (0-4). The former 3 features are components of the NAFLD activity score (NAS). A NAS of 5 or higher is indicative of NASH, while scores below 3 suggest simple steatosis, or NAFL[10]. Despite its accuracy, given the invasive nature of liver biopsy, associated sampling error, and high cost, there is a significant need for noninvasive techniques to diagnose pediatric NAFLD, NASH, and fibrosis.

NASH is a more advanced and active form of NAFLD and is characterized by the presence of lobular inflammation and hepatocellular ballooning[11]. Individuals with NASH are at an increased risk of liver fibrosis, with progressive scarring that can lead to cirrhosis. Adult studies have demonstrated that liver fibrosis is the most important histologic feature in determining transplant-free survival in adults with NAFLD[12].

This review summarizes the recent research on noninvasive serological biomarkers (serology-based noninvasive tests, NITs), composite scoring algorithms, and imaging biomarkers (imaging-based NITs) used in the diagnosis of pediatric NAFLD, NASH, and NAFLD with fibrosis.

SEROLOGICAL BIOMARKERS / SEROLOGY-BASED NONINVASIVE TESTS

NAFLD

Interleukins: Several studies have explored interleukins as potential noninvasive biomarkers for the diagnosis of NAFLD in children. Interleukin-1β (IL-1β) and IL-6 are secreted by various tissue types, but most abundantly by adipose tissue[13] (Table 1). IL-1β has been implicated in hepatocyte injury and the worsening of NASH[14] while IL-6 is involved in insulin signaling, the synthesis of acute phase proteins, and in regulating chronic inflammation[15]. IL-17 is produced primarily by T helper 17 (Th17)

Table 1 Cellular location(s) of synthesis of circulating biomarkers in nonalcoholic fatty liver disease

Serological biomarker	Cellular location(s) of synthesis
Adiponectin	Adipocytes
Adipo R2	Hepatocytes, skeletal muscle
ALT	Hepatocytes
Ang-2	Liver sinusoidal endothelial cells
CatD	Lysosomes
Chemerin	Adipocytes
CK18	Hepatocytes
FGF21	Hepatocytes
HA	Hepatic stellate cells
IL-1β	Adipocytes
IL-6	Adipocytes
IL-17	T helper 17 cells
IL-18	Macrophages
Leptin	Adipocytes
PIIINP	Released during procollagen processing
PRO-C3	Extracellular matrix
RBP4	Adipocytes, Hepatocytes
Resistin	Adipocytes
Visfatin	Adipocytes, Hepatocytes

IL: Interleukin; Adipo R2: Soluble adiponectin receptor 2; FGF: Fibroblast growth factor; RBP4: Retinol binding protein 4; ALT: Alanine aminotransferase; Ang-2: Angiopoietin-2; CK18: Cytokeratin 18; CatD: Cathepsin D; HA: Hyaluronic acid; PRO-C3: N-terminal type III collagen propeptide; PIIINP: Amino-terminal propeptide of type III procollagen. Serological biomarkers are listed in the order they appear in the text.

cells[16] and functions by linking T cell activation to neutrophil mobilization and activation. IL-17 has been shown to promote the progression of NASH and fibrosis in animal models[17]. A recent study found that levels of IL-1β, IL-6, and IL-17 were significantly elevated in obese children with NAFLD diagnosed through ultrasound compared to obese controls without NAFLD. These three biomarkers also had excellent diagnostic ability in distinguishing children with obesity and NAFLD from children with obesity without NAFLD. IL-1β had an area under the receiver operating characteristic (AUROC) of 0.94 (cutoff 11.74 pg/mL, sensitivity 84.6%, specificity 85.2%), IL-6 had an AUROC of 0.94 (cutoff 8.10 pg/mL, sensitivity 91.2%, specificity 80.1%), and IL-17 had an AUROC of 0.97 (cutoff 40.03 pg/mL, sensitivity 89.0%, specificity 93.8%)[18] (Table 2).

Flisiak-Jackiewicz *et al*[19] also evaluated IL-18 as a biomarker of liver steatosis in a cohort of 72 obese children with NAFLD diagnosed through magnetic spectroscopy (MRS). IL-18 is a proinflammatory cytokine associated with metabolic syndrome and hepatocyte injury. It is secreted primarily by macrophages, but also by endothelial cells, vascular smooth muscle cells, Kupffer cells, and adipocytes [20,21]. IL-18 was found to be significantly elevated in obese children with NAFLD compared to controls and had an AUROC of 0.68 and a positive predictive value (PPV) of 90% in differentiating between children with or without fatty liver in MRS[19] (cutoff 326.8 pg/mL, sensitivity 60%, specificity 75%). These findings suggest that Interleukins may be promising serology-based NITs in identifying the presence of NAFLD in children with comorbid obesity.

Adiponectin: Adiponectin is an adipokine with anti-inflammatory and insulin-sensitizing properties. In the liver, adiponectin triggers the peroxisome proliferator–activated receptor-α (PPAR-α) signaling pathway, leading to increased free fatty acid oxidation and reduced gluconeogenesis, giving it antisteatotic properties[22]. Studies in adults show that patients with NAFLD have lower levels of adiponectin compared to healthy controls, and that these levels are inversely associated with the degree of hepatic steatosis[23]. Multiple studies have found this correlation to be true in children with NAFLD as well. Boyraz *et al*[24] assessed adiponectin levels in 148 obese children, 63 of whom had liver steatosis diagnosed through ultrasound. The study found lower serum adiponectin levels in obese children with liver steatosis compared to obese controls. Adiponectin was able to differentiate children with advanced

Table 2 Serological biomarkers for the detection of nonalcoholic fatty liver disease

Ref.	Marker	Country	Categories Tested	Sample size (*n*)	Dx	Cutoff	AUROC (95%CI)	Sens (%)	Spec (%)	PPV (%)	NPV (%)	*P* value
Boyraz *et al*[24], 2013	**Adipo-nectin [μg/mL]**	Turkey	NAFLD *vs* no-NAFLD	Obese with NAFLD (*n* = 63), obese non-NAFLD (*n* = 85)	US	3.2	0.948 (0.924-0.972)	100	83.5			< 0.001
Boyraz *et al*[24], 2013	**Adipo-nectin [μg/mL]**	Turkey	Grade 3 *vs* Grade 1-2 steatosis	Obese with NAFLD (*n* = 63), obese non-NAFLD (*n* = 85)	US	2.56	0.809 (0.751-0.867)	84.2	63.6			< 0.001
Mohamed *et al*[25], 2017	**Adipo-nectin [μg/mL]**	Egypt	NAFLD *vs* no-NAFLD	NAFLD (*n* = 101), non-NAFLD controls (*n* = 57)	Biopsy	2.4	0.9213	74.3	96.5			< 0.001
Flisiak-Jackiewicz *et al*[19], 2018	**ALT**	Poland	NAFLD *vs* no-NAFLD	Obese with steatosis (*n* = 72), obese without steatosis (*n* = 20)	MRS		0.668 (0.514-0.822)					0.0325
Flisiak-Jackiewicz *et al*[19], 2018	**AST**	Poland	NAFLD *vs* no-NAFLD	Obese with steatosis (*n* = 72), obese without steatosis (*n* = 20)	MRS		0.683 (0.532-0.834)					0.0173
Mohamed *et al*[25], 2017	**Chemerin [ng/mL]**	Egypt	NAFLD *vs* no-NAFLD	NAFLD (*n* = 101), non-NAFLD controls (*n* = 57)	Biopsy	186.7	0.7836	56.4	87.7	88.9	52.6	< 0.001
Kłusek-Oksiuta *et al*[46], 2014	**Chemerin [ng/mL]**	Poland	NAFLD *vs* no-NAFLD	Steatosis (*n* = 33 *via* MRS)	MRS	190	0.7	75	58			0.04
Hua *et al*[38], 2019	**FGF-21 [pg/mL]**	Taiwan	Predicting high grade steatosis	Obese (*n* = 31), obese with liver steatosis (*n* = 83), controls (*n* = 89)	US	106.1	0.781 (0.687–0.874)	86.5	60			< 0.001
Hua *et al*[38], 2019	**FGF-21 + GGT**	Taiwan	Predicting high grade steatosis	Obese (*n* = 31), obese with liver steatosis (*n* = 83), controls (*n* = 89)	US	3.318	0.861 (0.786–0.937)	89.2	74.6			< 0.001
Hua *et al*[38], 2019	**FGF-21 + GGT + TG**	Taiwan	Predicting high grade steatosis	Obese (*n* = 31), obese with liver steatosis (*n* = 83), controls (*n* = 89)	US	5.403	0.871 (0.801–0.942)	83.8	82.5			< 0.001
Hua *et al*[38], 2019	**FGF-21 + GGT + TG**	Taiwan	Predicting high grade steatosis	Obese (*n* = 31), obese with liver steatosis (*n* = 83), controls (*n* = 89)	US	6.661	0.873 (0.801–0.945)	94.6	72.9			< 0.001
Flisiak-Jackiewicz *et al*[19], 2018	**GGT**	Poland	NAFLD vs. no-NAFLD	Obese with steatosis (*n* = 72), obese without steatosis (*n* = 20)	MRS		0.677 (0.521-0.832)					0.0257
Hua *et al*[38], 2019	**GGT [U/L]**	Taiwan	Predicting high grade steatosis	Obese (*n* = 31), obese with liver steatosis (*n* = 83), controls (*n* = 89)	US	21.5	0.840 (0.765–0.915)	82.5	70.5			< 0.001
Duan *et al*[18], 2022	**IL-17 [pg/mL]**	China	Obese with NAFLD *vs* obese	Obese with NAFLD (*n* = 176), obese non-NAFLD (*n* = 91)	US	40.03	0.97 (0.96-0.99)	89	93.8			< 0.001
Flisiak-Jackiewicz *et al*[19], 2018	**IL-18 [pg/mL]**	Poland	NAFLD *vs* no-NAFLD	Obese with steatosis (*n* = 72), obese without steatosis (*n* = 20)	MRS	326.8	0.680 (0.552-0.808)	60	75	34	60	0.0058

Flisiak-Jackiewicz *et al*[19], 2018	**IL18 + ALT + AST + GGT + TG**	Poland	NAFLD *vs* no-NAFLD	Obese with steatosis (n = 72), obese without steatosis (n = 20)	MRS		0.782 (0.678-0.887)	61	85	94	38	< 0.001
Duan *et al*[18], 2022	**IL-1β [pg/mL]**	China	Obese with NAFLD *vs* obese	Obese with NAFLD (n = 176), obese non-NAFLD (n = 91)	US	11.74	0.94 (0.91-0.97)	84.6	85.2			< 0.001
Duan *et al*[18], 2022	**IL-6 [pg/mL]**	China	Obese with NAFLD *vs* obese	Obese with NAFLD (n = 176), obese non-NAFLD (n = 91)	US	8.1	0.94 (0.91-0.96)	91.2	80.1			< 0.001
Boyraz *et al*[24], 2013	**RBP4 [μg/mL]**	Turkey	NAFLD *vs* no-NAFLD	Obese with NAFLD (n = 63), obese non-NAFLD (n = 85)	US	26	0.974 (0.960-0.988)	100	92.9			< 0.001
Boyraz *et al*[24], 2013	**RBP4 [μg/mL]**	Turkey	Grade 3 *vs* Grade 1-2 steatosis	Obese with NAFLD (n = 63), obese non-NAFLD (n = 85)	US	35	0.782 (0.726-0.838)	84.2	68.2			< 0.001
Boyraz *et al*[24], 2013	**Resistin [ng/mL]**	Turkey	NAFLD *vs* no-NAFLD	Obese with NAFLD (n = 63), obese non-NAFLD (n = 85)	US	12	0.884 (0.849-0.919)	100	77.7			< 0.001
Boyraz *et al*[24], 2013	**Resistin [ng/mL]**	Turkey	Grade 3 *vs* Grade 1-2 steatosis	Obese with NAFLD (n = 63), obese non-NAFLD (n = 85)	US	5.2	0.661 (0.586-0.736)	36.8	95.5			< 0.05
Flisiak-Jackiewicz *et al*[19], 2018	**TG**	Poland	NAFLD *vs* no-NAFLD	Obese with steatosis (n = 72), obese without steatosis (n = 20)	MRS		0.694 (0.574-0.815)					0.0015
Hua *et al*[38], 2019	**TG [mg/dL]**	Taiwan	Predicting high grade steatosis	Obese (n = 31), obese with liver steatosis (n = 83), controls (n = 89)	US	77	0.732 (0.639–0.824)	90.2	50			< 0.001
Elkabany *et al*[47], 2020	**Visfatin [ng/mL]**	Egypt	NAFLD *vs* no-NAFLD	Obese with NAFLD (n = 31), obese (n = 49), nonobese controls (n = 40)	US	18		83.9	81.4			

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AUROC: Area under the receiving operating characteristic; CI: Confidence interval; Dx: Diagnosis; IL, Interleukin; FGF: Fibroblast growth factor; GGT: Gamma-glutamyl transferase; MRS: Magnetic spectroscopy; NAFLD: Nonalcoholic fatty liver disease; NPV: Negative predictive value; PPV: Positive predictive value; RBP4: Retinol binding protein 4; Sens: Sensitivity; Spec: Specificity; TG: Triglycerides; US: Ultrasound.

liver steatosis from those with mild-moderate steatosis with an AUROC of 0.81 (cutoff 2.56 μg/mL, sensitivity 84.21%, specificity 63.64%). In addition, adiponectin was able to differentiate between the presence and absence of NAFLD in obese children with an AUROC of 0.95 (cutoff 3.2 μg/mL, sensitivity 100%, specificity 83.53%). Similarly, in a study of 101 obese children with biopsy-proven NAFLD, Mohamed *et al*[25] showed that adiponectin was able to discriminate between NAFLD patients and healthy controls with an AUROC of 0.92 (cutoff value 2.4 μg/mL, sensitivity 74.26%, specificity 96.49%). These studies suggest that adiponectin may be utilized in identifying NAFLD in children with and without obesity.

Soluble adiponectin receptor 2: While adiponectin receptor 2 (Adipo R2) has been studied in children with NAFLD, there are currently no studies evaluating its diagnostic ability in children with NAFLD. Adipo R2 is abundantly expressed in the liver and skeletal muscle and upon binding adiponectin, mediates fatty acid oxidation and glucose metabolism[26,27]. Aksoy *et al*[28] found in a cross-sectional

study of 51 obese/overweight children diagnosed with NAFLD through ultrasound that Adipo R2 levels were higher in obese children with NAFLD as opposed to obese controls. While adiponectin levels were similar in patients with and without NAFLD, this entire cohort of children had adiponectin levels below normal. The authors posit that this lower adiponectin level may have driven a compensatory increase in Adipo R2 expression. Studies have established that decreased hepatic Adipo R2 expression can lead to adiponectin resistance, which can subsequently contribute to NAFLD progression given adiponectin's antisteatotic properties. This is the basis behind the study of adiponectin receptor-sensitizing medications, such as thiazolidinediones, in patients with NASH[29]. Further research is needed to validate Adipo R2 as a clinically feasible diagnostic marker of pediatric NAFLD.

Fibroblast growth factor 21: Fibroblast growth factor 21 (FGF21) is a cytokine secreted primarily by hepatocytes, and to a lesser extent by pancreatic, testicular, duodenal, and adipose tissue[30,31]. Liver FGF21 regulates lipid metabolism by promoting lipolysis and reduces hepatic lipid accumulation in an insulin-dependent fashion. Multiple adult studies have found a positive correlation between hepatic steatosis and serum FGF21 levels[32,33]. However, studies in children have been less conclusive. One study found no correlation between FGF21 levels and NAFLD[34], one found greater FGF21 levels in obese children with NAFLD[35], and one showed lower FGF21 levels in children with NAFLD[36]. In a study by Alisi *et al*[36] of 84 children with biopsy-proven NAFLD (38% with NASH, 70% with > F0 fibrosis), median levels of FGF21 were significantly lower in NAFLD patients compared to controls and serum FGF21 levels were inversely associated with the probability of NASH and fibrosis. A 2016 study in mice reported similar findings, noting that FGF21 knockout mice were more prone to developing NASH[37]. A 2019 study in 203 children with steatosis diagnosed through ultrasound found that FGF21 had an AUROC of 0.78 (cutoff 106.10 pg/mL, sensitivity 86.5%, specificity 60%) for the prediction of high-grade liver steatosis in the obese and overweight subjects[38]. However, this AUROC increased when combined with other biomarkers. Composite algorithms are discussed below in further detail (see "NASH scores" and "Fibrosis scores").

Resistin and retinol binding protein 4: Boyraz *et al*[24] also explored resistin and resistin and Retinol Binding Protein 4 (RBP4) as serology-based NITs for pediatric NAFLD. Resistin is a proinflammatory adipokine mainly produced by adipose tissue, inflammatory cells, and hepatic stellate cells[39]. RBP4 is a member of the lipocalin family and is primarily expressed in the liver and adipose tissue[40]; it acts as a carrier of retinol in circulation[41]. Studies have demonstrated that RBP4 and resistin levels are higher in adults with NAFLD compared to controls[39,42]. In differentiating children with advanced steatosis from those with mild-moderate steatosis, resistin had an AUROC of 0.66 (specificity 92.5%) and RBP4 had an AUROC of 0.78 (sensitivity 84.2%). In differentiating children with obesity and NAFLD from controls, resistin and RBP4 had an AUROC of 0.88 (sensitivity 100%) and 0.97 (sensitivity 100%), respectively[24]. Further studies in larger cohorts are required to validate the results of this study and establish resistin and RBP4 as clinically feasible biomarkers for children with NAFLD.

Chemerin: Chemerin is an adipokine that enhances insulin-stimulated glucose uptake and insulin sensitivity of adipose tissue[43]. It is highly expressed in the liver and adipose tissue, however, its role in NAFLD is unclear[44] and its functional receptor is only expressed on adipocytes and inflammatory cells[45]. A prospective case-control study of 101 children with biopsy-proven NAFLD found a significantly higher serum chemerin concentration in obese children with NAFLD compared to non-obese controls. In differentiating obese children with NAFLD from controls, chemerin had an AUROC of 0.78 (cutoff value 186.7 ng/mL, sensitivity 56.44%, specificity 87.72%)[25]. Kłusek-Oksiuta *et al*[46] also investigated chemerin and found it was able to differentiate children with fatty liver diagnosed through MRS from those without with an AUROC of 0.70 with an optimal cutoff of 190 ng/mL (sensitivity 75%, specificity 58%). While chemerin shows promise as a noninvasive biomarker, it is not a liver-specific adipokine and therefore, its specificity for NAFLD needs to be further investigated.

Visfatin: Visfatin is an adipokine produced by hepatocytes and visceral adipose tissue with a role in glucose and lipid metabolism[44,47,48]. While Genc *et al*[49] suggested that visfatin may play a protective role against liver injury in NAFLD, they found no significant difference in visfatin levels between adults with NAFLD and healthy controls. An Iranian study found that children with obesity had higher serum visfatin levels compared to controls, especially when obesity was comorbid with metabolic syndrome or insulin resistance[50]. In a study of 80 children with obesity (31 of whom had NAFLD as diagnosed *via* ultrasound), serum visfatin levels were higher in children with dyslipidemia, NAFLD, elevated ALT, fibrosis stage 2–3, and steatosis stage 2–3. A visfatin cutoff of 18 ng/mL was reported to significantly detect the presence of NAFLD with high sensitivity (83.9%) and specificity (81.4%), making it a promising biomarker for monitoring NAFLD in children with obesity[47].

NASH

NASH is characterized histologically by steatosis, inflammation, and hepatocyte ballooning. Ideal serology-based NITs for NASH would need to highly correlate with these histologic components. This

section describes the serological biomarkers that have been investigated thus far in children with NASH (Table 3).

ALT: ALT, synthesized primarily within the cytosol of hepatocytes, is still commonly used in both the clinical setting and in clinical trials as an indicator of liver injury and inflammation. This is largely because ALT is widely available, relatively inexpensive, and requires only a small blood sample[51]. Current clinical practice guidelines note that ALT is the best screening test for children with NAFLD and that children older than 10 years with a BMI ≥ 85th percentile should be screened using ALT for NAFLD. In addition, the guidelines state that an ALT > 80 U/L or a persistently elevated ALT greater than twice the upper limit of normal should prompt an evaluation of NAFLD or other causes of chronic hepatitis[6]. However, Manco *et al*[52] demonstrated that children with NAFLD may present with normal ALT levels and Molleston *et al*[53] cautioned that ALT levels may underestimate liver injury in children with NAFLD. In their study of children with biopsy-proven NAFLD, children with normal and mildly elevated ALT showed significant histologic abnormalities including marked steatosis (50% and 24% in patients with mildly elevated and normal ALT, respectively) and advanced fibrosis (stage 3–4 in none of the patients with normal ALT, 9% in patients with mildly elevated ALT, 15% in those with elevated ALT). In addition, ALT did not significantly correlate with hepatocyte ballooning, inflammation, or NAS ≥ 4. This raises concerns about the use of ALT in screening children with NAFLD. Interestingly, a recent study by Arsik *et al*[54] evaluated mean ALT over 96 wk as a biomarker for monitoring change in liver histology in children with biopsy-proven NAFLD. Mean ALT was found to be a better predictor of NASH (AUROC 81.84, sensitivity 80.52%, specificity 82.99%) and NASH + fibrosis (AUROC 77.78, sensitivity 71.76%, specificity 80.81%) compared to change in NAS which had a lower AUROC of 0.63. These findings suggest that ALT may be better utilized as a tool for monitoring histologic change in children with NASH and fibrosis longitudinally rather than as a screening tool.

Angiopoeitin-2: Angiopoeitin-2 (Ang-2) is a potent regulator of vascular development and maturation and is synthesized in the liver, kidney, and endothelial cells. Within the liver, it is produced by liver sinusoidal endothelial cells which, when injured, may promote the progression of simple steatosis to NASH[55]. Studies in adults show elevated Ang-2 levels in patients with histological NASH compared to those with isolated steatosis and that Ang-2 levels are associated with steatosis, lobular inflammation, and ballooning, but not with fibrosis[56]. This finding was reproduced by Manco *et al*[57] who investigated levels of Ang-2 and cytokeratin-18 (CK18), an apoptotic marker, in 76 children with biopsy-proven NAFLD. Ang-2 was elevated in children with NAFLD and NASH compared to controls and was able to predict NASH with an AUROC of 0.911 (cutoff 135.4 ng/mL, sensitivity 85.7%, specificity 85.3%, PPV 83%, negative predictive value (NPV) 87.5%). Ang-2 had a poor predictive ability for differentiating fibrosis from non-fibrosis (AUROC 0.475). Ang-2 appears to be useful in predicting NASH, however, further research is required to increase the generalizability of the results published by Manco *et al*[57].

CK18: CK18 is a cytoskeletal protein expressed by cells of epithelial origin, including hepatocytes. It is released into the bloodstream during hepatocyte apoptosis as either the whole protein (CK18 M65), which is a measure of total cell death, or the caspase-3-cleaved fragment (CK18 M30), a measure of apoptotic death[58,59]. Several studies have evaluated the use of CK18 in adults with NAFLD in predicting NASH with AUROCs ranging from 0.71 to 0.93[29,60-64]. A meta-analysis of multiple cross-sectional studies showed that CK18 had a pooled AUROC of 0.82 (median sensitivity 78%, specificity 87%) in predicting NASH in adults with NAFLD[29]. A large multicenter study by Feldstein *et al*[64] in 139 adults with biopsy-proven NAFLD found CK18 fragments to have an AUROC of 0.83 (cutoff 279 U/L, sensitivity 71% and specificity 85%) in differentiating NASH from borderline/not NASH, further establishing CK18 as a promising biomarker for adult NASH.

Vos *et al*[65] was the first to study CK18 in a pediatric population in a cross-sectional study of 62 children (20 children with obesity and steatosis as diagnosed through ultrasound/CT/elevated ALT > 40 U/L; 6 of 20 had biopsy-proven NASH). CK18 levels were significantly elevated in children with suspected NAFLD compared to obese/normal weight controls and in a multiple regression analysis, had a prediction accuracy of 84.1% for NAFLD. Feldstein *et al*[66] studied CK18 in 201 children with biopsy-proven NAFLD (NASH (n = 140), no-NASH (n = 41) and found significantly higher CK18 Levels in children with NASH compared to those with isolated steatosis. The risk of having NASH on liver biopsy increased with increasing CK18 levels and CK18 had excellent accuracy in predicting the presence of NASH on liver biopsy with an AUROC of 0.933 (cutoff of 233 U/L had sensitivity 85% and specificity 86.9%, PPV 93.7%, NPV 71.6%). This AUROC was significantly higher than those of ALT (AUROC 0.635), AST (AUROC 0.651) or GGT (AUROC 0.672) alone. A study of 45 children with biopsy-proven NAFLD in 2010 found the median value of CK18 M30 was significantly higher in children with NAFLD compared to healthy controls. CK18 M30 had an AUROC of 0.85 in predicting NASH/borderline NASH from simple steatosis in patients with NAFLD (cutoff 207 IU/L, sensitivity 84%, specificity of 88%, PPV 90%, NPV 80%)[67].

In a cross-sectional study of 117 children with biopsy-proven NAFLD, greater decreases in serum CK18 levels were observed in children with histologic improvements compared to those without improvement at 1 and 2 years from baseline. However, change in ALT was found to be a better indicator of NASH resolution (AUROC 0.84) compared to CK18, which had an AUROC of 0.69 (P = 0.005).

Table 3 Serological biomarkers and composite scores for the detection of nonalcoholic steatohepatitis

Ref.	Marker	Country	Categories Tested	Sample size (*n*)	Dx	Cutoff	AUROC (95%CI)	Sens (%)	Spec (%)	PPV (%)	NPV (%)	*P* value
Feldstein *et al*[66], 2013	**ALT**	Italy	Diagnosing NASH	NASH (*n* = 140), non-NASH (*n* = 61)	Biopsy		0.635 (0.556, 0.715)					< 0.001
Walenbergh *et al* [70], 2015	**ALT**	Italy	Borderline NASH *vs* definite NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy		0.57					0.0011 (CatD *vs* ALT)
Walenbergh *et al* [70], 2015	**ALT**	Italy	Steatosis + Borderline NASH *vs* NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (n = 96)	Biopsy		0.53					< 0.001 (CatD *vs* ALT)
Walenbergh *et al* [70], 2015	**ALT**	Italy	Steatosis *vs* borderline NASH + NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy		0.66					0.103 (CatD *vs* ALT)
Walenbergh *et al* [70], 2015	**ALT [U/L]**	Italy	Steatosis *vs* NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy	> 64.5	0.59	61.5	68.4	72.7	56.5	0.0004 (CatD *vs* ALT)
Manco *et al*[57], 2022	**Ang-2 [ng/mL]**	Italy	Diagnosing NASH	NAFLD (*n* = 76), controls (*n* = 28, by ultrasound)	Biopsy	135.4	0.911 (0.844–0.979)	85.7	85.3	83	87.5	< 0.001
Feldstein *et al*[66], 2013	**AST**	Italy	Diagnosing NASH	NASH (*n* = 140), non-NASH (*n* = 61)	Biopsy		0.651 (0.573, 0.728)					< 0.001
Walenbergh *et al* [70], 2015	**CatD**	Italy	Borderline NASH *vs* definite NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy		0.85					
Walenbergh *et al* [70], 2015	**CatD**	Italy	Steatosis + Borderline NASH *vs* NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy		0.88					
Walenbergh *et al* [70], 2015	**CatD**	Italy	Steatosis *vs* borderline NASH + NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy		0.81					
Walenbergh *et al* [70], 2015	**CatD [pg/mL]**	Italy	Steatosis *vs* NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy	< 18445	0.94	100	89.5	92.9	100	
Vuppalanchi *et al* [68], 2014	**Change in ALT**	United States	Overall histologic improvement	NAFLD (*n* = 117)	Biopsy		0.79 (0.70-0.87)					
Vuppalanchi *et al* [68], 2014	**Change in ALT**	United States	Resolution of NASH	NAFLD (*n* = 117)	Biopsy		0.84 (0.76-0.93)					
Vuppalanchi *et al* [68], 2014	**Change in ALT + CK18**	United States	Overall histologic improvement	NAFLD (*n* = 117)	Biopsy		0.79 (0.71-0.87)					0.08 (CK18+ALT *vs* ALT)

Vuppalanchi *et al* [68], 2014	**Change in ALT + CK18**	United States	Resolution of NASH	NAFLD (n = 117)	Biopsy		0.83 (0.75-0.92)				0.92 (CK18+ALT *vs* ALT)
Vuppalanchi *et al* [68], 2014	**Change in CK18**	United States	Overall histologic improvement	NAFLD (n = 117)	Biopsy		0.72 (0.63-0.81)				0.42 (CK18 *vs* ALT)
Vuppa-lanchi *et al* [68], 2014	**Change in CK18**	United States	Resolution of NASH	NAFLD (n = 117)	Biopsy		0.69 (0.58-0.79)				0.005 (CK18 *vs* ALT)
Walenbergh *et al* [70], 2015	**CK18**	Italy	Borderline NASH *vs* definite NASH	NASH (n = 26), borderline NASH (n = 51), steatosis (n = 19), obese (n = 96)	Biopsy		0.57				0.0003 (CatD *vs* CK18)
Walenbergh *et al* [70], 2015	**CK18**	Italy	Steatosis + Borderline NASH *vs* NASH	NASH (n = 26), borderline NASH (n = 51), steatosis (n = 19), obese (n = 96)	Biopsy		0.52				< 0.0001 (CatD *vs* CK18)
Walenbergh *et al* [70], 2015	**CK18**	Italy	Steatosis *vs* borderline NASH + NASH	NASH (n = 26), borderline NASH (n = 51), steatosis (n = 19), obese (n = 96)	Biopsy		0.74				0.4299 (CatD *vs* CK18)
Manco *et al*[57], 2022	**CK18 [U/L]**	Italy	Diagnosing NASH	NAFLD (n = 76), controls (n = 28, by ultrasound)	Biopsy	352	0.827 (0.735–0.919)	77.1	73.2	71	78.9	< 0.001
Fitzpatrick *et al*[67], 2010	**CK18 [U/L]**	United Kingdom	Predicting NASH	NAFLD (n = 45), controls (n = 13)	Biopsy	207	0.85 (0.73–0.96)	84	88	90	80	
Feldstein *et al*[66], 2013	**CK18 [U/L]**	Italy	Diagnosing NASH	NASH (n = 140), non-NASH (n = 61)	Biopsy	233	0.9334	85	86.9	93.7	71.6	< 0.001
Walenbergh *et al* [70], 2015	**CK18 [U/L]**	Italy	Steatosis *vs* NASH	NASH (n = 26), borderline NASH (n = 51), steatosis (n = 19), obese (n = 96)	Biopsy	> 327.5	0.72	72	63.2	72.8	62.2	0.0225 (CatD *vs* CK18)
Feldstein *et al*[66], 2013	**GGT**	Italy	Diagnosing NASH	NASH (n = 140), non-NASH (n = 61)	Biopsy		0.672 (0.594-0.750)					< 0.001
Manco *et al*[95], 2007	**Leptin [ng/mL]**	Italy	Predicting NAFLD Activity Score	NAFLD (n = 72), F0 (n = 31), F1 (n = 41)	Biopsy	≤ 14.9	0.833	9	36	5	47	
Manco *et al*[95], 2007	**Leptin [ng/mL]**	Italy	Predicting NAFLD Activity Score	NAFLD (n = 72), F0 (n = 31), F1 (n = 41)	Biopsy	≥ 20.4		54	76	50	79	
Mosca *et al*[101], 2019	**PIIINP [ng/mL]**	Italy	Definite NASH *vs* No/Borderline NASH	No/borderline NASH (n = 115), definite NASH (n = 89)	Biopsy	> 7.60	0.737 (0.66-0.81)	62	91	85	75	
Manco *et al*[95], 2007	**TNF-α [pg/mL]**	Italy	Predicting NAFLD Activity Score	NAFLD (n = 72), F0 (n = 31), F1 (n = 41)	Biopsy	≤ 5.9	0.911	18	36	11	5	
Manco *et al*[95], 2007	**TNF-α [pg/mL]**	Italy	Predicting NAFLD Activity Score	NAFLD (n = 72), F0 (n = 31), F1 (n = 41)	Biopsy	≥ 7.9		82	96	90	96	

AUROC: Area under the receiver operating characteristic curve; ALT: Alanine aminotransferase; Ang-2: Angiopoietin-2; AST: Aspartate aminotransferase; CatD: Cathepsin D; CK18: Cytokeratin 18; CI: Confidence interval; Dx:

Diagnosis; GGT: Gamma-glutamyl transferase; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NPV: Negative predictive value; PPV: Positive predictive value; Sens: Sensitivity; Spec: Specificity; TNF-a: Tumor necrosis factor alpha.

Neither change in ALT, change in CK18, or change in CK18 + ALT had significantly different AUROCs for discriminating a ≥ 1 point decrease in steatosis, lobular inflammation, hepatocyte ballooning, or fibrosis stage[68]. Further research is required to determine the usefulness of CK18 Levels in tracking the progression of NASH over time. CK18 as a predictor of pediatric liver fibrosis is discussed in the section on diagnosing liver fibrosis below.

Cathepsin D: Cathepsin D (CatD) is a lysosomal protease that is ubiquitously distributed in high concentrations in the liver[69]. Thus far, it has only been evaluated as a biomarker for children in one study. Walenbergh *et al*[70] evaluated the predictive ability of CatD for hepatic inflammation in 96 children with biopsy-proven NAFLD (NASH (n = 26), borderline NASH (n = 51), steatosis (n = 19). The study found that plasma CatD was significantly lower in children with liver inflammation compared to those with steatosis, and had a negative correlation with increasing liver inflammation, steatosis, hepatocellular ballooning, and NAS. CatD had a high diagnostic accuracy in differentiating between NASH and steatosis with an AUROC of 0.94 and reached an AUROC of 0.998 when combined with CK18. A cutoff of < 18445 pg/mL had a sensitivity of 100% and specificity of 89.5% (PPV 92.9%, NPV 100%). CatD as a solo biomarker was also superior in differentiating NASH from steatosis in children with NAFLD compared to ALT (AUROC 0.59) and CK18 (AUROC 0.72). Additionally, CatD was able to accurately distinguish borderline NASH from definite NASH (AUROC 0.85), steatosis + borderline NASH from definite NASH (AUROC 0.88) and steatosis from borderline NASH + definite NASH (AUROC 0.81), better than ALT or CK18 could on their own. The combination of CatD with CK18 improved the discriminatory ability in each of the aforementioned categories[70]. Interestingly, a second study conducted by Walenbergh *et al*[71] in 2016 found that in 248 adults with biopsy-proven NAFLD, patients with NASH had increased levels of CatD compared to healthy controls. Following surgical intervention there was a reduction of plasma CatD compared to baseline. This suggests the presence of distinct pathophysiology between childhood and adulthood NASH.

Cytokines: Cytokines have also been studied as serology-based NITs for pediatric NASH. A cross-sectional NASH CRN study found that in 235 children with biopsy-proven NAFLD, certain cytokines were significantly associated with different histologic features of NASH. Children with definite NASH and lobular inflammation were found to have significantly higher levels of total (tPAI1) and activated tissue plasminogen activator 1 (aPAI1). In addition, PAI1 was able to significantly discriminate between borderline/definite NASH, definite NASH, lobular inflammation, and hepatocyte ballooning. IL-8 and soluble IL-2 receptor alpha (sIL-2Rα) were associated with fibrosis severity and with lobular and portal inflammation, respectively[72]. These findings suggest that these novel cytokines may be a useful tool in the diagnosis and management of NASH in children. However, more research is needed to validate these results and explore the utility of these biomarkers in clinical practice.

Fibrosis

Currently, liver biopsy remains the gold standard for diagnosing fibrosis in children with NAFLD.

However, there is a growing need for noninvasive, more cost-effective diagnostic methods. There have been many advances in recent years in developing noninvasive techniques for diagnosing liver fibrosis, including serological biomarkers, scores based on basic laboratory tests, and imaging modalities. This section discusses the serological biomarkers used in the diagnosis of liver fibrosis in pediatric NAFLD (Table 4). It is important to note that to date, two milestone studies have identified liver fibrosis as the strongest prognostic factor in predicting long-term outcomes in patients with NAFLD[12,73].

Fibrosis is most commonly scored based on the Metavir score, which includes 5 histologic categories: F0 (no fibrosis), F1 (portal fibrosis with no septae), F1 (portal fibrosis with few septae), F3 (numerous septae without cirrhosis), and F4 (cirrhosis). Any fibrosis refers to F1–F4, F2–F4 are considered significant fibrosis, and F3–F4 are considered advanced fibrosis[74].

Hyaluronic acid: Hyaluronic acid (HA) is a glycosaminoglycan polymer present in epithelial and connective tissue and constitutes a major component of the extracellular matrix (ECM). HA is abundant in the ECM of the liver. Within the liver, HA is primarily produced by activated hepatic stellate cells and degraded by sinusoidal endothelial cells[75]. In adults with NAFLD, HA has emerged as a good predictor of liver fibrosis[76-78]. In 2010, Nobili *et al*[79] were the first to evaluate HA as a predictive biomarker in children with NAFLD. This study included 100 children with biopsy-proven NAFLD, 65% of whom had ≥ stage 1 Liver fibrosis. The study found that serum HA was a good predictor of the degree of fibrosis in children with NAFLD, with HA ≥ 1200 ng/mL making the absence of fibrosis (F0) unlikely and HA ≥ 2100 ng/mL making significant fibrosis (≥ F2) highly likely. Serum HA as a diagnostic tool for liver fibrosis had an AUROC of 0.88 for any degree of fibrosis (F1-F4 *vs* F0) when using a cutoff of ≥ 1200 ng/mL (PPV 90%, NPV 53%) and an AUROC of 0.95 for significant fibrosis (≥ F2+ *vs* F0-F1) using a cutoff of 2100 ng/mL (PPV 40%, NPV 91%).

In 2011, Lebensztejn *et al*[80] found that HA was significantly higher in children with biopsy-proven NAFLD who had fibrosis compared to healthy controls. With a cutoff value at 19.1 ng/mL, HA had an AUROC of 0.672 (sensitivity 84%, specificity 55%, PPV 52%, NPV 86%) in differentiating children with NAFLD and fibrosis (F1–F3) from those without fibrosis (F0). When combined with CK18, the AUROC increased to 0.73 (sensitivity 74%, specificity 79%, PPV 56%, NPV 63%). Notably, 37% (19 of 52) of this cohort of children had fibrosis (F1–F3). Interestingly, the 2010 study by Fitzpatrick *et al*[67] that evaluated CK18 M30 and leptin as biomarkers in 45 children with biopsy-proven NAFLD did not find HA to be a reliable marker of NASH or fibrosis, despite 51.1% of the cohort having ≥ F2 (significant fibrosis).

N-terminal type III collagen propeptide: Fibrosis is a dynamic process that results from the imbalanced production and degradation of ECM proteins, leading to the continuous release of ECM-related proteins into the serum. N-terminal type III collagen propeptide (PRO-C3), a neo-epitope pro-peptide of type III collagen formation, has been studied as an independent predictor of the degree of fibrosis in adults with NAFLD[81]. One such adult study created a PRO-C3 based fibrosis algorithm, named ADAPT (age, presence of diabetes, PRO-C3, and platelet count), that had an AUROC of 0.86–0.87 in identifying patients with NAFLD and advanced fibrosis and superior to other fibrosis algorithms such as the Fibrosis-4 (FIB-4) score, NAFLD Fibrosis Score (NFS), and AST to Platelet Ratio index (APRI)[82]. Only one group has studied PRO-C3 as a serological marker of fibrosis in pediatric NAFLD. Cohen *et al*[83], in a study of 88 children with biopsy-proven NAFLD, found that PRO-C3 levels were similar between children with NAFLD and healthy controls, but significantly lower in children ≥ 15 years compared to children ≤ 10 years old. Amongst children with NAFLD, PRO-C3 levels were higher in children with advanced fibrosis (Ishak score ≥ 3) compared to children with no/mild fibrosis (Ishak score ≤ 2). However, these associations were not significant after adjusting for bone remodeling biomarkers, suggesting that PRO-C3 may not be a reliable biomarker for liver fibrosis until late adolescence, as it is influenced by age and pubertal growth.

CK18: While CK18 has been extensively studied as a marker of pediatric NASH, there is limited data on it as a marker for pediatric liver fibrosis. Lebensztejn *et al*[80] found that CK18 had an AUROC of 0.666 (cutoff 210 U/L, sensitivity 79%, specificity 60%, PPV 56%, NPV 82%) in differentiating children with fibrosis from those without fibrosis. When combined with HA, this AUROC increased to 0.73. In 2010, Fitzpatrick *et al*[67] found that CK18 M30 fragments were significantly higher in children with significant or severe fibrosis (≥ F2) compared to children with no/minimal fibrosis (< F2). CK18 M30 had an AUROC of 0.66 in predicting significant/severe fibrosis (≥ F2) (cutoff 200 IU/L, sensitivity 83%, specificity 40%). A more recent study by Mandelia *et al*[84] of 201 children with biopsy-proven NAFLD (68% of cohort with F1–F3 fibrosis) found CK18 levels to be significantly higher in children with F1–F3 compared to F0. Their study had an AUROC of 0.75 in predicting any fibrosis (F1–F3), AUROC 0.67 in predicting significant fibrosis (F2–F3) and AUROC 0.77 in predicting advanced fibrosis (F3). Mandelia *et al*[84] also generated a prediction model for fibrosis F1–F3 that combined CK18 with waist circumference percentile which reached an AUROC of 0.842 of differentiating any fibrosis (F1–F3) from no fibrosis (F0). Using this model, they propose that patients with a score of < 35 likely have no fibrosis (specificity 38%, sensitivity 97%, PPV 76%, NPV 86%) and patients with a score ≥ 82 likely have fibrosis (sensitivity 88%, specificity 59%, PPV 91%, NPV 51%).

Table 4 Serological biomarkers for the detection of fibrosis in nonalcoholic fatty liver disease

Ref.	Marker	Country	Categories Tested	Sample size (*n*)	Dx	Cutoff	AUROC (95%CI)	Sens (%)	Spec (%)	PPV (%)	NPV (%)	*P* value
Mandelia *et al*[84], 2016	**CK18**	Italy	F1-F3 *vs* F0	NAFLD (*n* = 201), F0 (*n* = 65), F1–F3 (*n* = 136)	Biopsy		0.75 (0.68-0.81)					
Mandelia *et al*[84], 2016	**CK18**	Italy	F2-F2 *vs* F0	NAFLD (*n* = 201), F0 (*n* = 65), F1–F3 (*n* = 136)	Biopsy		0.67 (0.54-0.80)					
Mandelia *et al*[84], 2016	**CK18**	Italy	F3 *vs* F0	NAFLD (*n* = 201), F0 (*n* = 65), F1–F3 (*n* = 136)	Biopsy		0.77 (0.56-0.97)					
Fitzpatrick *et al* [67], 2010	**CK18 [U/L]**	United Kingdom	Significant Fibrosis (≥ F2)	NAFLD (*n* = 45), healthy controls (*n* = 13)	Biopsy	200	0.66 (0.5-0.82)	83	40			
Lebensztejn *et al* [80], 2011	**CK18 [U/L]**	Poland	Fibrosis (F1-F3) *vs* F0	NAFLD (*n* = 52), NAFLD with obesity/overweight (*n* = 42), healthy non-obese controls (*n* = 25)	Biopsy	210	0.666	79	60	56	82	0.05
Nobili *et al*[79], 2010	**HA [ng/mL]**	Italy	F1 and F2+ *vs* F0	NAFLD (*n* = 100), F0 (*n* = 35), ≥ F1 (*n* = 65)	Biopsy	≥ 1200	0.88 (0.81–0.96)			90	50	
Nobili *et al*[79], 2010	**HA [ng/mL]**	Italy	F2+ *vs* F0 and F1	NAFLD (*n* = 100), F0 (*n* = 35), ≥ F1 (*n* = 65)	Biopsy	2100	0.95 (0.91–0.99)			40	90	
Lebensztejn *et al* [80], 2011	**HA [ng/mL]**	Poland	Fibrosis (F1-F3) *vs* F0	NAFLD (*n* = 52), NAFLD with obesity/overweight (*n* = 42), healthy non-obese controls (*n* = 25)	Biopsy	19.1	0.672	84	55	52	86	0.04
Lebensztejn *et al* [80], 2011	**HA + CK18**	Poland	Fibrosis (F1-F3) *vs* F0	NAFLD (*n* = 52), NAFLD with obesity/overweight (*n* = 42), healthy non-obese controls (*n* = 25)	Biopsy		0.73	74	79	56	63	0.002
Hamza *et al*[99], 2016	**PIIINP [ng/mL]**	Egypt	Presence of steatosis in obese children	Obese with NAFLD (*n* = 50), obese without NAFLD (*n* = 5), nonobese healthy controls (*n* = 30)	US	8.5		74	33			
Mosca *et al*[101], 2019	**PIIINP [ng/mL]**	Italy	Presence of ≥ F2	No/borderline NASH (*n* = 115), definite NASH (*n* = 89)	Biopsy	> 8.89	0.921 (0.87-0.97)	84	94	95	79	
Mosca *et al*[101], 2019	**PIIINP [ng/mL]**	Italy	Presence of F3	No/borderline NASH (*n* = 115), definite NASH (*n* = 89)	Biopsy	> 13.2	0.993 (0.98-1.0)	100	98	78	100	

AUROC: Area under the receiver operating characteristic curve; CK18: Cytokeratin 18; CI: Confidence interval; Dx: Diagnosis; F: Fibrosis stage; HA: Hyaluronic acid; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NPV: Negative predictive value; PIIINP: Amino-terminal propeptide of type III procollagen; PPV: Positive predictive value; Sens: Sensitivity; Spec: Specificity; US: Ultrasound.

ECM associated noninvasive tests: Several ECM biomarkers have been studied in adults with NAFLD as biomarkers of fibrosis, including laminin, chitinase-3-like protein 1 (YKL-40), amino-terminal propeptide of type III procollagen (PIIINP), and tissue inhibitor of metalloproteinases 1 (TIMP1). YKL-

40 is a glycoprotein with an unknown biological purpose, but it is known to promote growth in fibroblasts, chondrocytes, and synovial cells. During fibrogenesis, YKL-40 is released from the hepatic stellate cells[85]. Lebensztejn *et al*[80] are the only group to study laminin and YKL-40 in children with NAFLD and found that while they were significantly higher in NAFLD patients with fibrosis compared to healthy controls, neither correlated with fibrosis stage or were useful in predicting fibrosis in children with biopsy-proven NAFLD.

Leptin: Leptin is an adipocyte-derived hormone that plays a major role in the regulation of appetite and body fat mass and controls energy balance in the hypo- and normoleptinemic states[86,87]. Within the liver, leptin is thought to increase hepatic steatosis, steatohepatitis, and fibrosis[88]. Leptin has been established as an essential mediator of fibrosis in response to chronic liver injury. A 2002 study demonstrated that Leptin-deficient mice failed to develop fibrosis during steatohepatitis or in response to chronic toxic liver injury[89]. Leptin exerts its profibrotic effects by activating Kupffer cells and macrophages and stimulating endothelial cells to secrete transforming growth factor-β (TGF-β)[90]. In addition, leptin directly targets hepatic stellate cells through the stimulation of TIMP1 production[91]. Leptin also protects hepatic stellate cells from apoptosis, leading to a cycle that exacerbates its profibrotic effects[92,93]. Studies in adults with NAFLD have found that leptin levels increase in association with increased severity of hepatic steatosis and degree of fibrosis, especially in patients with a high BMI[94].

Studies have also been conducted evaluating the role of leptin in pediatric NAFLD. In a study of 72 children with biopsy-proven NAFLD (36 children each in the training set and validation set), Manco *et al*[95] found that leptin had an AUROC of 0.796-0.833 in predicting a NAS of ≥ 5. In the training set, a leptin cutoff of ≥ 20.4 ng/mL had sensitivity 54%, specificity 76%, PPV 50%, NPV 79%. A risk score that was developed by combining leptin with tumor necrosis factor α (TNF-α) had an AUROC of 0.964–0.985. The risk score showed high accuracy, with a cutoff of ≥ 13.5 having sensitivity 81%, specificity 92%, PPV 82%, NPV 92%. This is currently the only study that has done receiver operating characteristic (ROC) analysis regarding leptin in children with NAFLD. However, other studies have evaluated leptin in pediatric NAFLD with varying results. The most recent study by Brandt *et al*[96] found that in a cross-sectional study of 97 prepubertal children with obesity (34% of whom were diagnosed with hepatic steatosis through ultrasound), circulating leptin levels were negatively correlated with the degree of hepatic steatosis. However, Nobili *et al*[97] found that circulating leptin levels positively correlated with degree of hepatic steatosis, ballooning, and NAS (independently of age, BMI and gender) in the same cohort of 72 biopsy-proven NAFLD children studied by Manco *et al*[97]. Boyraz *et al*[24] similarly found that leptin levels were higher in obese children with steatosis, but that leptin was unable to differentiate obese children with steatosis from obese children without steatosis. A 2010 study by Fitzpatrick *et al*[67] in 45 children with biopsy-proven NAFLD found that leptin was able to accurately predict fibrosis grade but not degree of steatohepatitis. Leptin was able to distinguish.

PIIINP: PIIINP is a peptide released during the processing of procollagen. It was first studied in 1984 and found to be normal or slightly elevated in adults with NAFLD[98]. In a cross-sectional case-control study of 55 obese children (50 of whom were diagnosed with NAFLD through ultrasound), a PIIINP cutoff of 8.5 ng/mL yield a sensitivity of 74%, specificity 33% in differentiating cases from controls, suggesting that PIIINP may serve as a marker of hepatic steatosis[99]. A study of 172 adults with biopsy-proven NAFLD demonstrated that in patients with F0–F2 fibrosis, PIIINP had an AUROC of 0.77–0.82 in discriminating between NASH and simple steatosis, and in patients with F0–F3, an AUROC of 0.82–0.84. When considering patients with all levels of fibrosis, PIIINP was successful in distinguishing between those with simple steatosis and those with NASH or advanced fibrosis with an AUROC of 0.85-0.87[100]. Mosca *et al*[101] are the only study to date to evaluate PIIINP in a biopsy-proven cohort of 204 children with NAFLD. This study found that children with NASH had higher plasma PIIINP levels compared to children without NASH, and that PIIINP levels correlated with NAS and its constituent components. The risk of NASH and ≥ F2 progressively increased with increasing PIIINP levels (for every 3.6 ng/mL increase in PIIINP levels, the likelihood of having ≥ F2 increased by approximately 14 fold). PIIINP had an AUROC of 0.737 (sensitivity 62%, specificity 91%, PPV 85%, NPV 75%) in discriminating definite NASH from no/borderline NASH. This is higher than the discriminatory ability of the FIB-4 score or APRI (AUROC 0.6369 and 0.6826, respectively). PIIINP had an AUROC of 0.921 for ≥ F2 and 0.993 for F3. A cutoff of > 8.89 ng/mL had 84% sensitivity, 94% specificity, 95% PPV, 79% NPV for predicting ≥ F2, whereas a cutoff of > 13.2 ng/mL yielded 100% sensitivity, 98% specificity, 70% PPV, 100% NPV for predicting the presence of F3. These values were higher than those of FIB-4 (AUROC 0.7412 for ≥ F2 and AUROC 0.7687 for F3) and APRI (AUROC 0.7659 for ≥ F2 and AUROC 0.8535 for F3).

IMAGING-BASED BIOMARKERS / IMAGING-BASED NONINVASIVE TESTS

Ultrasound-based biomarkers

Steatosis (primarily controlled attenuation parameter, or CAP): The ultrasound-based FibroScan® can evaluate the severity of hepatic steatosis and fibrosis. Its best-known function is based on vibration-controlled transient elastography (VCTE) for fibrosis assessment, which works by sending a low-frequency ultrasound shear wave into the liver and measuring the velocity of the wave as it passes through liver tissue. Firmer tissue results in faster wave propagation. This measurement is converted into a liver stiffness measurement (LSM), expressed in kilopascals (kPa), which is able to assess the level of fibrosis in the liver[102]. Moreover, the FibroScan® is also able to assess liver steatosis through a measure called controlled attenuation parameter (CAP), expressed as decibels per meter (dB/m), which measures the amount of attenuation of the ultrasound wave as it passes through liver tissue. Higher values of CAP indicate a greater level of hepatic steatosis[103].

Kwon *et al*[104] evaluated the usefulness of FibroScan® in a Korean cohort of 59 obese children and 47 non-obese controls. The study found that children in the obese group had significantly higher levels of CAP and LSM compared to controls and that LSM had a strong positive correlation with conventional predictive indices for hepatic steatosis and fibrosis, including AST, ALT, and APRI. A recent study by Chaidez *et al*[105] found CAP to have outstanding discriminatory ability in differentiating steatosis grade 1–3 from grade 0 (AUROC 0.98) (Table 5). A CAP cutoff value of ≥ 259 dB/m had a sensitivity of 94%, specificity of 91%, PPV of 97%, and NPV of 91% for the prediction of steatosis grades 1–3.

Yang *et al*[106] evaluated the diagnostic efficacy of LSM in NAFLD and its subtypes, NAFL and NASH, in a cohort of 120 children with obesity. The results showed that LSM had an AUROC of 0.768 (sensitivity 70.5%, specificity 70.7%) for NAFLD, an AUROC of 0.674 (sensitivity 61.4%, specificity 64.5%) for NAFL, and an AUROC of 0.725 (sensitivity 64.7%, specificity 65.0%) for NASH. The study concluded that LSM has diagnostic efficacy for NAFLD and its subtypes in children with obesity with optimal predictive values for NAFLD being LSM > 4.65 kPa, for NAFL being LSM > 4.95 kPa, and for NASH being LSM > 5.15 kPa. It is important to note that LSM is typically used in the evaluation of fibrosis, however, in this study LSM was used for the evaluation of steatosis. This study also evaluated CAP with results showing that CAP had an AUROC of 0.757 (sensitivity 67.20%, specificity 67.20%) for NAFLD, an AUROC of 0.659 (sensitivity 59.10%, specificity 60.50%) for NAFL, and an AUROC of 0.722 (sensitivity 70.60%, specificity 72.80%) for NASH. The study concluded that CAP has diagnostic efficacy for NAFLD and its subtypes in children with obesity with optimal predictive values for NAFLD being CAP > 258.00 dB/m, for NAFL being CAP > 262.50 dB/m and for NASH being CAP > 276.00 dB/m.

NASH

Yang *et al*[106] also evaluated the ability of CAP and LSM in predicting NASH in children with obesity. They found that CAP had an AUROC of 0.722 (sensitivity 70.6%, specificity 72.8%) and LSM had an AUROC of 0.725 (sensitivity 64.7%, specificity 65%) in predicting NASH in children with obesity (Table 6). The optimal cutoff points were > 276 dB/m and > 5.15 kPa for CAP and LSM, respectively.

Fibrosis

Transient elastography/VCTE: Transient elastography (TE) has shown excellent performance in determining the severity of fibrosis in children with NAFLD in two large studies. In a cohort of 52 children with biopsy-proven NASH, Nobili *et al*[107] demonstrated that TE was able to predict any fibrosis (F1–F4) with an AUROC of 0.977 (cutoff of 5.1 kPa yielded sensitivity 97%, specificity 91%, PPV 97%, NPV 91%) (Table 6). It predicted significant fibrosis (F2-F4) with an AUROC of 0.992 and at a cutoff of 7.4 kPa, had sensitivity 100%, specificity 92%, PPV 80%, NPV 100%. TE was also able to predict advanced fibrosis (F3–F4) with an AUROC of 1.000 and at a cutoff of 10.2 kPa, had a sensitivity, specificity, PPV, and NPV all of 100%. Alkhouri *et al*[108] evaluated the ability of TE in predicting clinically significant (≥ F2) fibrosis in children with NAFLD. In their cohort of 67 children with biopsy-proven NAFLD (10 of whom had F2–F3 fibrosis), TE demonstrated an AUROC of 1.00 in predicting ≥ F2 fibrosis. A cutoff of 8.6 kPa yield 100% accuracy in predicting F0–F1 fibrosis, obviating the need for liver biopsy, and a cutoff of ≥ 8.6 kPa had 100% accuracy in predicting F2–F3 fibrosis, highlighting the need for liver biopsy. The ability of LSM to predict fibrosis stage was evaluated in a recent study by Chaidez *et al*[105] by comparing LSM to 4 dichotomized outcomes of the Ishak fibrosis scale: no fibrosis (F0) *vs* any fibrosis (F1-F6), mild fibrosis (F0-F1) *vs* moderate-to-severe fibrosis (F2-F6), mild-to-moderate fibrosis (F0-F2) *vs* severe fibrosis (F3-F6), and mild-to-severe fibrosis (F0-F3) *vs* very severe fibrosis (F4-F6). LSM had the strongest discriminatory ability in comparing mild-to-moderate fibrosis (F0-F2) with severe fibrosis (F3-F6) with an AUROC of 0.7 for the NAFLD group (n = 116), 0.77 for the non-NAFLD group (n = 90), and 0.73 for all participants (n = 206).

Acoustic radiation force impulse: Acoustic radiation force impulse (ARFI) is a noninvasive ultrasonography technique that uses short, high-intensity acoustic pulses to generate shear waves in the liver. These waves propagate at a speed proportional to the stiffness of the tissue, thus traveling faster as the degree of fibrosis increases. These waves are measured as shear wave velocity (SWV) and provide

Table 5 Imaging biomarkers for the detection of nonalcoholic fatty liver disease

Ref.	Marker	Country	Categories Tested	Sample size (*n*)	Dx	Cutoff	AUROC (95%CI)	Sens (%)	Spec (%)	PPV (%)	NPV (%)	*P* value
Yang *et al*[106], 2022	**CAP [dB/m]**	China	Predicting NAFL in with obesity	NAFLD (*n* = 61), Non-NAFLD (*n* = 59), NAFL (*n* = 44), NASH (*n* = 17)	US	> 262.5	0.659 (0.561-0.758)	59.1	60.5			0.0037
Yang *et al*[106], 2022	**CAP [dB/m]**	China	Predicting NAFL in with obesity	NAFLD (*n* = 61), Non-NAFLD (*n* = 59), NAFL (*n* = 44), NASH (*n* = 17)	US	> 258	0.757 (0.668-0.845)	67.2	67.2			<0.001
Chaidez *et al*[105], 2022	**CAP [dB/m]**	United States	S1-S3 *vs* S0	Total (*n* = 206), NAFLD (*n* = 116), Non-NAFLD (*n* = 90)	Biopsy	≥ 259	0.98 (0.96-0.99)	94	91	97	91	
Yang *et al*[106], 2022	**LSM [kPa]**	China	Predicting NAFL in with obesity	NAFLD (*n* = 61), Non-NAFLD (*n* = 59), NAFL (*n* = 44), NASH (*n* = 17)	US	> 4.95	0.674 (0.577-0.771)	61.4	64.5			0.0015
Yang *et al*[106], 2022	**LSM [kPa]**	China	Predicting NAFL in with obesity	NAFLD (*n* = 61), Non-NAFLD (*n* = 59), NAFL (*n* = 44), NASH (*n* = 17)	US	> 4.65	0.768 (0.684-0.852)	70.5	70.7			< 0.001
Trout *et al*[134], 2018	**MRE [kPa]**	United States	NAFLD stage 0-1 *vs* ≥ stage 2 fibrosis in patients with steatosis	Total (*n* = 86), Ludwig ≥ stage 2 (*n* = 51), steatosis (*n* = 44)	Biopsy	2.28	0.53 (0.35-0.71)	52.2	71.4			
Trout *et al*[134], 2018	**MRE [kPa]**	United States	NAFLD stage 0-1 *vs* ≥ stage 2 fibrosis in patients with steatosis	Total (*n* = 86), Ludwig ≥ stage 2 (*n* = 51), steatosis (*n* = 44)	Biopsy	0.94		13	100			
Middleton *et al* [121], 2018	**MRI-PDFF [%]**	United States	Grade 1 steatosis *vs* grade 2-3	Baseline MRI (*n* = 110), no baseline MRI (*n* = 59)	Biopsy	17.5	0.87 (0.80-0.94)	74	90	97	41	
Middleton *et al* [121], 2018	**MRI-PDFF [%]**	United States	Grade 1-2 steatosis *vs* grade 3	Baseline MRI (*n* = 110), no baseline MRI (*n* = 59)	Biopsy	23.3	0.79 (0.70-0.87)	60	90	88	65	
Middleton *et al* [121], 2018	**MRI-PDFF [%]**	United States	Decrease in steatosis grade	Baseline MRI (*n* = 110), no baseline MRI (*n* = 59)	Biopsy	-11	0.76 (0.66-0.87)	31	90	78	60	
Middleton *et al* [121], 2018	**MRI-PDFF [%]**	United States	Increase in steatosis grade	Baseline MRI (*n* = 110), no baseline MRI (*n* = 59)	Biopsy	5.5	0.83 (0.73-0.92)	40	90	33	92	
Zhao *et al*[123], 2019	**MRI-PDFF [%]**	China	Detecting ≥ S1	Total (*n* = 86), Obese/overweight (*n* = 65), healthy nonobese controls (*n* = 21)	MRS	5.1	0.991 (0.977-1.00)	95	100			
Di Martino *et al* [122], 2016	**MRI-PDFF [%]**	United States	Presence of steatosis	NASH (*n* = 27), healthy controls (*n* = 27)	Biopsy	3.5		89	88			
Di Martino *et al* [122], 2016	**MRS [%]**	United States	Presence of steatosis	NASH (*n* = 27), healthy controls (*n* = 27)	Biopsy	6		92.6	95.7			

AUROC: Area under the receiving operating characteristic; CAP: Controlled attenuation parameter; CI: Confidence interval; Dx: Diagnosis; LSM: Liver stiffness measurement; MRE: Magnetic resonance elastography; MRI: Magnetic resonance imaging; MRS: Magnetic spectroscopy; NAFL: Nonalcoholic fatty liver; NAFLD: Nonalcoholic fatty liver disease; NPV: Negative predictive value; PDFF: Proton density fat fraction; PPV: Positive predictive value; Sens: Sensitivity; Spec: Specificity; US: Ultrasound.

Table 6 Imaging biomarkers for the detection of nonalcoholic steatohepatitis and the detection of fibrosis in nonalcoholic fatty liver disease

Ref.	Marker	Country	Categories Tested	Sample size (*n*)	Dx	Cutoff	AUROC (95%CI)	Sens (%)	Spec (%)	PPV (%)	NPV (%)	*P* value
Yang *et al*[106], 2022	**CAP [dB/m]**	China	Predicting NASH in children with obesity	NAFLD (*n* = 61), Non-NAFLD (*n* = 59), NAFL (*n* = 44), NASH (*n* = 17)	US	> 276	0.722 (0.602-0.843)	70.6	72.8			0.0058
Chaidez *et al*[105], 2022	**LSM**	United States	F0-F2 *vs* F3-F6 (Ishak)	Total (*n* = 206)	Biopsy		0.73 (0.64-0.81)					
Chaidez *et al*[105], 2022	**LSM**	United States	F0-F2 *vs* F3-F6 (Ishak)	NAFLD (*n* = 116)	Biopsy		0.77 (0.67-0.88)					
Chaidez *et al*[105], 2022	**LSM**	United States	F0-F2 *vs* F3-F6 (Ishak)	Non-NAFLD (*n* = 90)	Biopsy		0.70 (0.56-0.83)					
Yang *et al*[106], 2022	**LSM [kPa]**	China	Predicting NASH in children with obesity	NAFLD (*n* = 61), Non-NAFLD (*n* = 59), NAFL (*n* = 44), NASH (*n* = 17)	US	> 5.15	0.725 (0.611-0.839)	64.7	65			0.0048
Xanthakos *et al* [132], 2014	**MRE [kPa]**	United States	F2-F4 *vs* F0-F1	Chronic liver disease (*n* = 35; 27 with NAFLD); F0-F1 (*n* = 27), F2-F4 (*n* = 8)	Biopsy	2.71	0.92 (0.79-1.00)	88	85			0.02
Schwimmer *et al* [133], 2017	**MRE [kPa]**	United States	Any Fibrosis (F0 *vs* F1-4)	F0 (*n* = 54), F1 (*n* = 24), F2 (*n* = 6), F3 (*n* = 5), F4 (*n* = 1)	Biopsy	≥ 2.77	0.77	44.4	90.7	76.2	71	
Schwimmer *et al* [133], 2017	**MRE [kPa]**	United States	Any Fibrosis (F0 *vs* F1-4)	F0 (*n* = 54), F1 (*n* = 24), F2 (*n* = 6), F3 (*n* = 5), F4 (*n* = 1)	Biopsy	≥ 2.69	0.79	47.2	88.9	73.9	71.6	
Schwimmer *et al* [133], 2017	**MRE [kPa]**	United States	Any Fibrosis (F0 *vs* F1-4)	F0 (*n* = 54), F1 (*n* = 24), F2 (*n* = 6), F3 (*n* = 5), F4 (*n* = 1)	Biopsy	≥ 2.78	0.772	44.4	90.7	76.2	71	
Schwimmer *et al* [133], 2017	**MRE [kPa]**	United States	Advanced Fibrosis (F0-2 *vs* F3-4)	F0 (*n* = 54), F1 (*n* = 24), F2 (*n* = 6), F3 (*n* = 5), F4 (*n* = 1)	Biopsy	≥ 3.05	0.925 (0.539-0.989)	50	91.7	30	96.2	
Schwimmer *et al* [133], 2017	**MRE [kPa]**	United States	Advanced Fibrosis (F0-2 *vs* F3-4)	F0 (*n* = 54), F1 (n = 24), F2 (*n* = 6), F3 (*n* = 5), F4 (*n* = 1)	Biopsy	≥ 3.03	0.879 (0.539-0.898)	33.3	94	28.6	95.2	
Schwimmer *et al* [133], 2017	**MRE [kPa]**	United States	Advanced Fibrosis (F0-2 *vs* F3-4)	F0 (*n* = 54), F1 (*n* = 24), F2 (*n* = 6), F3 (*n* = 5), F4 (*n* = 1)	Biopsy	≥ 3.33	0.894 (0.682-0.959)	33.3	90.5	20	95	
Trout *et al*[134], 2018	**MRE [kPa]**	United States	Ludwig stage 0-1 *vs* ≥ stage 2 fibrosis in total cohort	Total (*n* = 86; 48 with NAFLD), Ludwig ≥ stage 2 (*n* = 51), steatosis (*n* = 44)	Biopsy	2.27	0.70 (0.59-0.81)	68.6	74.3			
Trout *et al*[134], 2018	**MRE [kPa]**	United States	Ludwig stage 0-1 *vs* ≥ stage 2 fibrosis in total cohort	Total (*n* = 86; 48 with NAFLD), Ludwig ≥ stage 2 (*n* = 51), steatosis (*n* = 44)	Biopsy	1.67		35.3	91.4			
Trout *et al*[134], 2018	**MRE [kPa]**	United States	Ludwig stage 0-2 from ≥ stage 3 fibrosis	Total (*n* = 86; 48 with NAFLD), Ludwig ≥ stage 2 (*n* = 51),	Biopsy	6.55	0.90 (0.83-0.97)	85.7	77.8			

				steatosis (n = 44)							
Trout *et al*[134], 2018	**MRE [kPa]**	United States	Ludwig stage 0-2 from ≥ stage 3 fibrosis	Total (n = 86; 48 with NAFLD), Ludwig ≥ stage 2 (n = 51), steatosis (n = 44)	Biopsy	5.41		64.3	93.1		
Trout *et al*[134], 2018	**MRE [kPa]**	United States	Ludwig stage 0-1 *vs* ≥ stage 2 fibrosis in patients with steatosis (n = 41)	Total (n = 86; 48 with NAFLD), Ludwig ≥ stage 2 (n = 51), steatosis (n = 44)	Biopsy		0.53 (0.35-0.71)				
Trout *et al*[134], 2018	**MRE [kPa]**	United States	Ludwig stage 0-1 *vs* ≥ stage 2 fibrosis in patients without steatosis (n = 45)	Total (n = 86; 48 with NAFLD), Ludwig ≥ stage 2 (n = 51), steatosis (n = 44)	Biopsy		0.82 (0.67-0.96)				
Alkhouri *et al*[108], 2012	**PNFI**	Italy	≥ F2	F0-F1 (n = 57), F2-F3 (n = 10)	Biopsy	8.2	0.747 (0.632-0.820)				0.005
Nobili *et al*[107], 2008	**TE [kPa]**	Italy	≥ F1	F0 (n = 11), F1 (n = 27), F2 (n = 7), F3-4 (n = 5)	Biopsy	5.1	0.97 (0.90-0.99)	97	91	97	91
Nobili *et al*[107], 2008	**TE [kPa]**	Italy	≥ F2	F0 (n = 11), F1 (n = 27), F2 (n = 7), F3-4 (n = 5)	Biopsy	7.4	0.99 (0.92-0.99)	100	92	80	100
Nobili *et al*[107], 2008	**TE [kPa]**	Italy	≥ F3	F0 (n = 11), F1 (n = 27), F2 (n = 7), F3-4 (n = 5)	Biopsy	10.2	1.00 (0.94-1.00)	100	100	100	100
Alkhouri *et al*[108], 2012	**TE [kPa]**	Italy	≥ F2	F0-F1 (n = 57), F2-F3 (n = 10)	Biopsy	8.6	1.00 (0.981-1.00)				

AUROC: Area under the receiving operating characteristic; CAP: Controlled attenuation parameter; CI: Confidence interval; DX: Diagnosis; F: Fibrosis stage; LSM: Liver stiffness measurement; MRE: Magnetic resonance elastography; NAFL: Nonalcoholic fatty liver; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NPV: Negative predictive value; PNFI: Proton density fat fraction index; PPV: Positive predictive value; Sens: Sensitivity; Spec: Specificity; TE: Transient elastography.

information about the mechanical qualities of the liver being measured. A systematic review of 7 studies (723 adult patients with NAFLD) showed that ARFI had a diagnostic accuracy of 90% in detecting significant fibrosis (sensitivity 80%, specificity 85%)[109]. While there have been studies looking at ARFI in children with fibrosis and chronic liver disease, data on the performance of ARFI specifically in children with NAFLD are limited. One pediatric study found that ARFI values correlated strongly with AST/ALT ratios in obese children. In this study of 54 obese children, 90.7% had ARFI < 1.19 m/s (normal), 7.4% had ARFI values between 1.19 and 1.75 m/s, and 1.9% had ARFI > 1.75 m/s[110]. In another study of 148 school children (33.8% with NAFLD), ARFI values were found to correlate positively with hepatic steatosis grades. ARFI detected significant fibrosis (SWV > 1.60 m/s) in 7.5% of children, 6% of whom had a normal or mildly steatotic liver on ultrasound, suggesting that children with normal/mild steatosis on ultrasound may have significant fibrosis[111]. In a study of 39 children with various biopsy-proven liver etiologies, a SWV of 2.0 m/s had a sensitivity of 100% in detecting advanced fibrosis (≥ F3)[112]. A similar study found that in 52 children with chronic liver disease, ARFI was able to discriminate ≥ F1 fibrosis with an AUROC of 0.834 (sensitivity 78.9%, specificity 76.9%), ≤ F2 with an AUROC of 0.818 (sensitivity 87.5%, specificity 75%), and F4 with an AUROC of 0.983

(sensitivity 100%, specificity 96.7%)[113]. Further research is required to investigate ARFI as an imaging-based NIT in the pediatric NAFLD population.

Magnetic resonance imaging-based biomarkers

Steatosis (MRI-proton density fat fraction, or MRI-PDFF): Magnetic resonance imaging (MRI) is a noninvasive technique that can be used in the evaluation of hepatic steatosis. Magnetic resonance (MR)-based methods are able to measure hepatic fat as a continuous variable and typically measure the signal fat fraction, which is the fraction of the MR signal attributable to hepatic fat. However, this measure can be affected by numerous confounding variables and is scanner dependent. The PDFF removes these confounders and reflects the fraction of the liver proton density attributable to hepatic fat. This is a direct measure of hepatic fat content and is a fundamental property of the hepatic tissue[114,115].

Several studies have shown MRI-PDFF to strongly correlate with histology steatosis grade in adults [116-119]. A 2015 study in children with biopsy-proven NAFLD found that MRI-PDFF was significantly correlated with steatosis grade and that this correlation was influenced by sex and fibrosis stage. The correlation was stronger in girls compared to boys, and weaker in children with more severe fibrosis (F2–F4) compared to mild fibrosis (F0–F1)[120]. In 2018, a multicenter study in children with biopsy-proven NAFLD found that MRI-PDFF had a high diagnostic accuracy in predicting both histologic steatosis grade and change in histologic steatosis grade over time. It found that MRI-PDFF could discriminate between grade 1 steatosis and grade 2–3 steatosis with an AUROC of 0.87 (at a cutoff of 17.5%, it has a sensitivity of 74%, specificity of 90%, PPV of 97%, and NPV of 41%). It could also discriminate grade 1-2 steatosis from grade 3 steatosis with an AUROC of 0.79 (at a cutoff of 23.3%, it yielded the following: sensitivity 60%, specificity 90%, PPV 88%, NPV 65%). MRI-PDFF was able to classify improvement in steatosis grade with an AUROC of 0.76 (sensitivity 31%, specificity 90%, PPV 75%, NPV 60%), and worsening with an AUROC of 0.83 (sensitivity 40%, specificity 90%, PPV 33%, NPV 92%)[121].

A few studies have also investigated MRI-PDFF in discriminating between the presence and absence of steatosis. In 2016, a study of 27 children with biopsy-proven NASH found that a cutoff of 3.5% allowed MRI-PDFF to differentiate between children with NAFLD and healthy controls with a sensitivity of 89% and specificity of 88%[122]. A more recent study including 86 children and adolescents (65 overweight or obese), further investigated the accuracy of MRI in quantifying liver fat against a reference of MRS. MRI-PDFF predicted the presence of steatosis with an AUROC of 0.981 and at a cutoff of 5.4%, yield a sensitivity of 95% and specificity of 91.4%[123].

Recently, Jia *et al*[124] conducted a meta-analysis that showed that MRI-PDFF was accurately able to diagnose stage 1–3 steatosis with a summary sensitivity of 95%, specificity of 92% and hierarchical summary ROC (HSROC) of 0.96. MRI-PDFF was additionally found to be more accurate in assessing steatosis in children compared to TE, which had an HSROC of 0.94 with a sensitivity of 86% and specificity of 88% in differentiating S1–3 from S0. The high diagnostic accuracy and noninvasive nature of MRI-PDFF, therefore, make it a powerful tool in the evaluation of hepatic steatosis in children.

NASH: There are currently no MR-based imaging studies evaluating NASH in children. Even in adults, multiparametric MRE has only limited diagnostic accuracy for NASH[125]. However, a meta-analysis by Kim *et al*[126] which included 485 patients, 207 of whom had simple steatosis and 278 of whom had NASH found that MRI was able to detect NASH with an AUROC of 0.89 and had a pooled sensitivity of 87.4% and pooled specificity of 74.3%. Further research is needed to see whether MR is a feasible technique for diagnosing NASH in children.

Fibrosis (magnetic resonance elastography): Magnetic resonance elastography (MRE) is an MRI-based, noninvasive tool that can be used to diagnose fibrosis in patients with NAFLD and NASH. It uses propagating mechanical shear waves to determine the mechanical properties of hepatic tissue[127]. These shear waves propagate faster in stiffer tissue and the collected wave data is processed by an inversion algorithm that generates cross-sectional, quantitative depictions of the stiffness of hepatic tissue. Unlike ultrasound-based modalities, which provide localized measurements with limited penetration, MRE is able to provide quantitative maps of large regions of the abdomen at a greater depth, making results independent of abdominal wall fat deposition[128]. Studies have shown that MRE is able to accurately determine liver stiffness and assess fibrosis in adults with liver fibrosis[129,130], with an AUROC of 0.86 in predicting advanced fibrosis[131]. However, MRE has not reflected the high accuracy seen in adults in the pediatric population.

In a case-series of 35 children with 8 different biopsy-proven chronic liver diseases, Xanthakos *et al* [132] found that an MRE cutoff of 2.71 kPa had a sensitivity of 88% and specificity of 85% in discriminating F0–F1 from F2–F4 fibrosis. MRE had an AUROC of 0.92 in this study for detecting significant fibrosis. In a multicenter study of 90 pediatric patients, Schwimmer *et al*[133] evaluated the diagnostic utility of two-dimensional gradient-recalled echo MRE (2D GRE MRE) in conjunction with liver biopsy for fibrosis. The study participants, with a mean age of 13.1 ± 2.4 years and 73% male, underwent MRE within 6 mo of liver biopsy. The study found an AUROC of 0.77–0.79 for detection of any fibrosis (≥ F1) and a cutoff of 3.03–3.05 for detection of advanced fibrosis (≥ F3) with an AUROC of 0.88–0.93, sensitivity of 33.3%−50%, specificity of 91.7–94%, PPV of 28.6–30%, and NPV of 95.2–96.2%. The authors

Table 8 Composite scores for the detection of fibrosis in nonalcoholic fatty liver disease

Ref.	Scores	Country	Categories Tested	Sample size (*n*)	Dx	Cutoff	AUROC (95%CI)	Sens (%)	Spec (%)	PPV (%)	NPV (%)	*P* value
Mosca *et al* [101], 2019	**APRI**	Italy	Presence of ≥ F2	No/borderline NASH (*n* = 115), definite NASH (*n* = 89)	Biopsy	> 0.24	0.7659	80	70	92	43	
Mosca *et al* [101], 2019	**APRI**	Italy	Presence of F3	No/borderline NASH (*n* = 115), definite NASH (*n* = 89)	Biopsy	> 0.26	0.8535	100	49	100	100	
Mosca *et al* [142], 2022	**APRI**	Italy	> F1	NAFLD (*n* = 286), F0 (n = 105), F1 (*n* = 140), F2 (*n* = 31), F3 (*n* = 2)	Biopsy		0.619			62.8	52	
Mosca *et al* [142], 2022	**APRI**	Italy	> F2	NAFLD (*n* = 286), F0 (*n* = 105), F1 (*n* = 140), F2 (*n* = 31), F3 (*n* = 2)	Biopsy		0.74			86	78.1	
Mansoor *et al* [140], 2015	**APRI**	United States	Presence of F1-F4	NAFLD (*n* = 92)	Biopsy		0.800 (0.695-0.904)					
Mansoor *et al* [140], 2015	**APRI**	United States	Presence of F2-F4	NAFLD (*n* = 92)	Biopsy		0.666 (0.553-0.778)					
Mansoor *et al* [140], 2015	**APRI**	United States	Presence of F3-F4	NAFLD (*n* = 92)	Biopsy		0.628 (0.478-0.778)					
Mansoor *et al* [140], 2015	**AST/ALT ratio**	United States	Presence of F1-F4	NAFLD (*n* = 92)	Biopsy		0.572 (0.350, 0.793)					
Mansoor *et al* [140], 2015	**AST/ALT ratio**	United States	Presence of F2-F4	NAFLD (*n* = 92)	Biopsy		0.585 (0.466-0.703)					
Mansoor *et al* [140], 2015	**AST/ALT ratio**	United States	Presence of F3 - F4	NAFLD (*n* = 92)	Biopsy		0.441 (0.316-0.565)					
Mandelia *et al* [84], 2016	**CK18 + WC percentile**	Italy	Presence of ≥ F1	NAFLD (*n* = 201), F0 (*n* = 65), F1–F3 (*n* = 136)	Biopsy	≥ 35	0.84 (0.79-0.90)	97	38	76	86	
Mandelia *et al* [84], 2016	**CK18 + WC percentile**	Italy	Presence of ≥ F1	NAFLD (*n* = 201), F0 (*n* = 65), F1–F3 (*n* = 136)	Biopsy	> 82		59	88	91	51	
Gawrieh *et al* [150], 2021	**ELF**	United States	Any fibrosis (≥ F1)	NAFLD (*n* = 173), borderline/suspicious NASH (*n* = 73), definite NASH (*n* = 71)	Biopsy		0.60 (0.50–0.70)					0.11
Gawrieh *et al* [150], 2021	**ELF**	United States	Clinically significant (≥ F2)	NAFLD (*n* = 173), borderline/suspicious NASH (*n* = 73), definite NASH (*n* = 71)	Biopsy		0.70 (0.60–0.80)					< 0.001
Gawrieh *et al* [150], 2021	**ELF**	United States	Advanced fibrosis (≥ F3)	NAFLD (*n* = 173), borderline/suspicious NASH (*n* = 73), definite NASH (*n* = 71)	Biopsy		0.79 (0.69–0.89)					< 0.001
Chaidez *et al* [105], 2022	**FAST score**	United States	Significant liver disease (NAS ≥ 4 and Ishak ≥ 3) *vs* NAS < 4 / Ishak < 3)	Chronic liver disease (*n* = 206; 116 with NAFLD)	Biopsy	≥ 0.67	0.75 (0.56-0.94)	89	62			

Mosca *et al* [101], 2019	**FIB-4**	Italy	Presence of ≥ F2	No/borderline NASH (*n* = 115), definite NASH (*n* = 89)	Biopsy	> 0.22	0.7412	64	72	88	39	
Mosca *et al* [101], 2019	**FIB-4**	Italy	Presence of F3	No/borderline NASH (*n* = 115), definite NASH (*n* = 89)	Biopsy	> 0.24	0.7687	86	71	99	9	
Arsik *et al*[54], 2018	**Mean ALT over 96 weeks**	United States	Fibrosis	Fibrosis (*n* = 128), NASH (*n* = 131)	Biopsy		58.56	56.5	64.6			
Mosca *et al* [142], 2022	**FIB-4**	Italy	> F1	NAFLD (*n* = 286), F0 (*n* = 105), F1 (n = 140), F2 (*n* = 31), F3 (*n* = 2)	Biopsy		0.545			62	52	
Mosca *et al* [142], 2022	**FIB-4**	Italy	> F2	NAFLD (*n* = 286), F0 (*n* = 105), F1 (*n* = 140), F2 (*n* = 31), F3 (*n* = 2)	Biopsy		0.588					
Mansoor *et al* [140], 2015	**FIB-4**	United States	Presence of F1-F4	NAFLD (*n* = 92)	Biopsy		0.547 (0.375-0.719)					
Mansoor *et al* [140], 2015	**FIB-4**	United States	Presence of F2-F4	NAFLD (*n* = 92)	Biopsy		0.686 (0.576-0.797)					
Mansoor *et al* [140], 2015	**FIB-4**	United States	Presence of F3-F4	NAFLD (*n* = 92)	Biopsy		0.367 (0.231-0.503)					
Gawrieh *et al* [150], 2021	**HA**	United States	Any fibrosis (≥ F1)	NAFLD (*n* = 173), borderline/suspicious NASH (*n* = 73), definite NASH (*n* = 71)	Biopsy		0.57 (0.47–0.67)					0.32
Gawrieh *et al* [150], 2021	**HA**	United States	Significant fibrosis (≥ F2)	NAFLD (*n* = 173), borderline/suspicious NASH (*n* = 73), definite NASH (*n* = 71)	Biopsy		0.64 (0.54–0.74)					0.002
Gawrieh *et al* [150], 2021	**HA**	United States	Advanced fibrosis (≥ F3)	NAFLD (*n* = 173), borderline/suspicious NASH (*n* = 73), definite NASH (*n* = 71)	Biopsy		0.77 (0.66–0.88)					0.001
Mosca *et al* [142], 2022	**Hepamet**	Italy	≥ F2	NAFLD (*n* = 286), F0 (*n* = 105), F1 (*n* = 140), F2 (*n* = 31), F3 (*n* = 2)	Biopsy		0.73			88.8	76.6	
Mosca *et al* [142], 2022	**Hepamet**	Italy	> F1	NAFLD (*n* = 286), F0 (*n* = 105), F1 (*n* = 140), F2 (*n* = 31), F3 (*n* = 2)	Biopsy		0.778			63.2	61.3	
Mansoor *et al* [140], 2015	**NFS**	United States	Presence of F1-F4	NAFLD (*n* = 92)	Biopsy		0.470 (0.259-0.681)					
Mansoor *et al* [140], 2015	**NFS**	United States	Presence of F2-F4	NAFLD (*n* = 92)	Biopsy		0.554 (0.435-0.673)					
Mansoor *et al* [140], 2015	**NFS**	United States	Presence of F3-F4	NAFLD (*n* = 92)	Biopsy		0.521 (0.385-0.657)					
Mosca *et al* [142], 2022	**NFS**	Italy	> F1	NAFLD (*n* = 286), F0 (*n* = 105), F1 (*n* = 140), F2 (*n* = 31), F3 (*n* = 2)	Biopsy		0.537			62	52	
Mosca *et al* [142], 2022	**NFS**	Italy	> F2	NAFLD (*n* = 286), F0 (*n* = 105), F1 (*n* = 140), F2 (*n* = 31), F3 (*n* = 2)	Biopsy		0.6					
Gawrieh *et al*	**PIIINP**	United States	Any fibrosis (≥ F1)	NAFLD (*n* = 173), borderline/suspicious	Biopsy		0.55 (0.45–0.65)					0.18

[150], 2021				NASH (n = 73), definite NASH (n = 71)						
Gawrieh *et al* [150], 2021	**PIIINP**	United States	Clinically significant (≥ F2)	NAFLD (n = 173), borderline/suspicious NASH (n = 73), definite NASH (n = 71)	Biopsy		0.66 (0.57–0.75)			0.002
Gawrieh *et al* [150], 2021	**PIIINP**	United States	Advanced fibrosis (≥ F3)	NAFLD (n = 173), borderline/suspicious NASH (n = 73), definite NASH (n = 71)	Biopsy		0.65 (0.53–0.76)			0.06
Mosca *et al* [142], 2022	**PNFI**	Italy	> F1	NAFLD (n = 286), F0 (n = 105), F1 (n = 140), F2 (n = 31), F3 (n = 2)	Biopsy		0.81	90.3	75.4	
Nobili *et al* [148], 2009	**PNFI**	Italy	≥ F1	NAFLD (n = 203), Fibrosis (n = 141), no fibrosis (n = 62), stage 1 fibrosis (n = 115), stage 2 fibrosis (n = 9), stage 3 fibrosis (n = 17)	Biopsy	≥ 9	0.85 (0.80-0.90)	98.5		
Mosca *et al* [142], 2022	**PNFI**	Italy	> F2	NAFLD (n = 286), F0 (n = 105), F1 (n = 140), F2 (n = 31), F3 (n = 2)	Biopsy		0.84	97.5	72.6	
Gawrieh *et al* [150], 2021	**TIMP-1**	United States	Any fibrosis (≥ F1)	NAFLD (n = 173), borderline/suspicious NASH (n = 73), definite NASH (n = 71)	Biopsy		0.63 (0.54–0.72)			0.02
Gawrieh *et al* [150], 2021	**TIMP-1**	United States	Clinically significant (≥ F2)	NAFLD (n = 173), borderline/suspicious NASH (n = 73), definite NASH (n = 71)	Biopsy		0.63 (0.53–0.72)			0.01
Gawrieh *et al* [150], 2021	**TIMP-1**	United States	Advanced fibrosis (≥ F3)	NAFLD (n = 173), borderline/suspicious NASH (n = 73), definite NASH (n = 71)	Biopsy		0.76 (0.64–0.88)			< 0.001

ALT: Alanine aminotransferase; AUROC: Area under the receiver operating characteristic curve; AST: Aspartate aminotransferase; APRI: AST to platelet ratio index; CI: Confidence interval; Dx: Diagnosis; ELF: Enhanced liver fibrosis; F: Fibrosis stage; FAST: FibroScan-aspartate aminotransferase; FIB-4: Fibrosis-4, Fibrosis-4; HA: Hyaluronic acid; NAS: NAFLD activity score; NAFLD: Nonalcoholic fatty liver disease; NFS: NAFLD Fibrosis Score; NPV: Negative predictive value; PIIINP: Amino-terminal propeptide of type III procollagen; PNFI: Pediatric NAFLD Fibrosis Index; PPV: Positive predictive value; Sens: Sensitivity; Spec: Specificity; TIMP-1: Tissue inhibitor of metalloproteinase-1; WC, waist circumference.

caution that cutoffs validated in adult populations may not be appropriate for interpreting pediatric MRE results. Trout *et al*[134] found that MRE had an AUROC of 0.70 for differentiating Ludwig stage 0–1 from ≥ stage 2 fibrosis (defined as fibrosis with few bridges or septa)[135] in 86 children and young adults with a spectrum of biopsy-proven liver diseases. A cutoff of ≥ 2.27 kPa had sensitivity 68.6% and specificity 74.3% while a cutoff of ≥ 1.67 kPa had sensitivity 35.3%, specificity 91.4%. This study also found an AUROC of 0.90 for discriminating Ludwig stage 0–2 from ≥ Ludwig stage 3 (numerous bridges or septa). A cutoff of 5.41 kPa had a sensitivity of 64.3% and specificity of 93.1%. The study found that MRE was able to better distinguish between stage 0–1 and stage 2–4 fibrosis in patients without steatosis compared to patients with steatosis; suggesting that steatosis was playing a confounding effect in children with NAFLD[134]. A 2019 study in 69 children with biopsy-proven NAFLD found that MRE liver stiffness values (based on 2D GRE or 2D spin-echo echo-planar imaging pulse sequence) did not significantly differentiate ≥ F2 from[136].

NOVEL COMPOSITE SCORES

While several circulating biomarkers have shown promise in their diagnostic abilities for NAFLD, NASH, and fibrosis, none are currently being used in lieu of liver biopsy in the clinical setting. Multiple studies have demonstrated that novel composite algorithms that combine multiple serological biomarkers can sometimes have higher diagnostic accuracy than their solo components.

NASH SCORES

Manco *et al*[57] showed that a combination of Ang-2 and CK18 was able to predict NASH with a sensitivity of 71.4%, specificity of 100% (PPV 100%, NPV 80.4%), which was superior to Ang-2 or CK18 alone (Table 7). Similarly, combining CK18 with CatD was able to discriminate steatosis from NASH with an AUROC of 0.998, compared to CK18 (AUROC 0.72) or CatD (AUROC 0.94) alone[70].

A study by Kwon *et al*[137] looked at the use of bone formation biomarkers in children with NAFLD. Procollagen type 1 amino-terminal propeptide (P1NP) is a protein secreted by the ECM that has been implicated in liver disease in adults[138] and in liver fibrogenesis in animal models[139]. P1NP is typically elevated in children and adolescents compared to adults given their increased rate of bone formation. To correct for this, the researchers measured levels of serum osteocalcin, another marker of bone formation, and alkaline phosphatase (ALP), in the study cohort. The study found that the P1NP/osteocalcin ratio alone had a diagnostic capability for evaluating steatohepatitis (early fibrosis) with an AUROC of 0.782 (sensitivity 80.9%, specificity 76.9%). However, the diagnostic capability was higher when the ratio was multiplied by ALT, with an AUROC of 0.939 (sensitivity 83%, specificity 92.3%). Similarly, the P1NP/ALP ratio alone had a diagnostic capability with an AUROC of 0.788 (sensitivity 78.8%, specificity 81.3%), but it also showed better diagnostic capability when multiplied by ALT, with an AUROC of 0.894 (sensitivity 82.6%, specificity 92.9%)[137].

FIBROSIS SCORES

Liver fibrosis has been well-established as the most important determinant for survival in adults with NAFLD[12]. As end-stage liver disease [also known as cirrhosis (F4)] exists at the end of the fibrosis staging scale, higher fibrosis stages are more likely to progress to cirrhosis compared to earlier stages. Multiple scoring systems have been developed to determine the level of fibrosis in adults with NAFLD, including the AST/ALT ratio, APRI, FIB-4, and NFS. Studies in the last decade have shown that these scores may not be accurately used in the pediatric population for predicting fibrosis[140,141]. There have also been scores that have been developed specifically for children, including the pediatric NAFLD fibrosis index (PNFI) and pediatric NAFLD fibrosis score (PNFS). This section will discuss these simple scoring algorithms and novel composite algorithms for the diagnosis of fibrosis in children with NAFLD (Table 8).

Simple fibrosis scores

Pediatric studies have evaluated simple serum tests such as ALT, AST/ALT ratio, APRI, NFS, and FIB-4 for their diagnostic performance in detecting fibrosis. These tests have been well-studied in adult populations. One pediatric study reported poor diagnostic performance in detecting any fibrosis (≥ F1) for the AST/ALT ratio (AUROC 0.572), FIB-4 (AUROC 0.547), and NFS (AUROC 0.470). However, all three tests performed better for the detection of significant fibrosis (≥ F2): AST/ALT ratio (AUROC 0.585), FIB-4 (AUROC 0.686), and NFS (AUROC 0.554). APRI had the best performance of the surveyed tests, with an AUROC of 0.800 for the detection of any fibrosis (≥ F1) and 0.628-0.70 for ≥ F3-4 in the pediatric NAFLD population[140].

A recent study by Mosca *et al*[142] also evaluated APRI, FIB-4, NFS, and a score called Hepamet in predicting the degree of fibrosis in 286 children with biopsy-proven NAFLD. The Hepamet fibrosis score (HFS) is based on demographic and laboratory data (including sex, age, +/- diabetes, and serum lab values) and was developed to identify adult patients with NAFLD at risk for advanced fibrosis. HFS was able to discriminate between adults with and without advanced fibrosis with an AUROC 0.85 compared to NFS and FIB-4 with AUROC 0.80 (P = 0.0001)[143]. The study by Mosca *et al*[142] found that APRI had an AUROC of 0.61 (PPV 62.77%, NPV 52.01%) in identifying > F1 fibrosis in children with NAFLD compared to Hepamet which had an AUROC of 0.778 (PPV 63.24%, NPV 61.29%). NFS and FIB-4 both had poor accuracy for the diagnosis of fibrosis with both having AUROC 0.54 (PPV 62%, NPV 52%). APRI and Hepamet both had an AUROC of 0.74 in identifying the presence of > F2 fibrosis, higher than those for FIB-4 and NFS (AUROC 0.58–0.60). Interestingly, PNFI had a higher AUROC for identifying both > F1 (AUROC 0.81) and > F2 (AUROC 0.84) compared to these other scores. PNFI was the best noninvasive biomarker in the pediatric age, however, Hepamet showed promise[142].

Table 7 Composite scores for the detection of nonalcoholic steatohepatitis

Ref.	Scores	Country	Categories Tested	Sample size (*n*)	Dx	Cutoff	AUROC (95%CI)	Sens (%)	Spec (%)	PPV (%)	NPV (%)
Manco *et al* [57], 2022	**Ang-2 + CK18**	Italy	Diagnosing NASH	NAFLD (*n* = 76), healthy controls (*n* = 28, by ultrasound)	Biopsy			71.4	100	100	80.4
Mosca *et al* [101], 2019	**APRI**	Italy	Definite NASH *vs* No/Borderline NASH	No/borderline NASH (*n* = 115), definite NASH (*n* = 89)	Biopsy	> 0.24	0.6826	58	72	69	62
Walenbergh *et al*[70], 2015	**CatD + CK18**	Italy	Steatosis from NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy		0.998				
Walenbergh *et al*[70], 2015	**CatD + CK18**	Italy	Borderline NASH *vs* definite NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy		0.858				
Walenbergh *et al*[70], 2015	**CatD + CK18**	Italy	Steatosis + Borderline NASH *vs* NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy		0.892				
Walenbergh *et al*[70], 2015	**CatD + CK18**	Italy	Steatosis *vs* borderline NASH + NASH	NASH (*n* = 26), borderline NASH (*n* = 51), steatosis (*n* = 19), obese (*n* = 96)	Biopsy		0.85				
Mosca *et al* [101], 2019	**FIB-4**	Italy	Definite NASH *vs* No/Borderline NASH	No/borderline NASH (*n* = 115), definite NASH (*n* = 89)	Biopsy	> 0.22	0.6369	48	73	65	58
Arsik *et al* [54], 2018	**Mean ALT over 96 wk**	United States	NASH	Fibrosis (*n* = 128), NASH (*n* = 131)	Biopsy		81.84	80.5	83		
Arsik *et al* [54], 2018	**Mean ALT over 96 wk**	United States	NASH + Fibrosis	Fibrosis (*n* = 128), NASH (*n* = 131)	Biopsy		77.78	71.8	80.8		
Kwon *et al* [137], 2022	**P1NP/ALP ratio**	Korea	Presence of steatohepatitis	NAFLD (*n* = 60)	US	1.46	0.788 (0.658-0.918)	78.8	81.3		
Kwon *et al* [137], 2022	**P1NP/ALP ratio × ALT**	Korea	Presence of steatohepatitis	NAFLD (*n* = 60)	US	119.08	0.894 (0.812-0.977)	82.6	92.9		
Kwon *et al* [137], 2022	**P1NP/osteocalcin ratio**	Korea	Presence of steatohepatitis	NAFLD (*n* = 60)	US	3.54	0.782 (0.647-0.918)	80.9	76.9		
Kwon *et al* [137], 2022	**P1NP/Osteocalcin ratio × ALT**	Korea	Presence of steatohepatitis	NAFLD (*n* = 60)	US	305.38	0.939 (0.88-0.999)	83	92.3		
Manco *et al* [95], 2007	**Risk Score**	Italy	Predicting NAFLD Activity Score	NAFLD (*n* = 72), F0 (*n* = 31), F1 (*n* = 41)	Biopsy	≤ 12.9	0.985	9	2	4	33
Manco *et al* [95], 2007	**Risk Score**	Italy	Predicting NAFLD Activity Score	NAFLD (*n* = 72), F0 (*n* = 31), F1 (*n* = 41)	Biopsy	≥ 13.5		81	92	82	92

ALP: Alkaline phosphatase; ALT: Alanine transaminase; Ang-2: Angiopoietin-2; APRI: AST to platelet ratio index; AUROC: Area under the receiving operating characteristic; CatD: Cathepsin D; CK18: Cytokeratin 18; CI: Confidence interval; Dx: Diagnosis; F: Fibrosis stage; FIB-4: Fibrosis-4; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; P1NP: Procollagen type 1 amino-terminal propeptide; PPV: Positive predictive value; NPV: Negative predictive value; Risk Score: 0.440 + (1.454 × ln leptin) + (4.617 × ln TNF-α); Sens: Sensitivity; Spec: Specificity; TNF-α: Tumor necrosis factor-alpha; US: Ultrasound.

FibroScan-aspartate aminotransferase score

The FibroScan-aspartate aminotransferase (FAST) score is calculated using LSM, CAP, and serum AST and is used to predict liver disease severity in adults with NAFLD. In adults, it has been found to be an efficient way to non-invasively identify patients at risk of progressive NASH for clinical trials or treatments[144]. Chaidez *et al*[105] found that the FAST score had acceptable discriminatory ability for significant liver disease (NAS ≥ 4 and Ishak ≥ 3) with an AUROC of 0.75. At a cutoff of ≥ 0.67, it had a sensitivity 89% and specificity 62%.

Pediatric NAFLD fibrosis index

The Pediatric NAFLD fibrosis index (PNFI) was the first noninvasive fibrosis score created for children. Developed by Nobili *et al*[145] in a study of 136 children with biopsy-proven fibrosis, the PNFI uses age, waist circumference, and triglycerides to determine the degree of fibrosis. The PNFI had an AUROC of 0.85 in predicting liver fibrosis (≥ F1) and PNFI ≥ 9 had a PPV of 98.5%. However, the AUROC dropped to 0.41 in predicting ≥ F2 fibrosis in an external validation of the PNFI in a cohort of Korean children [141]. Interestingly, Alkhouri *et al*[108] found that PNFI had an AUROC of 0.747 in predicting the presence of clinically significant fibrosis (≥ F2) in children with NAFLD. A cutoff of PNFI > 8.2 provided 97% accuracy in predicting early fibrosis (F0–F1) and could be used to rule out patients with significant fibrosis. When combined with data for TE, the algorithm could predict the presence or absence of clinically significant fibrosis in 98% of children with NAFLD.

PNFS

The PNFS was developed in 2014 by Alkhouri *et al*[146] from a cohort of 242 children with biopsy-proven NAFLD. The PNFS uses ALT, alkaline phosphatase, platelet counts and GGT and was found to have an AUROC of 0.74 for the detection of advanced fibrosis (≥ F3). At a cutoff of 26%, PNFS had a sensitivity of 31%, specificity 92%, PPV 41% and NPV 88%. This scoring algorithm was found to be superior to APRI, FIB-4 and the NAFLD Activity Score (NAS). PNFS has not been validated by outside groups in the pediatric NAFLD population since being developed.

Enhanced liver fibrosis

The enhanced liver fibrosis (ELF) test was first characterized and validated in a cohort of adults with chronic liver disease[147]. ELF has been studied extensively in adults and shown to be an excellent marker of fibrosis in adults with chronic liver disease[76]. It is calculated using three serum biomarkers: HA, PIIINP, and TIMP1. Interestingly, these three markers have themselves been studied as serological biomarkers of fibrosis in children with NAFLD. In studies of adults with NAFLD/NASH, ELF has been validated and found to have an AUROC of 0.9 in distinguishing severe fibrosis, 0.82 for moderate fibrosis and 0.76 for no fibrosis[76]. In a pediatric study of 112 children with likely NAFLD, ELF was able to accurately predict the stage of fibrosis with an AUROC of 0.92 for any fibrosis (≥ stage 1), 0.92 for moderate-perisinusoidal fibrosis (≥ stage 1b), 0.90 for moderate-portal/periportal fibrosis (≥ stage 1c), and 0.99 for advanced fibrosis (≥ stage 3). The ELF test was found to have a high accuracy in predicting any fibrosis, with an AUROC of 0.92 (at an optimal cutoff value of 9.28, it had sensitivity of 88%, specificity of 81%, PPV of 90% and NPV of 77%)[148]. A later study by Alkhouri *et al*[149] found a lower optimal cutoff of 8.49 (sensitivity 76.9%, specificity 97%). Interestingly, when PNFI and ELF were combined, they were able to predict the presence or absence of fibrosis in 86.4% of children with NAFLD. It is important to note that ELF uses biomarkers that are not commonly available in a blood biochemistry panel, and therefore, potentially is less accessible than other fibrosis scores. Another study by Gawrieh *et al*[150] evaluated the relationship of the ELF score with histology in children from the Treatment of NAFLD In Children trial and found that ELF was significantly associated with severity of fibrosis at baseline and 2 years after treatment. In determining the presence of any fibrosis (≥ F1), ELF has an AUROC of 0.60, for ≥ F2 AUROC was 0.70, and for ≥ F3 AUROC was 0.79. ELF requires further validation in the pediatric NAFLD population prior to being implemented in clinical practice.

CONCLUSION

Pediatric NAFLD has increased in prevalence over the past decade in conjunction with the obesity epidemic. An early diagnosis of NAFLD, NASH, and fibrosis play a large part in preventing disease progression and tailoring management for patients. Therefore, it is very important that we develop and validate noninvasive methods of diagnosis for children with NAFLD.

Adiponectin, IL-1β, IL-6, and IL-17 all have strong diagnostic accuracy in identifying NAFLD in children with obesity with AUROC values exceeding 0.90. CK18 is extensively studied in children and appears to be the most promising serology-based NIT for the diagnosis of pediatric NASH. Given that adding CK18 to individual biomarkers, such as Ang-2 or CatD, significantly increases their diagnostic abilities, it stands to reason that further research into composite algorithms including CK18 will generate productive results. While select serological biomarkers (*e.g.* PIIINP and HA) have AUROCs >

0.90 in predicting advanced fibrosis, most of these serological markers perform poorly in detecting the presence of mild-moderate fibrosis, highlighting the need for further research in this area.

With regard to imaging, MRI-PDFF is superior to ultrasound-based CAP for the diagnosis of steatosis. While TE has excellent performance in differentiating stages of fibrosis, MRI-based methods that have weaker performance have an advantage when it comes to visualization of the liver both in terms of penetration depth and field of view. This is especially important given the rising number of children who have both NAFLD and (morbid) obesity. Future research should focus particularly on better noninvasive imaging modalities for the diagnosis of pediatric NASH, with excellent performance in diverse pediatric populations.

Currently, clinical practice in adults oftentimes utilizes a two-step screening approach for NAFLD prior to considering liver biopsy. In children with NAFLD, one could consider screening with ALT levels first in children with risk factors and, if above the gender-specific upper limit of normal, proceed to subsequent elastography with fat quantification through MRI-PDFF or ultrasound-based CAP measures. MRI-based methods would be preferred over ultrasound-based techniques in obese patients given the higher success rate. Clinicians would then proceed with liver biopsy if imaging reveals concerning findings and/or if ALT levels are persistently elevated > 80 U/L. Further studies are warranted to determine the cost-effectiveness of widespread implementation of elastography in the work-up of pediatric NAFLD.

FOOTNOTES

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MINIREVIEWS

Hypothyroidism and non-alcoholic fatty liver disease: A coincidence or a causal relationship?

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a global problem. It may be caused by metabolic and hormonal disorders, including hypothyroidism. However, non-thyroid causes of NAFLD in people with hypothyroidism, including improper eating behavior and low physical activity, should be acknowledged. This study aimed to present the current literature on whether the development of NAFLD is related to hypothyroidism or a typical consequence of an unhealthy lifestyle in people with hypothyroidism. The results of previous studies do not allow for an unequivocal determination of the pathogenetic relationship between hypothyroidism and NAFLD. Important non-thyroid-initiating factors include providing too many calories in relation to requirements, consuming excessive amounts of monosaccharides and saturated fats, being overweight, and maintaining low physical activity levels. The recommended nutritional model for both hypothyroidism and NAFLD may be the Mediterranean diet, which is rich in fruits and vegetables, polyunsaturated fatty acids, and vitamin E.

Key Words: Non-alcoholic fatty liver disease; Hypothyroidism; Lifestyle; Exercise; Feeding behavior; Body weight

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Core Tip: Non-alcoholic fatty liver disease affects 25% of the adult population worldwide; however, the causes of the disease remain unclear. This review answers the question of whether the development of non-alcoholic fatty liver disease is related to hypothyroidism or whether it is a typical consequence of an unhealthy lifestyle in people with hypothyroidism.

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INTRODUCTION

Global statistics indicate an increasing prevalence of non-alcoholic fatty liver disease (NAFLD) in society[1]. Currently, this disease affects approximately 25% of the adult population worldwide. It is often diagnosed in developed countries and is the second most common indication for liver transplantation[2].

The etiology of NAFLD is complex. NAFLD may be a result of excessive body weight, obesity, or carbohydrate and lipid metabolism disorders[3]. NAFLD is defined as a hepatic manifestation of metabolic syndrome[3,4]. The disorders mentioned are serious problems, which are related to improper eating habits and low physical activity, but may also have endocrine causes[5,6]. The most frequently described associations between NAFLD and endocrinopathies are polycystic ovary syndrome (PCOS) (PubMed: NAFLD PCOS - 93 publications in the last 5 years) and primary and secondary hypothyroidism (PubMed: NAFLD hypothyroidism - 86 publications in the last 5 years), which are the subject of the authors' considerations[7,8]. The accumulation of fatty compounds in the liver is associated with abnormal concentrations of cortisol, insulin, thyroxine, somatotropin, testosterone, and prolactin, which may be abnormal in PCOS and/or hypothyroidism[8]. The need for further research on thyroid-hepatic interdependence is still highlighted in publications from 2022. This is because of the insufficient amount of data available within the thematic scope[9-13].

Considering the relationship between non-alcoholic fatty liver disease, lifestyle, and endocrine disorders, including thyroid dysfunction, it is important to determine whether the development of NAFLD is related to hypothyroidism as its result or a typical consequence of an improper lifestyle of people with hypothyroidism. This study presents considerations within this scope based on the current literature.

LITERATURE REVIEW

This literature review of the PubMed electronic database included 54 scientific articles over 5 years and 11 articles published between 2012 and 2016, including meta-analyses, cohort, and experimental studies. The following words were used to search for publications between October 2022 and February 2023: NAFLD and hypothyroidism = 86 publications, NAFLD and physical activity = 1009 publications, NAFLD and lifestyle = 1258 publications, NAFLD and Mediterranean diets = 172 publications, and lipid metabolism and thyroid = 693 publications. The last search was conducted in February 2023.

LIPID METABOLISM IN NORMAL PHYSIOLOGY AND NAFLD

Hepatic lipid metabolism involves three substrate delivery mechanisms. The first is the absorption of fatty acids from chylomicrons formed during the absorption of lipids from food in the digestive system [14]. The second involves lipids stored in the adipocytes. Fatty acids supplied by food are stored as triglycerides in adipose tissue cells and in the liver. Triglyceride lipase affects the triglycerides in the adipose tissue, resulting in the release of fatty acids followed by their uptake by liver cells[14]. The third mechanism is de novo lipogenesis, in which hepatocytes absorb fatty acids due to the conversion of the consumed carbohydrates into fats[14].

Lipid metabolism in the liver can be classified into three transformations: oxidation of supplied fatty acids in the process of beta-oxidation, accumulation of fatty acids, or formation of protein complexes with fatty acid participation[14]. In NAFLD, fatty acid metabolism is disrupted. The accumulation of fatty compounds begins to increase, contrary to the synthesis and secretion of very-low-density lipoprotein (LDL) into the blood. *De novo* lipogenesis exceeds the efficiency of oxidation processes and the concentration of serum triglycerides of extrahepatic origin in the increases[14,15]. NAFLD is a

progressive disease that begins with simple NAFLD, which may coexist with non-alcoholic steatohepatitis (NASH). NASH may be associated with fibrosis, leading to cirrhosis of the organ and the development of hepatocellular carcinoma[16].

THYROID MECHANISMS OF METABOLIC CONTROL

Follicular thyroid cells produce and secrete thyroxine (T4) and triiodothyronine (T3) hormones. The control of their secretion depends on the concentrations of hormones of the hypothalamus (thyroliberin), and pituitary gland [tyrosine (TSH)]. The mechanism aimed at maintaining homeostasis is a negative feedback mechanism[17].

Thyroid hormones perform numerous functions, including metabolic control, maintenance of body temperature, regulation of hunger and satiety, and regulation of carbohydrate and lipid metabolism [18]. This is possible because of the localization of thyroid hormone receptors in the cells of many organs and tissues; for example, the liver (THR-beta), pancreas, adipose tissue (THR-alpha), and muscle tissue (THR-alpha)[18,19]. TSH receptors, which are located on thyroid cells, have also been located outside the thyroid gland in adipose tissue cells, hepatocytes, and ovaries[18,20].

Under the influence of thyroid hormones, the fat contained in adipocytes is broken down into free fatty acids, which are transported through the bloodstream to the hepatocytes by binding (1-L-fatty acid-binding protein and 36-CD36 fatty acid translocase) and transporting (fatty acid transport protein) proteins[19]. The function of these proteins is also regulated by thyroid hormones, which participate in de novo lipogenesis by enhancing gene expression, including the expression of Spot14[12,21]. Moreover, the activity of hepatic lipase, which breaks down fats into fatty acids, enabling their beta-oxidation, is dependent on the concentration of thyroid gland hormones[22].

HYPOTHYROIDISM AND ITS EFFECTS ON LIPID METABOLISM

Hypothyroidism is a disease in which the serum concentration of thyroid hormones is reduced[14,23]. This disease affects approximately 5% of the population[24]. Contributors of disease development include an insufficient supply of iodine, a component of thyroid hormones, or Hashimoto's disease. In the latter case, there is inflammatory lymphocytic infiltration and the production of antibodies against enzymes that enable the production of thyroid hormones, such as thyroid peroxidase and thyroglobulin [24]. Due to the formation of an insufficient amount of hormones, numerous disorders affecting the homeostasis of the body arise. The main common features of people with uncontrolled hypothyroidism are the occurrence of lipid disorders, increased blood cholesterol and triglyceride levels, and accumulation of fatty compounds in the liver[25,26]. Such disorders result from insufficient concentrations of thyroid hormones and excessive concentrations of TSH in the blood, which increase the production and secretion of T3 and T4 by the thyroid cells, according to the negative feedback effect. It should be noted that thyroxine supplementation significantly affects metabolism, causing an increase in the basic metabolic rate. This results in increased energy expenditure and, in the case of maintaining a negative caloric balance, may contribute to weight reduction[27].

In mouse models, elevated TSH levels in hypothyroidism have been observed to increase the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase in liver cells. This results in changes in cholesterol synthesis, which may lead to the accumulation of fatty compounds in the liver[28,29].

The regulation of lipid concentration in the body also occurs at a genetic level through transcription factors, including sterol regulatory element-binding protein (SREBP) and liver X receptor (LXR)[30,31]. LXR has also been detected in the liver, and is both a thyroid hormone receptor and a nuclear receptor. SREBP controls lipid synthesis, which is significantly influenced by thyroid hormone levels. The effect of the action of thyroid hormones on SREBP-2, an isoform of SREBP, is a decrease in the expression of the LDL receptor, which manifests as an increase in serum cholesterol levels. In cases of thyroid hormone deficiency, this situation is reversed[30].

In both overt and subclinical hypothyroidism, there is an increase in the concentration of angiopoietin-like proteins Angptl-3 and Angptl-8, which participate in lipid metabolism and inhibit the action of lipoprotein lipase. The weakened function of lipoprotein lipase can lead to fat accumulation in the liver and decrease LDL cholesterol breakdown. The results of studies on the occurrence of increased concentrations of Angplt3 and 8 are clear, and scientists suggest using these proteins in the detection of hypothyroidism, although high concentrations have also been observed in obesity and diabetes[32,33].

Fibroblast growth factor FGF-21, which is still under study, enhances the beta-oxidation of fatty acids in the liver (where it is produced) while slowing down the formation of triglycerides. In acute hypothyroidism caused by radioiodine treatment, there is an increase in FGF-21 concentration, which ultimately predisposes patients to hepatic steatosis by enhancing lipogenesis[34].

The control of hepatocyte autophagy is another function that involves thyroid hormones[35]. In autophagy within liver cells, NCoR1, a co-repressor of nuclear receptor 1 under typical conditions, is degraded. In hypothyroidism, beta-oxidation is reduced by affecting peroxisome proliferator-activated

alpha receptor, resulting in an increase in de novo lipogenesis[36].

The influence of thyroid hormones on lipid metabolism and liver function is a topic widely described in the scientific literature, and control mechanisms include multilevel interactions at molecular and cellular levels[37]. These relationships are still being researched, proving the importance of the subject [38,39].

NAFLD AS A RESULT OF METABOLIC DISORDERS CAUSED BY AN IMPROPER LIFESTYLE IN PEOPLE WITH HYPOTHYROIDISM

The relationship between NAFLD and hypothyroidism has been the subject of population studies in several countries. These results are sometimes contradictory, although most indicate the existence of a relationship[40]. In a cohort study involving 81166 German residents, a strong relationship between the diseases was found[41]. In contrast, no associations were found in a study involving 10116 Spanish residents, similar to the results of a retrospective study conducted among 18544 Korean residents[42, 43]. Therefore, it is important to consider the non-thyroid causes of NAFLD in patients with hypothyroidism. Possible associations were studied by Mansour-Ghanaei *et al*[44], who examined 333 Iranian patients with diabetes, lipid disorders, obesity, and PCOS. Participants who had additional NAFLD were also diagnosed with hypothyroidism significantly more often; had significantly higher body mass index (BMI) values; smoked cigarettes more frequently; had considerably higher concentrations of total cholesterol, glucose, uric acid, and abnormal eating habits, which are important due to the unquestionable adverse effects on the body[44].

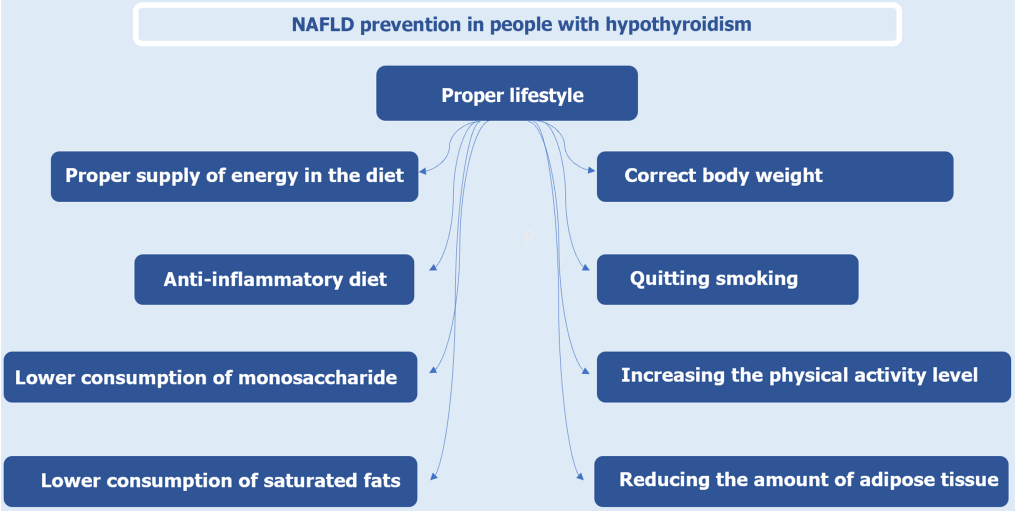
Due to the association between NAFLD and numerous metabolic disorders resulting from an improper lifestyle habits, metabolic dysfunction - associated fatty liver disease (MAFLD) has been distinguished as concurrent liver dysfunction and metabolic syndrome[45]. In turn, metabolic syndrome often accompanies hypothyroidism[46]. Biochemical and elastography screening tests are important for detecting the presence of metabolic disorders and MAFLD in people with hypothyroidism[47].

Proper eating behavior as an important element of NAFLD prevention

A significant risk factor for NAFLD is improper eating behavior, resulting in an excessive supply of energy in the diet, excessive consumption of monosaccharides (especially fructose), and saturated fats [38]. The diet of patients diagnosed with NAFLD should primarily limit the supply of monosaccharides because they intensify de novo lipogenesis[48]. Their consumption should also be limited because of the prevalence of insulin resistance in people with NAFLD, which is also observed in people with hypothyroidism[49]. Thyroid hormones affect glucose metabolism in the liver by activating glucose transporter 2, resulting in the release of glucose into the bloodstream[50]. The above mechanism is disrupted by thyroid hormone deficiency[50]. Insulin resistance, an imbalance in the proportion of serum insulin and glucose concentrations, activates SREBP-1c, resulting in the intensification of de novo lipogenesis, leading to fatty liver[51]. The Mediterranean diet, rich in vegetables, polyunsaturated fatty acids, and vitamin E, may be the recommended nutritional model for both hypothyroidism and NAFLD because of its proven anti-inflammatory effect and low glycemic index, which are important in the case of insulin resistance[52-56].

Proper body weight as an important element of NAFLD prevention

Excessive body weight caused by an increased amount of adipose tissue is an abnormality that may contribute to the development of obesity - a serious disease in which metabolic disorders (including lipid disorders) are associated with endocrine disorders and have a negative impact on all areas of a patient's life[55,56]. Scientists have emphasized that excessive body weight and obesity predict a worse course of NAFLD[57,58]. Obesity is also associated with hypothyroidism, as proven in a cohort study of 9,011 Chinese residents. In addition, researchers proved that obesity in younger men was an independent risk factor for hypothyroidism, while in older men, metabolic disorders were a significant factor in its pathogenesis[56]. There is equivocal evidence of a high correlation between BMI and TSH concentration[59,60]. Such conclusions were reached by Amin *et al*[59], who examined the effect of weight reduction on thyroid function and NAFLD in obese Egyptian adolescents during an intervention. It has been calculated that for an increase in TSH of 10 mIU/L, the BMI increases by 5.28 kg/m^2[59]. Decreased body weight, TSH concentration, and percentage of people with fatty liver have been observed after lifestyle modifications, including altered physical activity and eating habits among adolescents[59]. Du *et al*[60] who studied the effect of thyroid hormones on central obesity, showed a positive correlation between the central obesity index (waist-hip Ratio, WHR), level of TSH, and BMI. The results of this study indicate the association between thyroid hormones and body weight and body fat distribution, which is due to the hypometabolic state caused by a thyroid hormone deficiency[60]. Researchers have suggested that hepatic steatosis may have a negative impact on thyroid function rather than hypothyroidism, which has a negative impact on liver function[61].



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Figure 1 Non-alcoholic fatty liver disease prevention in people with hypothyroidism: importance of proper lifestyle. The most important lifestyle elements to prevent the development of non-alcoholic fatty liver disease include: proper supply of energy in the diet, anti-inflammatory diet, lower consumption of monosaccharides, lower consumption of saturated fats, correct body weight, quitting smoking, increasing the physical activity level, reducing the amount of adipose tissue. NAFLD: Non-alcoholic fatty liver disease.

Physical activity as an important element of NAFLD prevention

According to studies on the impact of lifestyle on the development of NAFLD[62]. The positive effect of physical activity is manifested by body weight reduction and increased insulin sensitivity in cells[55, 63]. Additionally, physical activity prevents muscle mass reduction, which is a risk factor for NAFLD [60]. Increasing the level of physical activity is also recommended for people without excessive body weight but with NAFLD, in whom the researchers believed the cause of the disease to be an elevated TSH concentration[63,64]. Moreover, in a Chinese population study, which included 5,154 people, hypothyroidism was significantly more common in people who did not undertake physical activity than in euthyroid people[65].

A summary of the above information about importance of proper lifestyle in people with hypothyroidism is presented in Figure 1.

CONCLUSION

Hypothyroidism and NAFLD coexist, although their interdependence is not a cause-and-effect relationship.

The pathomechanism of excessive fat accumulation in the liver is complex, and its important non-thyroid-initiating factors are as follows: (1) Providing too many calories in relation to requirements; (2) consuming excessive amounts of monosaccharides and saturated fats; (3) being overweight; and (4) keeping a low physical activity level.

The results of the previous studies do not allow for an unequivocal determination of the pathogenetic relationship between hypothyroidism and NAFLD.

Further research is necessary to answer the questions posed in the title. Among the patients with coexisting NAFLD and hypothyroidism, environmental factors and those dependent on human choice are more important.

FOOTNOTES

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MINIREVIEWS

Sarcopenia in chronic viral hepatitis: From concept to clinical relevance

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Abstract

Although the frequency of metabolic risk factors for cirrhosis and hepatocellular carcinoma (HCC) is increasing, chronic hepatitis B (CHB) and chronic hepatitis C (CHC) remain the most relevant risk factors for advanced liver disease worldwide. In addition to liver damage, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are associated with a myriad of extrahepatic manifestations including mixed cryoglobulinaemia, lymphoproliferative disorders, renal disease, insulin resistance, type 2 diabetes, sicca syndrome, rheumatoid arthritis-like polyarthritis, and autoantibody production. Recently, the list has grown to include sarcopenia. Loss of muscle mass or muscle function is a critical feature of malnutrition in cirrhotic patients and has been found in approximately 23.0%-60.0% of patients with advanced liver disease. Nonetheless, among published

studies, there is significant heterogeneity in the aetiologies of hepatic diseases and measurement methods used to determine sarcopenia. In particular, the interaction between sarcopenia, CHB and CHC has not been completely clarified in a real-world setting. Sarcopenia can result from a complex and multifaceted virus-host-environment interplay in individuals chronically infected with HBV or HCV. Thus, in the present review, we provide an overview of the concept, prevalence, clinical relevance, and potential mechanisms of sarcopenia in patients with chronic viral hepatitis, with an emphasis on clinical outcomes, which have been associated with skeletal muscle loss in these patients. A comprehensive overview of sarcopenia in individuals chronically infected with HBV or HCV, independent of the stage of the liver disease, will reinforce the necessity of an integrated medical/nutritional/physical education approach in the daily clinical care of patients with CHB and CHC.

Key Words: Chronic hepatitis B; Chronic hepatitis C; Sarcopenia; Skeletal muscle loss; Cirrhosis; Clinical outcomes

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Core Tip: Sarcopenia is a key feature of malnutrition in liver cirrhosis and has been found in approximately 23.0%-60.0% of patients with advanced hepatic disease. Skeletal muscle loss is associated with poor quality of life and increased mortality, which are significant cirrhosis-related complications. In individuals chronically infected with hepatitis B virus or hepatitis C virus, the muscle-liver-immune crosstalk during the development of sarcopenia has not been completely clarified. Based on these findings, an overview of the concept, prevalence, clinical relevance, and potential mechanisms of sarcopenia in patients with chronic viral hepatitis is of utmost importance.

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INTRODUCTION

Globally, chronic hepatitis B (CHB) and chronic hepatitis C (CHC) were responsible for almost 96.0% of the 1.3 million deaths related to hepatitis viruses in 2015[1,2]. Two-thirds of the global burden of cirrhosis could be attributed to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections[2] and approximately 720000 deaths involving chronically infected individuals have occurred, mostly from cirrhosis and hepatocellular carcinoma (HCC)[1,2].

HBV and HCV affect hepatocytes and can cause both acute and chronic diseases[3]. Individuals with long-term chronic infections have a considerable risk of developing cirrhosis and HCC during their lifetime[3]. Although the frequency of metabolic risk factors for cirrhosis and HCC, such as metabolic syndrome, obesity, type II diabetes, and non-alcoholic fatty liver disease (NAFLD) is increasing, HBV and HCV are currently the most relevant global risk factors for severe hepatic diseases[4,5].

In addition to potential liver diseases, HCV infection is associated with several extrahepatic manifestations, including mixed cryoglobulinaemia, lymphoproliferative disorders, renal disease, insulin resistance, type 2 diabetes, sicca syndrome, rheumatoid arthritis-like polyarthritis, and autoantibody production[6]. Similar to CHC, CHB can be associated with extrahepatic systemic and/or autoimmune manifestations such as systemic vasculitis, glomerulonephritis, and cutaneous manifestations[7].

Numerous studies have demonstrated that both CHB and CHC are associated with nutritional disorders, especially in hepatic cirrhosis, and patients with impaired metabolic function of the liver are at a high risk of malnutrition. This nutritional abnormality has been identified in 13.0%-70.0% of patients with liver disease[8,9] and it is associated with poor quality of life[10-12] and relevant cirrhosis-related complications such as sepsis[13], refractory ascites[14], hepatic encephalopathy[15,16], spontaneous bacterial peritonitis[17], reduced survival[18], and high mortality[19-21]. Taken together, malnourishment and liver cirrhosis contribute to skeletal muscle wasting, an important marker of malnutrition. Loss of muscle mass or muscle function is the key feature of malnutrition in cirrhotic patients and has been found in approximately 23.0%-60.0% of patients with advanced liver disease[9,22-25].

Sarcopenia has been considered a relevant topic in clinical hepatology settings, and a comprehensive overview of skeletal muscle loss in individuals chronically infected with HBV or HCV, independent of

the stage of the liver disease, will strengthen an integrated medical/nutritional/physical education approach in the daily clinical care of patients with CHB and CHC.

Thus, we first contextualised our review in relation to the connection between liver and nutrient metabolism. We then briefly reviewed the origin of the concept of sarcopenia along with the progress in understanding viral hepatitis biology and its related clinical manifestations. Finally, we performed a review to identify and summarise available data on the prevalence and clinical implications of sarcopenia in patients with chronic viral hepatitis.

Essential crosstalk between liver metabolic functions and nutrient metabolism in the body

It is well known that liver plays a central role in the metabolism of nutrients, including macronutrients/micronutrients, vitamin storage and processing, and oxidant/antioxidant balance[26-29]. Hepatic dysfunction can impair the entire spectrum of metabolic and nutritional processes in the body. Therefore, liver diseases are strongly associated with nutritional disorders[9,23,25]. In fat metabolism, hepatocytes break down fats to generate energy[30]. In carbohydrate metabolism, hepatic cells are capable of storing or releasing glucose and contribute to maintaining a constant blood glucose level in circulation[31].

Additionally, the liver is crucial for maintaining protein and nitrogen metabolism[32]. Hepatic cells perform important functions in the balance between protein synthesis and degradation. In healthy individuals, the blood ammonia level originating from amino acid metabolism is controlled by functional hepatic glutamine metabolism and urea cycle in the liver[33,34]. In the presence of cirrhosis, hepatocyte dysfunction is associated with a state of overall protein deficiency and hyperammonaemia. In this setting, glutamine synthesis from glutamate in skeletal muscle mass plays a significant compensatory role in ammonia disposal[33-35].

Although glutamine synthetase activity is low in skeletal muscle[36] because of its large mass, skeletal muscle is quantitatively the most important site of glutamine synthesis. Ammonia uptake by appendicular muscle has been measured in patients with acute liver failure[37] and was estimated to be 100 nmoL/100 g/min. In chronic liver disease, skeletal muscle also functions as an important extrahepatic site for the removal of ammonia[34,38].

Skeletal muscle encompasses 30.0%–40.0% of the total body mass; thus, this organ is the primary protein store in the human body[39]. The protein turnover balance is responsible for maintaining normal skeletal muscle mass[40]. Increased plasma ammonia levels have been linked to sarcopenia, as a potential mediator of muscle depletion in cirrhosis. Several investigations, including those using animal models, have demonstrated that hyperammonaemia stimulates myostatin expression[41-43]. Myokine is a well known inhibitor of protein synthesis[12]. Furthermore, hyperammonaemia results in muscle mitochondrial dysfunction, increased formation of reactive oxygen species, and oxidative stress, which impair muscle function and repair[44].

This evidence sheds light on the potential pathophysiological mechanisms involved in the liver-muscle axis in hepatic fibrosis[12,35]. Various investigations have shown that skeletal muscle wasting is associated with the progression and poor prognosis of chronic hepatopathy[9,17-21,45-47].

Definition of sarcopenia in different scenarios – from the aging process to hepatic diseases

Sarcopenia and chronic viral hepatitis timelines: Understanding the potential interactions between muscle, liver, and chronic viral hepatitis. The neurologist MacDonald Critchley wrote a manuscript 90 years ago titled "The neurology of old age"[48], which is recognised as the first publication demonstrating age-related skeletal muscle loss. Later, in 1970, Nathan Shock conducted the Baltimore Study of Aging, in which functional changes with age were observed in physiological systems such as sensory, cardiovascular, respiratory, and renal systems[49,50]. However, the term sarcopenia from the Greek words "sarx" (flesh or muscle) and "penia" (loss) was first coined by Rosenberg in the late 1980s. According to the author, no decline is more dramatic or potentially more functionally significant than the loss of muscle mass with advancing age[50]. Thus, in the first stage of concept elaboration, sarcopenia was operationally described as a gradual loss of muscle mass based on methods estimating muscle mass[50]. A pioneering study by Baumgartner *et al*[51] (1998) described sarcopenia as when the appendicular skeletal muscle mass measured by the dual-energy X-ray absorptiometry (DXA) and adjusted for squared height, was less than two standard deviations below the sex-specific means of healthy young adult individuals. However, following studies have shown that the loss of muscle function, defined as muscle strength and power, is two to five times higher than muscle mass wasting and is significantly linked to adverse outcomes[52,53].

The European Working Group on Sarcopenia in Older People (EWGSOP) defined sarcopenia as a syndrome characterised by gradual and generalised loss of skeletal muscle mass and strength[54,55]. Finally, in 2016, sarcopenia was recognised as a disease in the 10th Edition of the International Classification of Diseases with the ICD code 10 - M62.84: Muscle insufficiency[56,57].

Although sarcopenia was originally recognised as an age-related loss of skeletal muscle mass, this clinical condition has been expanded to include loss of muscle function; in addition, it is related to a broad range of chronic diseases[58,59]. Translating this definition into the hepatic disease panorama, several studies have demonstrated that sarcopenia is of utmost significance[9,17-21,58]. Nevertheless, in literature, the term sarcopenia is marked by multiple definitions, diverse methods used to measure

skeletal muscle mass, and heterogeneous study designs enrolling patients with cirrhosis of different aetiologies[58,59]. In addition, the major part of the investigations targeting sarcopenia in patients with hepatic diseases evaluated only the skeletal muscle mass[58,59]. Despite these conundrums, we have to bear in mind that most of the cirrhotic patients with skeletal muscle loss included in previous investigations had chronic viral hepatitis.

Although many studies have shown a relationship between liver damage and sarcopenia, the mechanisms underlying skeletal muscle injury have not been completely clarified. Sarcopenia can result from a complex and multifaceted virus-host-environment interplay in individuals chronically infected with HBV or HCV.

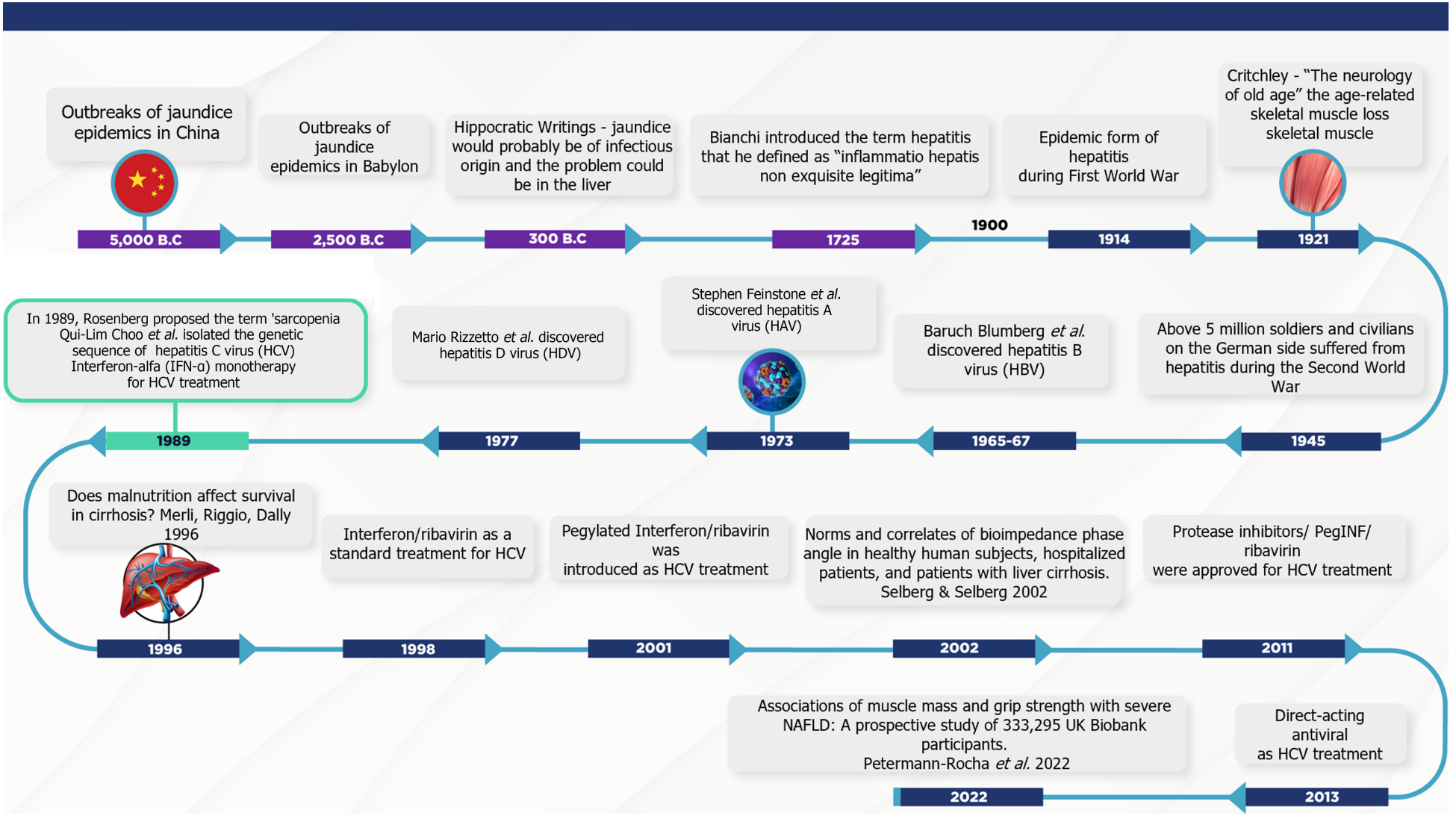
Concerning the overlap between viral hepatitis[60-64] and sarcopenia timelines[48-51,65-67] (Figure 1), even before Rosenberg proposed the term sarcopenia[50], Storch (1984) reported a clinical case of 'lupoid' hepatitis with the detection of hepatitis B core antigen (HBcAg) in motor endplates and cross-striations of skeletal muscle in a 12-year-old female patient[67]. Although the diagnostic significance and causes of the described findings were unclear, extrahepatic deposition of this viral marker has been proposed as an indicator of HBV replication in skeletal muscle[67]. Subsequently, inclusion body myositis, a chronic progressive inflammatory myopathy in the elderly, was associated with HCV infection[68,69].

In particular, the prevalence of sarcopenia has been associated with the progression of liver fibrosis [24,70,71]. A study by Hiraoka *et al*[71] (2016) in Japan, using computed tomography and handgrip strength (based on the EWGSOP criteria), found sarcopenia in 7.1%, 11.8%, and 21.9% of the patients with chronic viral hepatitis B and/or C without cirrhosis, with compensated (Child-Turcotte–Pugh A), and decompensated (Child-Turcotte–Pugh B/C) cirrhosis, respectively. Bering *et al*[72] (2017), also using the reference values recommended by EWGSOP, identified the presence of sarcopenia in 7.1% and 11.8% of the non-cirrhotic and compensated cirrhotic (Child–Turcotte–Pugh A) Brazilian patients, respectively. In both studies, 7.0% of the CHC subjects had sarcopenia prior to the onset of cirrhosis[71,72]. In line with these findings, a cross-sectional study from the National Health Examination and Nutrition showed that low muscle mass, as evaluated by mid-upper arm circumference measurements, antedates the development of cirrhosis in American patients with CHC[73]. Taken together, these data shed light on putative risk factors for skeletal muscle loss other than advanced hepatopathy-related factors. Among the potential predictors, virus, host, and environmental factors, such as viral load/genotype, nutritional status, and immune response should be highlighted.

Currently, advancements in direct-acting antiviral agents (DAAs) have resulted in outstanding improvements in the management of patients chronically infected with HCV, with sustained virological response rates that surpass 95.0% in real-life scenarios[74]. Treatment with DAAs is safe and effective and has been associated with liver and non-liver benefits, such as the prevention of hepatic disease progression and improvements in quality of life scores[74,75]. However, a study showed that DAA-induced clearance does not completely restore the altered cytokine and chemokine milieu in CHC patients[76]. Hence, in these individuals, cytokine and chemokine signatures vary depending on the stage of the liver disease and the response to antiviral therapy[77-80]. This knowledge can be transferred to the muscle-liver axis in the context of HCV eradication, especially with the introduction of interferon-free (IFN-free) treatments in clinical practice. More recently, results from interventional studies have demonstrated that HCV eradication by DAAs suppresses skeletal muscle loss in patients with CHC[81-84], suggesting a direct role of the virus in muscle mass depletion. However, the role played by the host immune response, especially pro-inflammatory effects, on skeletal muscle cells in CHC should not be overlooked[6,77-79,85]. Future longitudinal and multicentre studies are required to reduce this gap in knowledge.

Concerning extrahepatic manifestations of HCV infection, several studies suggest that an imbalance between pro-inflammatory and anti-inflammatory cytokines might induce immune activation in sites outside the liver, and consequently, generate a wide range of systemic symptoms and signals, including myalgia, weakness, fatigue, nausea, abdominal pain, weight loss, arthralgia, purpura, Raynaud's phenomenon, xerostomia, dry eyes, depressive feelings, and anxious mood[6,86]. Therefore, CHC has been identified as a systemic disease, and 40%-74% of patients chronically infected with HCV may develop at least one non-liver manifestation throughout the clinical course of the infection[86].

It is generally acknowledged that the mechanisms involved in HCV-related extrahepatic manifestations are attributable to antibody- and cell-mediated immune responses[87-90]. Among these mechanisms, cryoglobulinaemia (type II cryoglobulin) is associated with chronic HCV[6,86]. Cryoglobulins are immunoglobulins that precipitate *in vitro* at temperatures < 37 °C and solubilise upon warming. HCV can trigger the expansion of B cell clones that secrete monoclonal IgM with rheumatoid factor activity. IgM then binds to polyclonal IgG molecules, which recognise HCV antigens. The resulting immunocomplexes activate complement proteins, which bind cell receptors on endothelial cells, leading to the recruitment of mononuclear and polymorphonuclear cells resulting in vasculitis. Vasculitis may occur in the brain, skin, joints, kidneys, lungs, heart, and digestive tract[6,86]. Another site that may be affected by immune-mediated occurrence is the skeletal muscle. Although secondary sarcopenia is frequently identified in patients with cirrhosis, the mechanisms underlying the interaction between the loss of skeletal mass, inflammatory mediators, and chronic viral hepatitis are still unclear. Given the potential role of circulating pro-inflammatory cytokines in mediating age-related sarcopenia



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Figure 1 Timeline of the knowledge evolution in the chronic viral hepatitis and sarcopenia fields.

[91,92], the effects of these inflammatory mediators on the pathogenesis of skeletal muscle loss occurring in HBV and HCV should be evaluated.

Other potential predictors of sarcopenia in chronic viral hepatitis should be considered, such as environmental factors, which are strongly linked to nutritional disorders and muscle homeostasis. Several lifestyle aspects in individuals with CHB and CHC may contribute to muscle damage, such as dietary patterns, diet-related non-communicable diseases, sedentarism, cigarette smoking, alcohol, and non-alcohol substance use. Analysing data from the Korean National Health and Nutrition Examination Surveys 2008-2011, Han *et al*[93] observed that sarcopenia was independently associated with liver fibrosis in patients with CHB. The authors also observed that when the study population was stratified according to metabolic factors, sarcopenia was independently associated with fibrosis among subgroups with obesity, insulin resistance, metabolic syndrome, and liver steatosis.

More recently, Santos *et al*[94] (2022), used DXA, handgrip strength, and Timed Up and Go test to show that in patients with CHB, the presence of metabolic-associated fatty liver disease and central obesity was associated with low muscle mass and strength. Although secondary sarcopenia is a well-known predictor of liver fibrosis in patients with NAFLD[95], the interaction between sarcopenia and CHB is poorly understood. These findings encourage the evaluation of metabolic and skeletal muscle loss among individuals chronically infected with HBV and reinforce the need for further large-scale case-control studies.

Few studies have examined the effects of antiviral treatment on muscle mass in CHB patients. In an investigation centred on the measurement of psoas major muscle using computed tomography before and after long-term entecavir therapy, no significant change in the muscular area was identified in any of the patients, but a significant increase was detected in the group of patients with serum albumin < 4 g/dL before treatment[96]. In contrast, Kim *et al*[97] (2020) investigated the dynamic association between changes in fibrosis and muscle mass during antiviral therapy and reported that appendicular skeletal muscle mass (ASM) significantly decreased during treatment of HBV infection.

Approximately 462 million adults worldwide are underweight, whereas 1.9 billion are either overweight or obese[98,99]. According to the World Health Organization definition, the double burden of malnutrition is characterised by the coexistence of undernutrition along with overweight, obesity, or diet-related non-communicable diseases within individuals, households, and populations across the course of life[98,99]. Furthermore, a growing body of evidence has shown that excessive food intake and lack of physical exercise, considered serious characteristics of the modern lifestyle, have also been verified in patients with liver disease[100,101]. Health professionals face a great challenge particularly in the management of CHB and/or CHC patients, because malnutrition and overweight can simultaneously be present in a patient[47,102]. Sarcopenic obesity, which is characterised by a decrease in ASM and excess body fat, is associated with increased mortality and influences the metabolic profile and physical performance compared with clinical manifestations alone[47,100]. Consequently, an improvement in the comprehension of body composition and nutritional status of chronically infected HBV and HCV individuals, regardless of the severity of the liver disease, is highly relevant for clinicians, dieticians, and specialists in hepatic diseases[101,102].

PREVALENCE AND CLINICAL IMPLICATIONS ASSOCIATED WITH SARCOPENIA IN PATIENTS WITH CHRONIC VIRAL HEPATITIS

Sarcopenia is a relevant risk factor for adverse outcomes in cirrhotic patients[12,18]. As mentioned earlier, among the objectives of this review, we aimed to identify and summarise the available data on the prevalence and adverse clinical outcomes of sarcopenia in patients with chronic viral hepatitis. The steps involved in the review process are as follows:

Literature search

We first performed a sequential electronic search using PubMed, Embase, Biblioteca Virtual em Saúde, Cochrane Library, Scopus, Web of Science, and Cumulative Index to Nursing and Allied Health on September 1, 2022 to identify published scientific reports on sarcopenia in patients with chronic viral hepatitis. The search included studies that were published between January 1995 and September 2022. To do the research, a combination of the following descriptors was used: "hepatitis C", "chronic hepatitis C", "hepatitis B", "chronic hepatitis B", "sarcopenia", "low muscle mass", "sarcopenic obesity", "skeletal muscle mass", and "skeletal muscle" (Supplementary material).

The eligibility of the articles was evaluated by two independent reviewers (MPPC and TPV). Duplicate articles were excluded from the analysis. The articles were selected by title, abstract, and full text in separate and sequential steps, following the predefined inclusion and exclusion criteria. To evaluate whether the articles met all previously established criteria, each article was analysed individually. A third reviewer resolved the disagreements between the two reviewers.

Eligibility criteria

We used the Patients, Intervention, Comparison, Outcome model to develop literature search strategies [103]. Eligible manuscripts included adults aged ≥ 18 years who were chronically infected with HBV or HCV. We also considered the following conditions: presence of inpatients and outpatients, sample size of at least 30 subjects, and loss of skeletal muscle mass and/or function as the variable of interest. In addition, the clinical outcomes included infectious and noninfectious complications (clinical outcomes), increased length of hospital stay, mortality, survival, and health-related quality of life scores. Moreover, data on the prevalence of low skeletal muscle mass and/or function, including pre-sarcopenia, sarcopenia, and sarcopenic obesity, independent of the grade of liver fibrosis, were also assessed.

Quality assessment

The methodological quality of the studies was assessed by two independent reviewers using the Joanna Briggs Institute Critical Appraisal tools applicable to each specific study design[104]. Each criterion was assessed as ''yes'' (fulfilled), ''no'' (not fulfilled), or ''unclear''. Any differences in opinion between the reviewers regarding the methodological quality were resolved by consensus through direct discussion. Disagreements were resolved through discussion with a third research member.

A total of 1427 articles were identified in the aforementioned databases. After discarding duplicates, non-English language papers, and non-relevant articles, 17 full-length published articles were selected for appraisal and were retained in the current mini-review (Supplementary material).

Prevalence, definitions, and clinical outcomes associated with sarcopenia

One of the most remarkable consequences of aging is the involuntary loss of muscle mass, strength, and function, termed sarcopenia[54-56]. Various attempts have been made to apply this operational definition to hepatic disease settings, as summarised in Table 1[47,71-73,93,94,105-109,110-114,115]. The designs of the 17 included studies were retrospective cohort (n = 8), cross-sectional (n = 8), and prospective cohort (n = 1). Most of the studies were performed in Asia (7/17, six in Japan and one in Korea[71,93,108,109,111,112,114]) and America (four in the United States[73,106,107,115], two in Canada [47,105], and two in South America[72,94]), while one each was performed in Europe[113], and in Australia/Oceania[110]. The overall sarcopenia prevalence varied from 3.8% to 53.7% in the 17 studies.

The median age of the participants ranged from 49.2 to 70.5 years[112,113]. One study included only men[110] while all the others were mixed-sex investigations, with the number of women varying between 26[72] and 9287[73]. Different aetiologies of liver diseases were observed in these studies[47,71-73,93,94,105-109,110-114,115]; with respect to chronic viral hepatitis, two studies included only patients chronically infected with HBV[93,94] and HCV[72]. In 53.0% of the investigations, sarcopenia was diagnosed according to one of the four consensus diagnostic criteria for age-related sarcopenia proposed by the Asian Working Group for Sarcopenia, EWGSOP, the Foundation for the National Institutes of Health Sarcopenia Project, and the Japan Society of Hepatology[71,72,93,108,111,112,114,115]. Muscle mass was measured using computed tomography in the majority of studies (8/17 studies [47,71,105-107,109,111,113]), followed by bioelectrical impedance analysis (4/17[108,112,114,115]), DXA (4/17 studies[72,93,94,110]), and anthropometric measurements (1/17 studies[73]). Muscle strength was measured using handgrip strength in 5/17 studies[71,72,94,112,114]. Physical performance was evaluated in two studies[94,112].

The studies reported results for approximately six different types of outcomes: mortality (five studies [47,105,106,110,113]), decreased survival (four studies[108,109,113,114]), severity of liver fibrosis (two studies[71,93]), osteopenia/osteoporosis and vertebral fractures (two studies[72,112]), while one each identified poor quality of life[111], and malnutrition[72]. In HBV scenario, sarcopenia was associated with metabolic derangements, central obesity, and metabolic syndrome[93,94].

DISCUSSION

Globally, sarcopenia is a research hotspot [116] and its clinical significance in patients with chronic liver disease is of utmost importance. In cirrhosis, sarcopenia intensely affects the health status and health-related quality of life [10,13-17,18-21]. Muscle wasting that affects cirrhotic patients is accelerated, and losses greater than 3.0% annually have been related to adverse outcomes[59].

Despite the awareness and clinical recognition of sarcopenia in cirrhotic patients, large heterogeneity permeates studies focused on sarcopenia in these individuals. It should be highlighted that in literature, the term sarcopenia is marked by multiple definitions, diverse measurement methods, and heterogeneous study designs enrolling patients with cirrhosis of diverse aetiologies and different stages of liver fibrosis[59]. In addition, most investigations targeting sarcopenia in patients with hepatic diseases have evaluated only skeletal muscle mass[59].

In the current review, the overall prevalence of sarcopenia varied from 3.8% to 53.7%. This difference can be attributed to the different criteria used to detect sarcopenia. In patients with chronic liver disease, there is neither a gold-standard definition nor a universal operational criterion for identifying sarcopenic cases. Additionally, the aetiology of the liver disease and severity of hepatic fibrosis varied

Table 1 Summary of main studies investigating the association between sarcopenia and adverse clinical outcomes in the context of chronic hepatopathies including patients chronically infected with hepatitis B or C virus, *n* (%)

Ref.	Study location	Study design	Diagnosis of sarcopenia	Study population (*n*)	Age, yr[1]	Sex (M/ F) (*n*)	Aetiology of liver disease, *n* (%)	Overall prevalence of sarcopenia (%)	Prevalence of sarcopenia according to the severity of the liver disease (%)	Clinical outcome/main results
Montano-Loza *et al* [105], 2012	Canada	Retrospective cohort	CT at the level of the third lumbar vertebrae (L3 SMI, ≤ 38.5 cm^2/m^2 for women and ≤ 52.4 cm^2/m^2 for men)	112 cirrhotic patients evaluated for LT	54.0 ± 1.0	78/34	Alcohol 25 (22.0); HCV 32 (29.0); Alcohol + HCV 18 (16.0); HBV 2 (2.0); Autoimmune 21 (19.0); Others 14 (13.0)	40	Not mentioned	Sarcopenia, Child-Pugh score, and MELD score were associated with mortality
Krell *et al* [106], 2013	United States	Retrospective cohort	CT-measured psoas muscle; Sex-stratified TPA terciles; Criteria for cutoff: Lowest TPA tercile	207 adult patients who underwent LT	51.7 ± 9.8	129/78	HCV 54 (26.1); HBV 9 (4.4); Alcohol 30 (14.5); Autoimmune 47 (22.7); NASH 8 (3.9); HCC 52 (25.1); Others 28 (13.5); More than one indication for liver transplantation 21 (10.1)	-	Not mentioned	Sarcopenia was associated with a heightened risk for post-transplant infections and mortality
Gowda *et al*[73], 2014	United States	Cross-sectional	MUAC below the 10^{th} percentile for age- and sex-matched reference values	18513 NHANES participants	HCV –39.3 ± 8.5; HCV + 47 ± 5.8	HCV-8923/9287; HCV+ 197/106	303 (1.6%) had CHC	Low MUAC HCV+ 42/303 (13.8); HCV-1220/18210 (6.7)	HCV+ without significant liver fibrosis (APRI ≤ 1.5)	CHC was associated with low MUAC, even in the absence of advanced liver disease
Yadav *et al* [107], 2015	United States	Prospective cohort	CT at the level of the third lumbar vertebrae; (L3 SMI, ≤ 38.5 cm^2/m^2 for women and ≤ 52.4 cm^2/m^2 for men)	213 cirrhotic patients evaluated for LT	55.3 ± 8.6	129/84	HCV 94 (44.0); Alcohol 34 (16.0); NASH 29 (13.6); PBC/PBS 16 (7.5); Cryptogenic cirrhosis 13 (6.1); Others 26 (12.2)	22.2	Not mentioned	Sarcopenia was not associated with mortality, poor quality of life, and functional capacity
Hiraoka *et al*[71], 2016	Japan	Cross-sectional	CT-measured psoas muscle and HGS-measured muscle strength AWGS and EWGSOP criteria	807	67.1 ± 10.0	466/341	HCV 511 (63.3); HBV 134 (16.6); HBV and HCV 3 (3.7); Alcohol 45 (5.6); Others 114 (14.1); Previous or current HCC 256 (31.7)	3.9–16.7 (AGWS); 7.1–21.9 (EWGSOP)	[CH, LC Child-Pugh (A, and B/C)]; AGWS; 3.9, 4.8, 16.7; EWGSOP; 7.1, 11.8, 21.9	Prevalence of sarcopenia increased with the progression of chronic liver disease
Montano-Loza *et al* [47], 2016	Canada	Retrospective cohort	CT at the level of the third lumbar vertebrae; (L3 SMI, ≤ 41.0 cm^2/m^2 for women and ≤ 53.0 cm^2/m^2 for men)	678	56.0 ± 1.0 to 58.0 ± 1.0	457/221	HCV 269 (40.0), alcohol 153 (23.0), NASH and cryptogenic cirrhosis 96 (14.0); Autoimmune liver disease 55 (8.0); HBV 43 (6.0); Others not specified 5 (1.0); Concomitant HCC 291 (43.0)	Sarcopenia 292 (43.0), Sarcopenic obesity 135 (20.0), Myosteatosis 353 (52.0), Sarcopenia and myosteatosis 176 (26.0)	Child-Pugh (A, B, C); Sarcopenia 12.7, 51.0, 36.3; Sarcopenic obesity; 8.9, 47.4, 43.7; Myosteatosis 12.2, 51.0, 36.8	Sarcopenia and myosteatosis were independently associated with a higher long-term mortality in cirrhosis

Nishikawa *et al*[108], 2017	Japan	Cross-sectional	BIA-measured upper limb skeletal muscle mass (kg) AWGS cutoff (SMI, ≤ 7.0 kg/m^2 for men and ≤ 5.7 kg/m^2 for women)	383	65.2 ± 10.3	205/178	HBV 32 (8.3); HCV 235 (61.4); Others 116 (30.3)	136 (35.5)	No association with Child-Pugh score	Sarcopenia was associated with low overall survival in male patients
Bering *et al* [72], 2018	Brazil	Cross-sectional	DXA-measured ASMI with EWGSOP cutoff (ASMI, ≤ 7.26 kg/m^2 for men and ≤ 5.45 kg/m^2 for women)HGS-measured muscle strength - EWGSOP criteria	104	50.5 ± 11.3	78/26	CHC patients without cirrhosis 70 (67.3), with compensated cirrhosis 34 (32.7)	Low muscle strength 29 (27.9), Low ASMI 15 (14.4); Sarcopenia 9 (8.7); Sarcopenic obesity 3 (3.8)	Sarcopenia without cirrhosis 5 (7.1) with compensated cirrhosis 4 (11.8)	Sarcopenia was associated with bone mineral content and malnutrition. BMI was normal in 88.9% of sarcopenic patients and in all patients with sarcopenic obesity. The mid-arm muscle circumference was positively correlated with ASMI
Han *et al* [93], 2018	Korea	Cross-sectional	DXA-measured ASMI with sarcopenia defined as the lowest quintile for sex-specific sarcopenia index cutoff values (< 0.89 for men and < 0.58 for women) modified from the criteria, were adapted from the FNIH Consensus	506	Non-sarcopenic 48.5 ± 12.9; Sarcopenic 48.5 ± 12.9	258/248	CHB significant fibrosis according to FIB4without sarcopenia160/407 (39.3)with sarcopenia57/99 (57.6)	99 (19.6)	Not mentioned	Sarcopenia was associated with significant fibrosis, specifically in CHB patients with obesity, insulin resistance, metabolic syndrome, and liver steatosis
Kamo *et al* [109], 2019	Japan	Retrospective cohort	CT at the level of the third lumbar vertebrae; Sarcopenic obesity as the combination of low SMI (< 40.31 cm^2/m^2 for men; < 30.88 cm^2/m^2 for women) and either VFA ≥ 100 cm^2 or BMI ≥ 25 kg/m^2	277	54.0 [18.0–69.0]	134/143	HCC 74 (26.7), HCV and/or HBV 60 (21.7), Cholestatic disease 56 (20.2); Others 87 (31.4)	Groups divided according to SMI and VFA or BMI; Without sarcopenia/non-obesity (NN); *n* = 167 (60.0)/*n* = 179 (65.0); Without sarcopenia/obesity (NO); *n* = 55 (20.0)/*n* = 43 (15.0); Sarcopenia/ non- obesity (SN); *n* = 46 (17.0)/*n* = 49 (18.0); Sarcopenia/obesity (SO); *n* = 9 (3.0)/*n* = 6 (2.0)	Groups divided according to SMI and VFA Child-Pugh A, B/C; Sarcopenia/ non-obesity (SN); 13 (28.3)/33 (71.7); Sarcopenia/obesity (SO) 4 (44.4)/5 (55.6); Groups divided according to SMI and BMIChild-Pugh A, B/C; Sarcopenia/ non-obesity (SN); 12 (24.5)/37 (75.5); Sarcopenia/obesity (SO) 5 (8.3)/1 (1.7)	Patients with sarcopenic obesity showed worse survival after LDLT compared to non-sarcopenic/non- obesity patients
Sinclair *et al*[110], 2019	Australia	Retrospective cohort	DXA-measured ASMI - cutoff (ASMI, ≤ 7.26 kg/m^2 for men)	420	55.4 [49.1–59.4]	Male, 420	HCC 119 (28.3), HCV 102 (24.3), Alcoholic cirrhosis 53 (12.6), Primary sclerosing cholangitis 43 (10.2), NAFLD 26 (6.2); Others autoimmune and metabolic conditions, 77 (18.3)	130 (30.9)	Not mentioned	Low ASMI is strongly associated with mortality in men awaiting liver transplantation
Ohashi *et al*[111], 2019	Japan	Cross-sectional	CT at the level of the third lumbar vertebrae; JHS criteria (L3 SMI, ≤ 38.0 cm^2/m^2 for women and ≤ 42.0 cm^2/m^2 for men)	335	69.5 ± 10.2	169/166	HCV 139 (41.5), HBV 57 (17.0), NAFLD 44 (13.1), Alcoholic liver disease 40 (11.9) Others 55 (16.4)HCC 86/335	180 (53.7)	Child-Pugh A, B, C169 (94.0), 10 (5.5), 1 (0.5)	Sarcopenia was associated with low scores of quality of life using the Medical Outcomes Short-Form Health Survey (SF-36)

							(25.7)			
Saeki *et al* [112], 2019	Japan	Cross-sectional	BIA-measured SMISarcopenia was diagnosed using the following criteria: JSH criteria: Low HGS (< 26 kg for men and < 18 kg for women) and low SMI (< 7.0 kg/m^2 for men and < 5.7 kg/m^2 for women); AWGS criteria: Low HGS (< 26 kg for men and < 18 kg for women) and/or low gait speed (≤ 0.8 m/s both for men and women) and low SMI (< 7.0 kg/m^2 for men and < 5.7 kg/m^2 for women); FWGSOP2 criteria: Low HGS (< 27 kg for men and < 16 kg for women) and low SMI (< 7.0 kg/m^2 for men and < 5.5 kg/m^2 for women). Low gait speed (≤ 0.8 m/s for both men and women) is an indicator for defining severe sarcopenia	142	70.5 [58.8–76.0]	90/52	HCV 45 (31.7), HBV 16 (11.3), Alcoholic liver disease 48 (33.8); Others 33 (23.2)	JSH or AWGS criteria; 48 (33.8); EWGSOP2 criteria; 40 (28.2)	Child-Pugh A/B, C; 32 (66.7)/16 (33.3)	Sarcopenia, osteoporosis, osteosarcopenia, and vertebral fracture were highly prevalent and closely associated with one another in patients with liver cirrhosis. Specifically, patients with osteosarcopenia had the highest risk of vertebral fractures
Pinto dos Santos *et al* [113], 2019	Germany	Retrospective cohort	CT-measured PMA and bilateral ESA as well as the combined PSMA. Muscle areas were subsequently normalised to the patient's height squared - PMI, ESI, and PSMI	368	49.2 [36.9–61.5]	255/113	HCC 164 (44.6), Alcoholic liver disease 147 (39.9), HCV 91 (24.7), HBV 55 (14.9), Biliary liver disease 38 (10.3) Others (11.1)	Median PSMI was used to divide the study population into high and low muscle index subgroups, which were further compared	Child-Pugh A, B, C; 53 (14.4), 92 (25.0), 197 (53.5)	Sarcopenia was a predictor of early post-OLT survival in male patients
Nishikawa *et al*[114], 2021	Japan	Retrospective cohort	BIA-measured SMI; Sarcopenia was diagnosed using criteria: JSH criteria: low HGS (< 26 kg for men and < 18 kg for women) and SMI (< 7.0 kg/m^2 for men and < 5.7 kg/m^2 for women); AWGS criteria: Low calf circumference (CC) (< 34 cm for men and < 33 cm for women); Japanese criteria: High waist circumference (WC) (> 85 cm for men and > 90 cm for women)	631 CLD	65.0 [52.0–71.0]	309/322	HCV 286 (45.3), HBV 90 (14.3), Others 255 (40.4)	Sarcopenia; Low HGS + Low SMI; 73/631 (11.6); Low HGS; men 49 (15.9); women 89 (27.6); Low SMI; men 76 (24.6); women 107 (33.2); Low CC; men 49 (15.9); women 81 (25.2); High WC; men 106 (66.7); women 103 (32.0)	Not mentioned	Multivariate analysis showed that men, presence of LC, presence of HCC, low-GS, low-CC, serum albumin, estimated glomerular filtration rate, hepatitis B virus, and hepatitis C virus were significant factors contributing to the overall survival. CC can be an alternative marker for muscle mass in CLD patients
Van Dongen *et al*[115], 2022	United States	Retrospective cohort	BIA-measured SMI; EWGSOP2 criteria: With sarcopenia if their SMI > 1 SD below the gender-specific meanfor young adults (aged 20–39 y) in NHANES III (≥ 36.7% in men and ≥ 26.6% in women)	12032 NHANES participants (NHANES III, 1988–1994); 4200 (34.9%) CLD; 7832 (65.1%)	NAFLD 46.01(0.47); ALD 43.92 (1.33); HCV 39.49 (0.94); HBV 41.12 (1.70); Control;	6049/5983	NAFLD 3238 (77.1%); ALD 685 (16.3%); HCV 218 (5.2%); HBV 59 (1.4%)	Prevalence of sarcopenia was higher among NAFLD than other; CLDs and controls (40.7% in NAFLD, 27.2% in ALD, 22.4% in HCV, 16.8% in HBV, and 18.5% in controls)	Not mentioned	Among 4 patients with CLDs and the controls, all-cause cumulative mortality was: 35.2% HCV, 34.7% ALD, and 29.6% NAFLD. The presence of sarcopenia was associated with a higher risk of all-cause

				controls	41.56 (0.40)					mortality only among subjects with NAFLD. Attainment of ideal LS7 metrics (ideal body mass index, ideal blood pressure, ideal physical activity, and ideal glycaemic control) provides protection against sarcopenia in NAFLD
Santos *et al* [94], 2022	Brazil	Cross-sectional	DXA-measured ALM_{BMI} and patients in the first sex-specific quintile (< 0.767 for men and < 0.501 for women) were considered to have low ALM_{BMI} adapted from FNIH Consensus criteria, HGS-measured muscle strength, and physical performance - TUG	105 CHB outpatients	48.5 ± 12.0	61/44	105 CHB outpatients without cirrhosis 76.2% with compensated cirrhosis 23.8%	-	Not mentioned	MAFLD and central obesity were associated with low muscle mass and strength in patients with chronic hepatitis B, independent of the stage of the liver disease

[1]Mean (SD) or standard error of the mean (SEM) or median [interquartile range 25^{th}–75^{th} percentile.

HBV: Hepatitis B virus; HCV: Hepatitis C virus; SD: Standard deviation; F: Female; M: Male; CT: Computerised tomography; L: Lumbar; SMI: Skeletal muscle index; LT: Liver transplantation; MELD: Model for End-stage Liver Disease; TPA: Total psoas area; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma; MUAC: Mid-upper arm circumference; NHANES: The National Health and Nutrition Examination Survey; APRI: Aspartate aminotransferase to Platelet Ratio Index; CHC: Chronic hepatitis C; PBC: Primary biliary cholangitis; PBS: Primary sclerosing cholangitis; HGS: Handgrip strength; CH: Chronic hepatitis; LC: Liver cirrhosis; AWGS: Asian Working Group for Sarcopenia; EWSOP: The European Working on Sarcopenia in Older People; BIA: Bioelectrical impedance analysis; ASMI: Appendicular skeletal muscle mass index; DXA: Dual-energy X-ray absorptiometry; FNIH Consensus: Foundation for the National Institutes of Health Biomarkers Consortium Sarcopenia Project consensus; BMI: Body mass index; FIB4: Fibrosis-4 index; VFA: Visceral fatty area; LDLT: Living donor liver transplantation; NAFLD: Non-alcoholic fatty liver disease; JSH: Japan Society of Hepatology; PMA: Psoas muscle area; ESA: Erector spinae muscle area; PSMA: Paraspinal muscle area; PMI: Psoas muscle index; ESI: Erector spinae muscle index; PSMI: Paraspinal muscle index; CC: Calf circumference; WC: Waist circumference; CLD: Chronic liver disease; NHANES III, 1988–1994, the Third National Health and Nutrition Examination Survey; ALD: Alcohol-associated liver disease; LS7: Life's Simple 7 health metrics; ALM_{BMI}: Appendicular lean mass was adjusted for BMI; TUG: Timed Up and- Go test; MAFLD: Metabolic-associated fatty liver disease; OLT: Orthotopic liver transplantation.

among the investigations included in this minireview. Using computed tomography and hand grip strength, Hiraoka *et al*[71] (2016) (based on EWGSOP1 criteria) found that sarcopenia was present in 7.1%, 11.8%, and 21.9% of Japanese patients with chronic B and/or C viral hepatitis with non-cirrhosis, compensated cirrhosis (Child-Turcotte-Pugh A), and decompensated cirrhosis (Child-Turcotte-Pugh B/C), respectively. The authors observed that the prevalence of sarcopenia increased with the progression of hepatic fibrosis. Of particular concern was the finding that patients with CHC had sarcopenia prior to the onset of cirrhosis[71]. These findings reinforce the need for further research focusing on the biological mechanisms underlying the concurrent occurrence of sarcopenia in patients with chronic viral hepatitis.

Concerning the clinical outcomes associated with sarcopenia in patients chronically infected with HBV or HCV, skeletal muscle loss has been considered an independent prognostic marker of mortality in cirrhotic patients and is associated with an increased risk of complications, such as sepsis[13], refractory ascites[14], hepatic encephalopathy[15,16], and spontaneous bacterial peritonitis[17].

Considering other clinical implications of sarcopenia in patients with CHC, an association between skeletal muscle loss and bone loss was verified, independent of the severity of liver fibrosis[72]. In cirrhosis settings, bone disorders have been linked to hypogonadism[117], vitamin D deficiency[118],

and low levels of insulin-like growth factor[119]. Nevertheless, little is known about the bone status of patients with CHC, especially before the onset of cirrhosis. Among the potential factors, chronic inflammation, inadequate diet and nutrition, and weight and muscle loss may contribute to low bone mineral density in subjects chronically infected with HCV. Taken together, muscle mass and muscle strength stimulate osteogenesis through connections between the bone and skeletal muscle[120]. In addition, skeletal muscle mass is recognised as an independent predictor of bone mineral density in healthy[121] and diseased individuals[122,123].

In the current review, metabolic derangements, central obesity, and metabolic syndrome were associated with sarcopenia in patients with CHB[93,94]. However, there are few studies exploring skeletal muscle loss in CHB patients. To date, among the various aetiologies implicated in liver diseases, liver-muscle interaction has been the most studied in patients with NAFLD/NASH[66,70]. Of particular concern in fatty liver disease is the fact that various evidence point to the complexity of the mechanisms implicated in skeletal muscle damage. In a previous investigation, Lee *et al*. observed that up to 12.0% of patients diagnosed with NAFLD had sarcopenia independent of obesity and insulin resistance, and approximately 30.0% of sarcopenic individuals without metabolic syndrome and obesity had NAFLD [124,125].

CONCLUSION

There is no universal consensus regarding the diagnosis of sarcopenia in patients with chronic viral hepatitis. Although the prevalence of sarcopenia increased in parallel with the progression of hepatic fibrosis, interestingly, sarcopenia was observed in patients chronically infected with HCV before the onset of cirrhosis. Even in studies not focused on evaluating only patients with chronic viral hepatitis, relevant adverse health-related outcomes were associated with sarcopenia in CHB or CHC patients. These findings highlight the importance of addressing skeletal muscle mass and strength loss in patients with chronic viral hepatitis. Effective strategies should be implemented to screen for sarcopenia in these patients, independent of the stage of the liver disease. An integrated medical/nutritional/physical education approach will enable greater understanding of the significance of musculoskeletal changes in patients chronically infected with HBV or HCV.

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Janaina Luz Narciso-Schiavon, Leonardo Lucca Schiavon

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Abstract

Celiac disease (CD) is a chronic inflammatory intestinal disorder mediated by the ingestion of gluten in genetically susceptible individuals. Liver involvement in CD has been widely described, and active screening for CD is recommended in patients with liver diseases, particularly in those with autoimmune disorders, fatty liver in the absence of metabolic syndrome, noncirrhotic intrahepatic portal hypertension, cryptogenic cirrhosis, and in the context of liver transplantation. Non-alcoholic fatty liver disease is estimated to affect approximately 25% of the world's adult population and is the world's leading cause of chronic liver disease. In view of both diseases' global significance, and to their correlation, this study reviews the available literature on fatty liver and CD and verifies particularities of the clinical setting.

Key Words: Fatty liver; Non-alcoholic fatty liver disease; Celiac disease; Transaminases; Aspartate aminotransferase; Alanine aminotransferase

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Core Tip: In view of fatty liver and celiac disease (CD) global significance, and to their correlation, this study reviews the available literature on fatty liver and CD and verifies particularities of the clinical setting.

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INTRODUCTION

Celiac disease (CD) is a chronic inflammatory intestinal disorder mediated by the ingestion of gluten in genetically susceptible individuals. It is relatively common and affects 0.7%–1.4% of the global population[1]. Its diagnosis relies on a combination of serologic testing for anti-tissue transglutaminase, anti-endomysial, and/or anti-deamidated gliadin peptide antibodies, as well as typical findings of villous atrophy and intraepithelial lymphocytosis in duodenal biopsies[2]. The classical clinical manifestations of CD, related to the gastrointestinal tract, are seen in 50%-60% of all cases. Non-classical CD accounts for 40%-50%, and is characterized by systemic involvement including musculoskeletal, neurological, endocrine, kidney, heart, lung, and liver manifestations, concomitant with other autoimmune diseases and malignancies[3]. Liver involvement in CD has been widely described in case reports and case series in the past fifty years. Presently, active screening for CD is recommended in patients with liver diseases, particularly in those with autoimmune disorders, fatty liver in the absence of metabolic syndrome, noncirrhotic intrahepatic portal hypertension, cryptogenic cirrhosis, and in the context of liver transplantation[4]. Non-alcoholic fatty liver disease (NAFLD), estimated to affect approximately 25% of the world's adult population, is the world's leading cause of chronic liver disease [5]. Due to both diseases' global significance, and to their correlation[6], this study aims to review the available literature on fatty liver and CD, and verify what has already been published on this subject in order to define the clinical particularities of their coexistence.

CELIAC DISEASE IN PATIENTS WITH FATTY LIVER

NAFLD refers to a spectrum of diseases of the liver ranging from simple steatosis (fatty infiltration of the liver) to nonalcoholic steatohepatitis (steatosis with inflammation and hepatocyte necrosis) to cirrhosis[7]. Massive hepatic steatosis complicating adult celiac has been described since the 1980s. These cases had a marked increase in aminotransferases, sometimes coursing with jaundice and transitory liver failure, presenting complete resolution of the liver condition after a gluten-free diet (GFD)[8-11]. A few authors have investigated the diagnosis of CD in patients with fatty liver by employing different screening methods (Table 1)[12-18]. The methodologies differ between the studies, both with regard to the diagnosis of fatty liver and the serological and histological diagnosis of CD. The prevalence of reactive celiac antibodies varies from 2% to 13%[12-16]. CD has been described as more prevalent in NAFLD patients with body mass index value < 27 kg/m^2[16] and < 25 kg/m^2[17].

A clinical picture of undiagnosed chronic diarrhea, bloating, refractory anemia, dermatitis herpetiformis, suboptimal body mass index (< 24) or nutritional deficiency (vitamin B12, vitamin D or folic acid) in patients with NAFLD are associated with high likelihood of CD[18]. Liver biochemistry and celiac antibodies become normal after a GFD[12,14,16]. When either abdominal ultrasound or liver biopsy are performed after treatment, steatosis resolution is observed[12,16]. Concerning this topic, controversy persists as to whether the presence of fatty liver is a hepatic manifestation of CD[19]. Some claim that the hepatic manifestation of CD would be a nonspecific chronic hepatitis[13], called by some authors celiac hepatitis[20]. Furthermore, it is not known whether liver disease associated with CD has the potential to progress to liver cirrhosis, although it has been reported that CD is associated not only with cirrhosis of various etiologies, but also with cryptogenic cirrhosis[21-24]. The association between metabolic cirrhosis and refractory CD has also been reported[25].

FATTY LIVER IN PATIENTS WITH CELIAC DISEASE

Zali *et al*[30] followed up on 98 patients with CD, and observed that 2% of the patients with CD fulfilled the diagnostic criteria for metabolic syndrome at diagnosis, while 29.5% of the patients met the criteria after 12 mo of GFD (P < 0.01; OR: 20). Agarwal *et al*[26] evaluated 44 naïve patients with CD, and observed that patients having fatty liver increased from 6 patients (14.3%) at baseline to 13 (29.5%) after one year of GFD (P = 0.002). Ciccone *et al*[27] evaluated the incidence of hepatic steatosis at diagnosis and during follow-up of 185 patients with CD. Hepatic steatosis was found by ultrasound in three patients (1.6%) at CD diagnosis. At the end of the follow-up period (median = 7 years; range 1–36), the prevalence of hepatic steatosis was significantly higher than at the time of CD diagnosis (n = 20; 11.0%) (P < 0.001). A Swedish nationwide study of over 26000 patients with CD demonstrated an increased risk of NAFLD in both children and adults with CD. The relative risk was highly increased in the first year of follow-up, but remained statistically significant even 15 years after CD diagnosis[28]. Tovoli *et al*[29] evaluated 202 celiac patients under a GFD and evidenced the diagnosis of NAFLD in 34.7% when compared with 21.8% in 333 controls. Curiously, in normal-weight patients the higher prevalence of NAFLD was even more evident than in the controls (20% *vs* 5.8%, P = 0.001). On the other hand, this difference was not observed in the overweight population (67.8% *vs* 55.4%, P = 0.202), suggesting that traditional metabolic risk factors may mask the effects of the GFD in these patients. Due to the above stated evidence, monitoring aminotransferases levels periodically in celiac patients under GFD is

Table 1 Celiac disease in patients with fatty liver

Ref.	Country	Patients	Ultrasound	Liver biopsy	AGA	tTG	EmA	Duodenal biopsy	Response with GFD
Grieco *et al*[12], 2001	Italy	30	(+)	(+)	N/A	N/A	4	Negative	Complete
Nehra *et al*[13], 2001	United States	47	N/A	(+)	N/A	N/A	1	N/A	N/A
Bardella *et al*[14], 2004	Italy	59	N/A	(+)	N/A	6	2	2/6	Partial
Lo Iacono *et al*[15] , 2005	Italy	121	N/A	(+)	N/A	20	4	4/4	Partial
Rahimi *et al*[16], 2011	Iran	316	Either/or	Either/or	N/A	8	7	7/8	Complete
Bakhshipour *et al* [17], 2013	Iran	403	(+)	N/A	N/A	14	N/A	12/14	Partial
Kamal *et al*[18], 2018	Egypt	613	(+)	(-)	N/A	160	68	181	Partial

GFD: Gluten-free diet; N/A: Not available; AGA: Anti-gliadin antibody; tTG: Tissue transglutaminase antibody; EmA: Endomysial antibody; Complete response to GFD: Normalization of liver enzymes, celiac antibodies and normal abdominal ultrasound; Partial response to GFD: Normalization of liver enzymes and celiac antibodies.

recommended, especially in patients gaining weight[30].

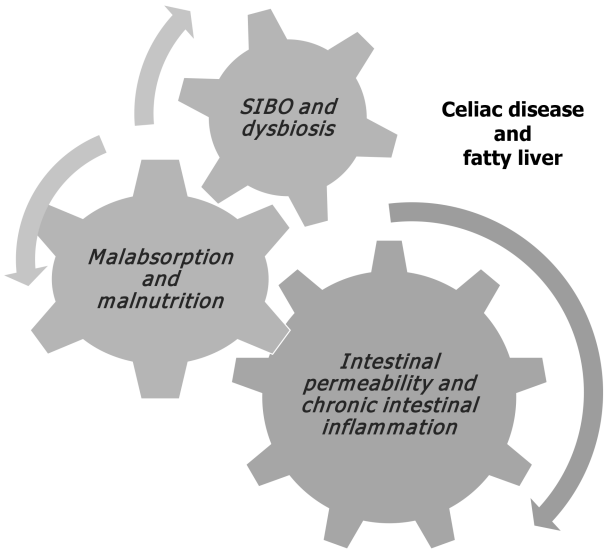
Long-term treatment with proton pump inhibitors (PPI) is associated with excessive weight gain[31, 32]. Imperatore *et al*[33] evaluated 301 patients with newly diagnosed CD, where 4.3% were diagnosed with metabolic syndrome and 25.9% presented with hepatic steatosis at the time of CD diagnosis; 32.8% had long-term exposure to PPI during the study period. After one year, 23.9% of the patients had developed metabolic syndrome and 37.2% had developed hepatic steatosis. Upon multivariate analysis, HOMA-IR (OR: 9.7; P = 0.001) and PPI exposure (OR: 9.2; P = 0.001) were the only factors associated with the occurrence of hepatic steatosis in celiac patients.

PATHOPHYSIOLOGICAL MECHANISMS THAT LINK DISEASES

The mechanisms leading both CD and GFD to the metabolic alterations such as the increase in body weight and body mass index, blood triglyceride and cholesterol levels and blood glucose levels, as well as the development of NAFLD remain to be clarified[34]. In non-celiac patients, insulin resistance leads to fat accumulation resulting in steatosis and oxidative stress, determines lipid peroxidation and increases cytokine production, that results in inflammation and necrosis[35]. In celiac patients, malabsorption and long-standing malnutrition, increased intestinal permeability, chronic intestinal inflammation, small intestinal bacterial overgrowth and/or dysbiosis have been suggested to have possible roles in establishing celiac hepatitis in CD (Figures 1 and 2)[36,37].

Malabsorption and long-standing malnutrition

At diagnosis, CD patients have lower body mass index than the general population due to malabsorption[38,39]. Kwashiorkor and dietary protein deficiency may occur associated with fatty liver on liver biopsy[40]. In patients with significantly reduced intestinal absortive surface, the ability to assimilate dietary protein may be severely reduced; intestinal malabsorption per se has been associated with hepatic steatosis after jejunoileal bypass in patients with morbid obesity[41,42] and also in patients with inflammatory bowel disease, specially after extensive intestinal resections[43]. It has been hypothesized that malabsorption in CD might lead to chronic deficiency of a lipotropic factor, and that fatty liver may occur with an associated pyridoxine deficiency[44]. In addition to malnutrition itself, qualitative and quantitative changes in the intestinal microflora occur in protein-energy malnutrition [45], however the subject of dysbiosis will be addressed below. Weight changes are common in patients suffering from CD after commencing a GFD[46,47], and the GFD dietary behavior of CD patients correlates with NAFLD[48]. A possible explanation would be the ingestion of gluten substitute products paired with the hyperphagic compensatory status that usually follows malabsorption inducing weight gain[29]. There is evidence that GFD can determine a higher intake of simple sugars, proteins and saturated fat and a lower intake of complex carbohydrates and fibers[49,50]. These changes can contribute to the development of hypertension, dyslipidemia, diabetes mellitus, metabolic syndrome



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Figure 1 Pathophysiological mechanisms associated with fatty liver in patients with celiac disease. SIBO: Small intestinal bacterial overgrowth.

and hepatic steatosis[51].

Intestinal permeability and chronic intestinal inflammation

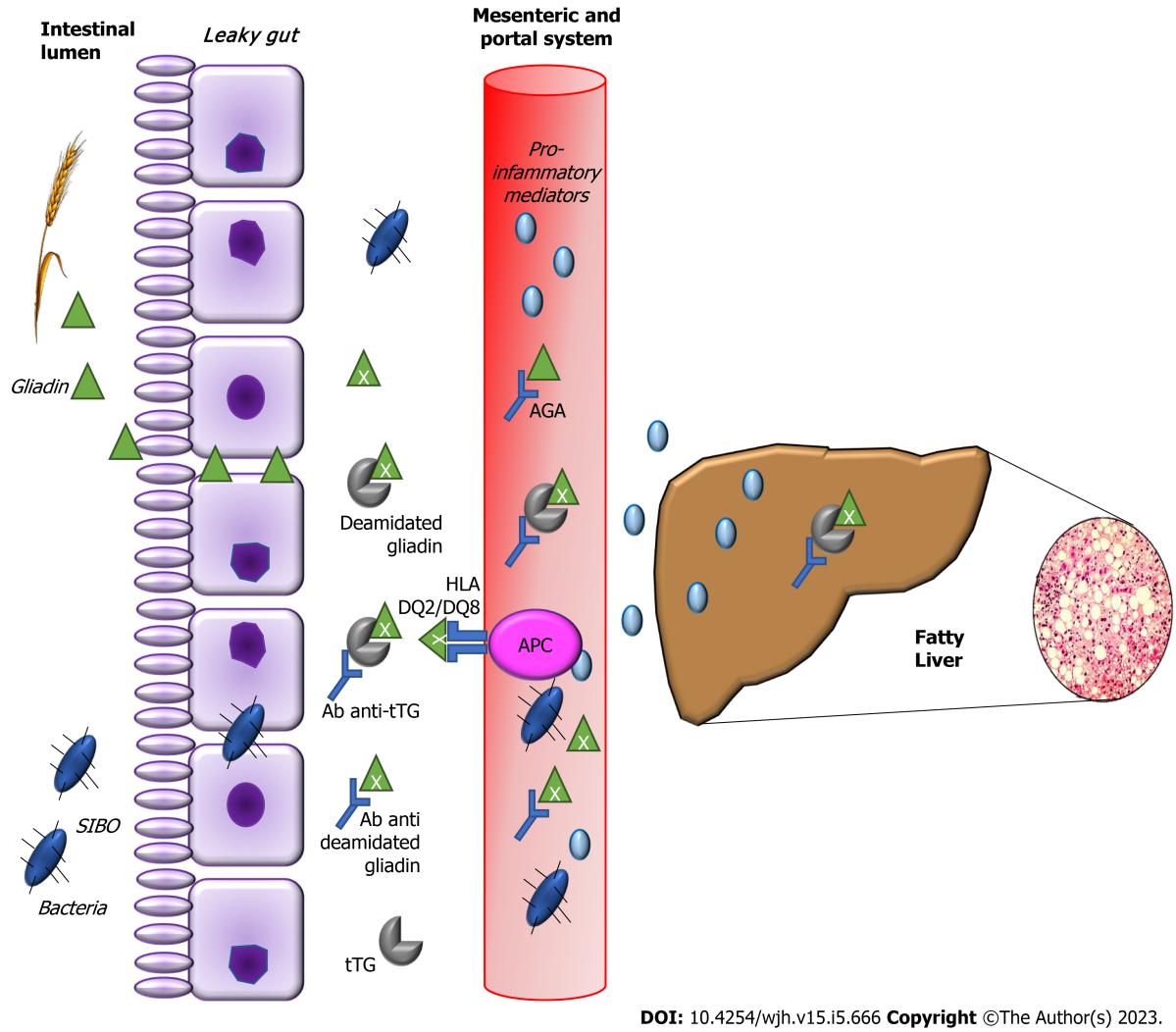
Intestinal permeability is increased in CD[52] and may favor the absorption of antigens by the intestine, *via* the portal circulation[53]. It is known that intestinal permeability is increased in NAFLD, as is bacterial overgrowth in the small intestine, and that these factors are associated with the severity of hepatic steatosis. The increase in intestinal permeability appears to be the key in the contribution of the gut-liver axis to the development of NAFLD[54]. The term gut–liver axis refers to a close anatomical, metabolic, and immunologic link between the gut and liver. The liver and intestine are tightly connected *via* the mesenteric and portal system, which supplies the liver not only with nutrients but also with gut derived food, bacterial antigens, and bacterial metabolic products. The liver portal circulation, derived from the mesenteric vessels, are the afferent part of the gut–liver axis[55]. Patients with concomitant NAFLD and CD present advanced intestinal inflammation and villous atrophy and higher levels of proinflammatory cytokines than those with CD alone, which suggests advanced intestinal injury when both diseases are present in one individual[18]. Moreover, the intestines and the liver are characterized by shared lymphocyte homing and recruitment pathways. Gut-derived T-lymphocytes may also contribute to hepato-biliary inflammation[56]. Additionally, patients with concomitant NAFLD and CD reveal higher levels of hepatic steatosis, liver stiffness, hepatic fibrosis progression rates and profibrotic mediators compared with those with either NAFLD or CD alone[18].

Small intestinal bacterial overgrowth and/or dysbiosis

The diagnostic standard for small intestinal bacterial overgrowth (SIBO) is the detection of > 10^5 colony forming units of bacteria per ml of jejunal fluid. The difficulty in collecting jejunal fluid has led to the development of new diagnostic methods, one of them being the hydrogen breath test[57]. Wigg *et al*[58] observed that small intestinal bacterial overgrowth was present in 50% of patients with NAFLD and 22% of control subjects (P = 0.048), while intestinal permeability and serum endotoxin levels were similar in the two groups. For the CD patients, gliadin may impair the balance between intestinal microflora and the human body. Through digestive process, large quantity of undegraded gliadin reaches the intestines, delivers abundant substrates for different bacteria, contributes the reproduction of gliadin-degrading bacteria and breaks the steady state of intestinal microbiota[59]. Rubio-Tapia *et al*[60] observed a prevalence of SIBO of 9.3% diagnosed by quantitative culture of intestinal aspirate in CD patients. The association between SIBO and CD occurs mainly in patients who are newly diagnosed and beginning a GFD, and specially in those with nonresponsive CD[61,62].

WHEN TO INVESTIGATE

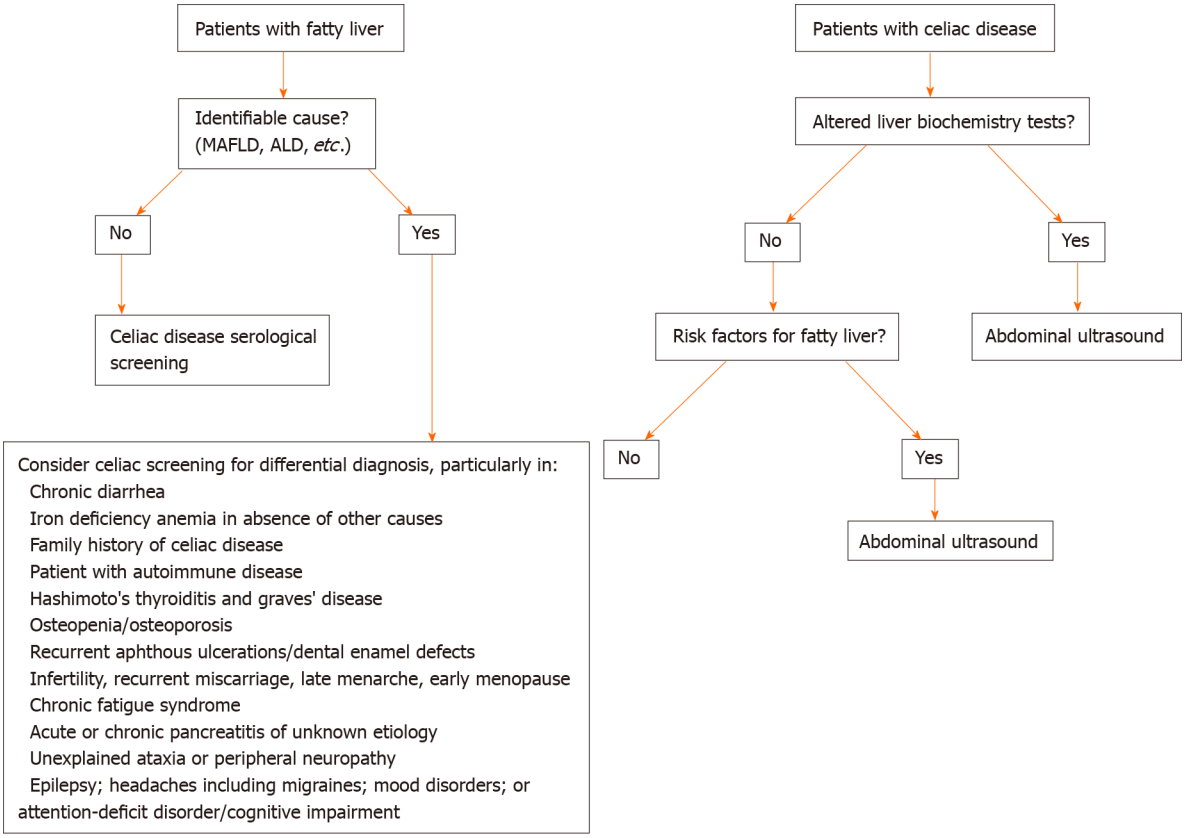
Screening all patients with NAFLD for CD is controversial. Nonetheless, clinical suspicion may arise from the presence of classical malabsorption symptoms or low body mass index, leading to active



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Figure 2 Molecular mechanisms that link fatty liver and celiac disease. Mucosal epithelium of the small intestine is the barrier between gut lumen and microbiota. The intestinal microflora is a primary source of the endotoxins produced by Gram-negative bacteria, normally crossing the mucosa in small amounts and entering the mesenteric and portal system. Celiac patients present increased intestinal permeability. Consequently, there are increased gut derived products and endotoxins in portal stream and increased levels of endotoxin-mediated cytokines in the liver. Ab anti-tTG: Antibody against tissue transglutaminase; AGA: Anti-gliadin antibody; APC: Antigen presenting cells; HLA: Human leukocyte antigen; SIBO: Small intestinal bacterial overgrowth; tTG: Tissue transglutaminase antibody.

screening of celiac antibodies and early diagnosis. The GFD may improve liver tests and liver steatosis in patients with NAFLD and CD, but it remains controversial whether this effect is independent of nutritional factors. Given the biological complexity and clinical heterogeneity of NAFLD and its comorbidities, the identification of the precise drivers of such disease would aid the development of targeted therapeutics[35]. International NAFLD guidelines[63,64] recommend the investigation of other diseases that may occur with liver steatosis and that have a treatment different from NAFLD, even in the presence of metabolic risk factors. From our point of view, CD represents a disease for which diagnosis requires targeted treatment to benefit the patient. This approach not only reduces the risk of developing more severe celiac-related liver injuries, but from a systemic point of view it is known that a GFD can prevent celiac complications such as intestinal malignancies and several autoimmune diseases. Screening for CD is justified in subjects with and without known risk factors for NAFLD. Priority groups include, individuals with chronic diarrhea, iron deficiency anaemia in absence of other causes, family history of CD, patients with autoimmune disease, Hashimoto's thyroiditis and Graves' disease, osteopenia or osteoporosis, recurrent aphthous ulcerations/dental enamel defects, infertility, recurrent miscarriage, late menarche, early menopause, chronic fatigue syndrome, acute or chronic pancreatitis after excluding other known causes and neurological symptoms such as unexplained ataxia or peripheral neuropathy, epilepsy, headaches including migraines, mood disorders, or attention-deficit disorder/cognitive impairment[65]. On the other hand, it is not yet well defined whether it is necessary to investigate fatty liver in all patients with CD, as liver changes may be resolved with GFD. However, it is imperative to investigate fatty liver when liver biochemical tests persist elevated despite GFD. Figure 3 demonstrates an algorithm proposal for CD screening in patients with fatty liver and also for fatty liver screening among celiac patients.



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Figure 3 Proposed screening algorithm for celiac disease in patients with fatty liver and vice versa. MAFLD: Metabolic associated fatty liver disease; ALD: Alcoholic liver disease.

CONCLUSIONS AND FUTURE DIRECTIONS

CD and NAFLD are a common association and prompt recognition of both diseases is crucial for adequacy of treatment and to improve care. Although a direct cause-effect relationship can be clearly observed in some patients with CD that develop NAFLD as a result of malabsorption; a subtler mechanism, in which CD acts more as a cofactor capable of changing the natural history of NAFLD, has recently been suggested. Therefore, screening for CD should be strongly considered in these patients, although there are no data that exactly define the priority groups. Future investigations focusing on the pathophysiological mechanisms, particularly on the role of changes in the microbiota and intestinal permeability, may help in understanding the interference of one disease on the other. In addition, longitudinal studies evaluating the progression of these patients, particularly the impact of the GFD on NAFLD outcomes, are essential to support the clinical decision-making process.

FOOTNOTES

Author contributions: Narciso-Schiavon JL designed the research, collected clinical data, analyzed the data and wrote the paper; Schiavon LL designed the research and reviewed the paper; all authors have read and approved the final version of the manuscript.

Conflict-of-interest statement: All authors do not have any conflicts of interest.

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MINIREVIEWS

Current guidelines for diagnosis and management of hepatic involvement in hereditary hemorrhagic teleangiectasia

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Abstract

Hereditary hemorrhagic teleangiectasia (HHT), also known as Rendu-Osler-Weber syndrome, is the most common cause of hepatic vascular malformations in adults. Different vascular shunts (arteriovenous, arterioportal or portovenous) lead to different clinical manifestations. Even though no hepatic-related symptoms are reported in the majority of cases, the severity of liver disease could lead to refractory medical conditions, in some cases requiring liver transplantation. The aim of this manuscript is to provide an updated overview of the current evidence regarding the diagnosis and treatment of HHT liver involvement and liver-related complications.

Key Words: Hereditary hemorrhagic teleangiectasia; Rendu-Osler-Weber syndrome; Hepatic vascular malformations; Liver

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Core Tip: Hereditary hemorrhagic teleangiectasia (HHT) is the most common cause of hepatic vascular malformation in adults. Although liver involvement is common in HHT, most patients do not present any hepatic-related symptoms. Unfortunately, some patients have severe forms of disease with refractory medical conditions related to the hepatic vascular malformations. For those patients the only definitive treatment available at present is liver transplantation.

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INTRODUCTION

Hereditary hemorrhagic teleangiectasia (HHT) or Rendu-Osler-Weber syndrome is a rare autosomal dominant disorder characterized by mucocutaneous teleangiectases and systemic vascular malformations (VMs). HHT can be ruled in by using the Curaçao criteria (recurrent epistaxis, multiple mucosal/cutaneous teleangiectases, visceral VMs and first-degree relative with HHT); if at least 3 of these criteria are met, the diagnosis of HHT is considered to be definite (Table 1)[1,2].

Molecular genetic test is useful in order to detect gene mutations. Endoglin (ENG, on chromosome 9) and activin A receptor type II-like 1 (ACVRL1, on chromosome 12) genes are involved in approximately 90% of cases and are responsible of HHT1 and HHT2, respectively. In addition to these two genes, mutation of SMAD4 has been identified in patients with the association of juvenile polyposis and HHT (PJ-HHT syndrome, approximately 2% of cases) in which anemia is the predominant symptom due to gastrointestinal bleeding. Mutations of GDF2 and RASA-1 genes have also been described but they are extremely rare (Table 2)[3,4].

Loss of function mutations in ENG and ACVRL1 cause anomalous angiogenesis leading to VMs development[2]. One of the primary mechanisms underlying aberrant vascular endothelial growth factor (VEGF)-related angiogenesis in HHT patients appears to be the overactivation of phosphatidylinositol 3-kinase (PI3K) signaling in endothelial cells[5]. High VEGF levels drive VMs development in mouse models and its normalization suppresses progression of these anomalous vascular structures[6,7].

HHT1 is more frequent in Mediterranean countries and it is characterized by a higher incidence of pulmonary and brain VMs, while HHT2 is more frequent in Northern Europe and North America with a higher incidence of hepatic VMs[8]. No significant difference was found in age at debut of symptoms and the severity of epistaxis between patients with HHT1 and HHT2. On the other hand gastrointestinal bleeding was reported to be more common in patients with HHT1[9]. HHT2 is associated to a higher risk of symptomatic liver disease[10].

HHT represents the most common cause of congenital hepatic vascular malformations in adults, and liver involvement is a commonly observed feature in the disease (Table 2)[11-13]; the mean age at diagnosis is 48 years[14].

Whilst more than 90% of cases do not present any hepatic-related symptoms, patients affected by HHT are susceptible to developing a range of clinical condition with varying presentations depending on the type of hepatic VM[15]. In some cases, the severity of clinical conditions requires liver transplantation. Women seems to have a more frequent (female prevalence 4.5 fold higher than males) and more severe liver involvement in both HHT1 and HHT2[16].

In the following paragraphs we will discuss the diagnostic and therapeutic approach for liver involvement in HHT patients.

HEPATIC VMS CLASSIFICATION AND CLINICAL MANIFESTATIONS

Three types of hepatic VMs have been described based on liver vascular anatomy: arteriovenous (the most frequent, between hepatic artery and hepatic vein), arterioportal (between hepatic artery and portal vein) and portovenous (between portal vein and hepatic vein)[15]. These different subtypes of hepatic vascular shunting usually coexist and affect the liver diffusely[17]. HHT liver involvement is a continuous process from small teleangiectases to very large VMs; size change during follow up has been observed in 21% of patients[10].

Arteriovenous shunts could cause high output cardiac failure (HOCF), ischemic cholangitis and mesenteric ischemia. Arterioportal shunts could cause portal hypertension, but also biliary ischemia. Portovenous shunts could cause HOCF, but also portosystemic encephalopathy[15]. Generally, one of them predominates functionally, but fluctuation from a clinical condition to another is common.

HOCF is the most common complication of HHT liver involvement and it generally starts being clinically significant when intrahepatic shunt output is > 20% of cardiac output[18]. HOCF is associated to an increased risk of atrial fibrillation and the associated increased pulmonary blood flow secondary to liver VMs may lead to the development of post-capillary pulmonary arterial hypertension. Less frequently, HHT patients may develop a pre-capillary pulmonary arterial hypertension that seems to be related to the remodeling of small pulmonary arteries caused by ENG and ACVRL1 gene mutations with histologic features broadly similar to those observed in idiopathic pulmonary arterial

Table 1 Curaçao diagnostic criteria of hereditary hemorrhagic teleangiectasia

Curaçao criteria	Description
Epistaxis	Spontaneous and recurrent
Teleangiectases	Multiple, at characteristic sites: Lips, oral cavity, fingers, nose
Visceral lesions	GI telangiectasia, pulmonary, hepatic, cerebral or spinal AVMs
Family history	A first degree relative with HHT
Number of criteria	**HHT diagnosis**
3-4	Definite
2	Possible
0-1	Unlikely

AVMs: Arteriovenous malformations; HHT: Hereditary hemorrhagic teleangiectasia.

Table 2 Genes responsible for hereditary hemorrhagic teleangiectasia, phenotypes and liver involvement prevalence

Gene	Protein	Location	Phenotype	Liver involvement prevalence
ENG	Endoglin	9q34.11	HHT1	7.6%-43.0%
ACVLR1	ALK1	12q13.13	HHT2	40.6%-57.6%
MADH4	Smad4	18q21.1	PJ-HHT	33.3%
GDF2	BMP9	10q11.22	HHT-like	Unknown
RASA-1	p120-RasGAP	5q14.3	CM-AVM	Unknown

ACVLR1: Activin A receptor type II-like 1; ALK1: Activin-like receptor kinase 1; BMP9: Bone morphogenetic protein 9; CM-AVM: Capillary malformation–arteriovenous malformation syndrome; GDF2: Growth differentiation factor 2; ENG: Endoglin; MADH4: Mothers against decapentaplegic homolog 4; p120-RasGAP: p120-Ras GTPase activating protein; PJ: Juvenile polyposis; RASA-1: Ras p21 protein activator 1; Smad4: Small mother against decapentaplegic.

hypertension. Right heart catheterization is essential to differentiate between the two forms[19].

Arteriovenous shunting can cause a blood steal with secondary bile ducts ischemia; this phenomenon is facilitated by the vascular anatomy of the biliary system, which derives its blood supply solely from the hepatic artery *via* the peribiliary plexus. Biliary ischemia can subsequently evolve in biliary strictures and dilations (secondary sclerosing cholangitis), secondary infection of the biliary system (infectious cholangitis), bilomas or biliary cysts (mimicking Caroli's disease), and elevation of serum alkaline phosphatase and gamma glutamyl transpeptidase. In the more severe forms, ischemia also affects hepatocytes causing hepatocellular necrosis leading to hepatic hemorrhage and bile leak[18,20].

Modification of normal liver perfusion may increase hepatocytes regenerative activity leading to development of focal nodular hyperplasia (FNH), 100 times more frequent in HHT patients than in general population, or nodular regenerative hyperplasia (NRH). In NRH the liver parenchyma undergoes a diffuse transformation into multiple regenerative nodules with hepatocytes arranged in plates, without fibrosis separating nodules.

Therefore, portal hypertension in HHT patients may be pre-hepatic, due to the increased blood flow from arterioportal VMs, or pre-sinusoidal, due to NRH (a well-known cause of non-cirrhotic intra-hepatic portal hypertension).

Hepatocellular regeneration nodules may be associated with minimal perisinusoidal and portal fibrosis which can mimic cirrhosis on imaging and lead to being diagnosed incorrectly[18,21].

This appearance is commonly defined "pseudocirrhosis" since there is no significant liver fibrosis, liver function tests are generally normal and the risk of hepatocellular carcinoma is not as increased as for liver cirrhosis[22].

Xu *et al*[23] reported that hepatic involvement in HHT and Budd-Chiari syndrome (BCS) may be linked, suggesting a shared pathogenetic mechanism characterized by vascular dysplasia and a thrombophilic condition induced by HHT that would eventually lead to BCS[24]. Nonetheless further studies are needed to evaluate the possible relationship between these two diseases.

Several disease progression predictors have been identified. Singh *et al*[25] proposed a clinical scoring system for the estimation of the probability of clinically significant liver disease in HHT patients. This score uses readily available information such as patient gender, age, hemoglobin and alkaline

phosphatase at presentation, but is currently not widely recognized and still need to be validated (Table 3).

IMAGING SCREENING AND STADIATION

Screening for liver VMs should be offered to adults with a definite or suspected diagnosis of HHT[26] and the imaging test of choice for screening is Doppler ultrasound[27] for its accuracy in detecting hepatic VMs[28], its availability, repeatability, low cost and interobserver agreement[29-31]. In addition, Doppler ultrasound allows to establish the grade of severity of liver involvement and therefore correlates with patient outcomes and predictors of clinical outcomes[27].

Regarding the follow-up of hepatic VMs there are no standardized protocols nor consensus; ultrasound is usually repeated every 1 or 2 years according to the severity of liver involvement and is generally determined case by case.

Caselitz *et al*[28] defined major and minor criteria required for the diagnosis of liver VMs in HHT by Doppler ultrasound: A dilated common hepatic artery (> 7 mm) and intrahepatic arterial hypervascularization are the two major criteria; minor criteria are either Vmax in hepatic artery > 110 cm/s, low resistivity index (RI) of the proper hepatic artery (*i.e.* < 0.60), Vmax of portal vein > 25 cm/s and/or a tortuous course of extrahepatic hepatic artery. Presence of liver VMs in HHT is defined by two major criteria or one major criterion and two minor criteria[28]. According to Buscarini *et al*[27] severity grading ranges from 0.5 to 4 (Table 4)[32].

Hepatic artery dilation > 4 mm is a very sensitive parameter to differentiate HHT patients with or without liver involvement from the very early stages (Figure 1A)[32]; despite cirrhosis and hypervascular liver tumors may cause a dilation of hepatic artery, this rarely exceeds the upper normal limit as in HHT patients.

Peripheral subcapsular spots (identified by color Doppler) with high-velocity arterial blood flow and low RI are suggestive of small peripheral VMs, which are usually found from early stage in HHT patients with liver involvement (Figure 1B)[27].

Common hepatic artery dilation is also a predictor of HOCF development in patients with liver VMs [33]. A high velocity flow with low RI in intrahepatic branches of hepatic artery is highly suggestive of intrahepatic arterioportal shunt; furthermore, hepatic artery to portal vein shunts commonly cause pulsatility of portal flow with phasic or continuous reversal (Figure 1C). Arteriovenous shunts, on the other hand, usually result in a change in the Doppler waveform of hepatic veins (from triphasic to biphasic or even continuous patterns in severe involvements)[27,32].

FNH is common in HHT patients with liver involvement, and it generally appears as an isoechoic nodular lesion in liver parenchyma.

In those cases where the liver involvement is more severe, common findings are nodular and irregular liver surface with a coarse echo-pattern, previously known as pseudocirrhosis[34], as well as portal vein and hepatic vein dilation[27,32].

Multiphase contrast-enhanced abdominal computed tomography (CT) has an excellent yield and accuracy in defining liver vascular malformations and it is easily reproducible across different centers (Figure 2), however, it does not correlate with liver VMs severity and clinical presentations and is therefore recommended only if the expertise in detecting liver VMs using Doppler US is unavailable[17,35]. Nonetheless, it is widely used in complicated liver vascular malformation which are considered for liver transplantation[14] as it has the advantage of great accuracy in detecting biliary complications (*i.e.* necrotizing cholangitis with formation of bilomas)[32]; it is able to characterize the complexity of hepatic vascular alterations, the different types of shunts and parenchymal perfusion disorders[36,37] and it has great accuracy in differentiation between FNH from regenerative nodules[38].

Magnetic resonance imaging (MRI) of the liver shows great accuracy in characterizing focal liver lesions and in detecting liver VMs (they are better depicted on MRI angiograms and dynamic MRI images outlining a map of anomalous vessels)[39]. MRI is as accurate as multirow CT scan, with the advantage of the absence of ionizing radiations; nonetheless due its high cost and low availability it is recommended for diagnosis and follow-up of liver AVMs only when expertise in Doppler US is lacking [26,36].

The role of contrast-enhanced ultrasound (CEUS) with sulfur hexafluoride-filled microbubbles has been recently investigated in a cohort of 18 patients with HHT regarding macro and micro-circulation showing a higher percentage of hepatic VMs (especially of arterioportal shunts) than what is reported in literature[40]. However, CEUS seems to add no further information to Doppler US evaluation that still has great accuracy and sensitivity. It should also be noted that the use of sulfur hexafluoride-filled microbubbles is contraindicated in patients with right-to-left shunts and may result in an unjustified risk considering the high percentage of pulmonary VMs in HHT patients[32].

Liver biopsy is generally not necessary for diagnosis of hepatic VMs due to the increased bleeding risk related to a percutaneous procedure. Therefore, hepatic nodules in HHT patients should be characterized non-invasively when possible. If a biopsy is needed, always consider the increased risk of bleeding in HHT patients[14,26].

Table 3 Clinical Scoring Index for clinical probability of significant liver disease in hereditary hemorrhagic teleangiectasia patients[25]

Criteria		Points
Age at presentation (yr)	> 47	1
	≤ 47	0
Sex	Female	1
	Male	0
Hb at presentation (g/dL)	< 8	3
	8-12	2
	12-16	1
	> 16	0
ALP at presentation (IU/L)	> 300	4
	225-300	3
	150-224	2
	75-149	1
	> 75	0
Clinical Scoring Index	**Clinical probability of significant liver disease**	
≤ 2	Low	(0.4%-3.2%)
3-6	Intermediate	(8.2%-64.1%)
≥ 7	High	(82.9%-93.0%)

ALP: Alkaline phosphatase; Hb: Hemoglobin.

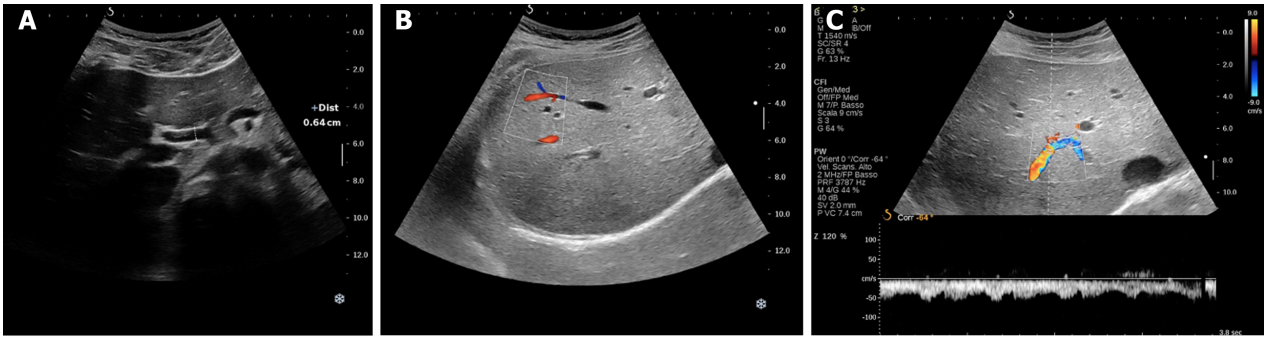
Table 4 Doppler ultrasound grading of hepatic vascular malformations in hereditary hemorrhagic teleangiectasia patients[24]

VMs grade	Doppler US findings
0.5	HA diameter 5-6 mm and/or
	PFV > 80 cm/sec and/or
	HA RI < 0.55 and/or
	Peripheral hepatic hypervascularization
1	HA dilation > 6 mm (only extrahepatic) and
	PFV > 80 cm/sec and/or
	HA RI < 0.55 and/or
2	HA dilation intra- and extrahepatic and
	PFV > 80 cm/sec
	Possible flow abnormality in portal and/or hepatic veins
3	Complex changes in HA and its branches with marked flow abnormalities
	Flow abnormality in portal and/or hepatic veins
4	Decompensation of arteriovenous shunt with dilatation of portal and/or hepatic vein and marked flow abnormalities in both arteries and vein/s

HA: Hepatic artery; PFV: Peak flow velocity; RI: Resistivity index; US: Ultrasound; VMs: Vascular malformations.

LIVER TRANSPLANTATION

The first case of liver transplantation (LT) for HHT was reported in 1995[41]. Nowadays, LT is the recommended surgical option for severe hepatic involvement in HHT patients[26]. The main indication for LT are refractory HOCF and ischemic cholangitis (67.5% and 39.7% of cases, respectively)[42].



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Figure 1 Ultrasound findings in hereditary hemorrhagic teleangiectasia. A: Hepatic artery dilation; B: Peripheral hepatic hypervascularization; C: Pulsatile flow in right portal branch related to arteriovenous malformation.



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Figure 2 Multiple arteriovenous malformations and enlarged hepatic artery in contrast-enhanced computed tomography scan.

A recent systematic review by Riera-Mestre *et al*[42], reported 83 cases of LT for HHT worldwide. Perioperative complications within 30 days were described in 33.7% of patients (mainly bleeding complications) and a survival rate of 88% at six years has been reported.

While ischemic cholangitis is considered an urgent indication to LT, the best timing for transplantation in a patient with HOCF has not been defined yet.

MELD score was designed for cirrhotic patients and is widely used in defining the LT waitlist priority; HHT patients are exempt from being scored and should be included and prioritized in LT waitlist regardless of MELD score[43]. Right heart catheterization should always be performed in patients with HHT being evaluated for LT, to exclude severe pulmonary hypertension; LT can be undertaken if pulmonary vascular resistance is < 240 dynes · sec · cm^{-5} (< 3 Woods units)[14].

LT for HHT patients constitutes a more complex surgical procedure compared to other indications for LT and is characterized by higher blood transfusion requirement and more perioperative complications.

The hepatic artery in HHT patients may be dilated, tortuous and/or aneurysmatic and arterial graft anastomosis could be more challenging. Moreover, there is a high incidence of hepatic artery thrombosis after LT (about 7% of cases) that could result in need for re-transplantation. The presence of high-flow extrahepatic arterial teleangiectases may cause an arterial steal, so an attentive stadiation of disease before transplantation and an intraoperatively ultrasound arterial flow measurement through the anastomosis are strongly suggested[42,44].

The hyperdynamic state following recipient hepatic artery dissection constitutes a potential risk of bleeding in any extrahepatic site of VMs. Fatal pulmonary bleeding has been described in two patients, so embolization of pulmonary VMs should be considered before LT[45,46].

Intrahepatic relapse of HHT lesions is a late but common event after LT. The median recurrence time is 127 mo and can occur up to 19 years after LT; the estimated cumulative risk of recurrence at 5, 10, 15 and 20 years is 0%, 16.7%, 47.9% and 87%, respectively[42,47-49]. For this reason, these patients require a life-long follow-up.

The pathophysiology of recurrence in the transplanted liver is still unclear. Presence of endothelial cells of recipient origin in the transplanted liver has been recently described[48]. Microchimerism after LT is a well-known phenomenon, but in this case the liver graft repopulation by patient endothelial cells

may lead to an aberrant angiogenesis causing the recurrence of the disease[50].

An mTOR inhibitor-based immunosuppressive regimen after LT may reduce hepatic VMs recurrence by blocking the PI3K signaling pathway[51].

ENDOVASCULAR AND SURGICAL TREATMENTS

Hepatic VMs are generally considered not suitable for endovascular or surgical approach due to the high morbidity and mortality rates.

Transarterial embolization is generally used for treating HOCF and portal hypertension. This procedure is performed in multiple stages (one to five sessions); among the several protocols proposed, the most used one consists in an initial embolization of vascular bed with a mixture of polyvinyl alcohol followed by embolization with microcoils. Arterial branches of right and left lobe have to be embolized in different sessions[52].

A peri-procedural infusion of analgesics, anti-emetics and steroids is generally advised; some authors also consider a peri- and post-procedural prophylactic antibiotic coverage[53].

The most common complications are biliary or hepatic necrosis that occur in 20%-60% of cases[53]; need for emergent LT and death is reported in up to 10% of cases[54].

Regarding the high risk of ischemic hepatic damage, transarterial embolization is generally contraindicated in patients with signs of biliary involvement[14].

There have been very few published accounts of transjugular intrahepatic portosystemic shunt (TIPS) as portal decompressive intervention. The high risk of worsening the cardiac output and the high bleeding risk related to the puncture lead to consider this treatment largely unsuccessful and so not recommended[55,56].

Hepatic artery banding and/or ligation are other potential approaches for managing HOCF due to hepatic VMs. Banding consists in the diameter reduction by one third to a half of the pre-operative diameter of common hepatic artery and potentially lobar arteries; ligation consists in closure of feeding arteries of the lobe predominantly involved by VMs.

The diameter reduction achieved with arterial banding should be sufficient to reduce liver hyperperfusion, without causing ischemic hepatobiliary damage. Banding should be guided by colorDoppler ultrasound with a desired hepatic artery flow of 330 ± 80 mL/min[57]; another indirect parameter of sufficient arterial banding is the return of arterialized areas of liver surface to normal red color[58].

CT angiography is always recommended before surgery in order to investigate extra-hepatic vascular anatomy. If appropriate, collateral circulation arising from superior mesenteric or left gastric arteries could also be ligated and enlarged gastroduodenal artery banding may also be considered[58].

Based on the risk of hepatic necrosis, these procedures are contraindicated in case of significant portovenous shunting[59].

For a long time, hepatic artery ligation or banding has been used in limited number of cases due to the high rate of ischemic cholangitis and undefined long-term survival[57,60,61]. Lui *et al*[58] recently reported a series of 13 patients treated with hepatic artery ligation/banding with a low rate of peri- and post-operative complications (only two patients experienced cholangitis, who were treated conservatively), improvement of symptoms and good survival outcome (only one patient died in a median follow-up of 50 mo). Authors advise against dissecting malformed and tortuous vessels around extrahepatic biliary tract in order to reduce the risk of ischemic damage and against dividing perihepatic ligaments in order to preserve arterial flow to the liver.

Conventional hepatic surgery, like segmental resection or hemi-hepatectomy, is anecdotal[62] or reported for hepatic shunting in non-HHT patients[63] and for non VM indications in HHT patients[64,65]. This approach could be considered in very selected patients with symptomatic disease and very large VMs localized in a single segment/lobe, but such kind of indication should be given with caution.

Considering the high complication and mortality rates, together with their palliative role, endovascular and surgical treatments are still generally not recommended and should be proposed only in severely symptomatic patients that are not transplant candidates and have failed medical therapy; these approaches should be deliberated by a multidisciplinary team and should be performed only by expert physicians in referral centers[14].

MEDICAL TREATMENTS

First-line medical treatment, such as management of anemia with iron replacement therapy or management of mild bleedings with antifibrinolytics, concerns almost all HHT patients but it is not the aim of this paper, so it will not be discussed further. At the same time, first-line medical treatment for hepatic VM-related HOCF should be evaluated and managed by physicians with expertise in that field (such as cardiologists) and it goes beyond the purpose of this paper.

Management of portal hypertension follows the same principles as in patients without HHT[66,67], but non-selective beta-blockers should be used with caution in patients with HOCF, although they still are the drugs of choice[26].

Similarly, the management of portosystemic encephalopathy follows the same principles as in cirrhotic patients without HHT (*i.e.* lactulose and rifaximin)[26,68].

Infectious complications, such as cholangitis and hepatic abscesses, generally require antibiotic therapy. Large biliary duct obstruction is uncommon in HHT patients, and endoscopic retrograde cholangiopancreatography with stenting is not indicated[26], because it seems to increase the risk of infection in ischemic ducts and the risk of hemobilia[69,70].

Over the last few decades, research has primarily focused on utilizing antiangiogenetic drugs with the aim of targeting the aberrant angiogenesis causing VM formation and endothelial frailty. Several molecules have been investigated and multiple clinical trials are ongoing (such as thalidomide[71,72], tacrolimus[73], sorafenib[74], pazopanib[75,76], doxycycline[77,78] and others) with interesting results on nasal and gastrointestinal bleeding control, but the only molecule that has been studied for HOCF related to hepatic VMs is bevacizumab.

Bevacizumab is a humanized monoclonal antibody which exerts its antiangiogenic activity by inhibiting the VEGF. In 2012, its efficacy has been prospectively investigated in HHT patients with HOCF related to liver VMs resulting in a decrease cardiac output[79]; a reduced or delayed need for transplantation has also been described[80]. Bevacizumab has also demonstrated a reduction in nasal and gastrointestinal bleedings resulting in an improvement of anemia, decrease of blood transfusion need and better quality of life[81,82].

Numerous dosing schedules have been investigated, but the most common dose for initiation was 5 mg/kg every 2 wk for a total of 6 injections; infusion duration should be of at least 30 min (first administration should be given in at least 60 min to assess patient drug tolerance)[79]. Despite a high inter-patient bleeding-free interval, almost all patients relapse after a year of discontinuation of bevacizumab and they may require maintenance therapy or may repeat a new administration cycle that could become lifelong[83]. To date, there are no prospective studies concerning maintenance therapy; the dosing schedule should therefore be determined based on patient response and tolerance[81,83].

Similarly, the safety of long-term bevacizumab administration has not been prospectively evaluated. However, it could be inferred indirectly from prolonged administration of the drug for other indication.

The most frequent adverse events are generally mild and infusion-related, such as headache, nausea and vomiting, asthenia, abdominal pain, muscle pain, diarrhea and rash[79].

A major concern among drug-related adverse events is addressed to arterial hypertension, venous thrombosis and hemoptysis from pulmonary VMs[81,84]. Therefore, it is crucial to assess patients prothrombotic conditions prior to starting therapy with bevacizumab, and pulmonary VMs screening and treatment should be performed according to guidelines as for every HHT patient. Other potentially serious adverse events are gastrointestinal perforation and proteinuria[84]. Since a delay in wound healing has been reported during antiangiogenetic treatment, it is recommended to stop bevacizumab 6-8 wk before surgery and to restart it only if wounds are totally healed.

Bevacizumab is contraindicated in patients with severe arteriopathy, a history of ischemic complications, recent deep vein thrombosis (< 6 mo) or recent severe infection (< 1 mo) and should be used with caution in patients with non-post-capillary pulmonary hypertension[85]. It is also contraindicated in pregnancy, so effective contraceptive measures should be adopted by women in childbearing age during treatment and for six months after discontinuation[85].

A recent international expert consensus paper suggests a monitoring protocol for HHT patients treated with bevacizumab which consists in regular clinical examination (blood pressure measurement, epistaxis monitoring, blood transfusion require recording, adverse events collection) laboratory (blood cell count, liver and kidney function, ferritin, proteinuria) and scheduled echocardiography with cardiac index measurement[85].

To date, there is not sufficient available evidence from randomized control trials and bevacizumab is not market-authorized for HHT, but international expert consensus recommends considering intravenous bevacizumab for severe and refractory nasal and/or gastrointestinal bleeding and for HOCF secondary to hepatic VMs not sufficiently responder to first-line medical therapy[26,85,86]. Based on the rates of minimal or partial response to bevacizumab and the recurrence after drug discontinuation, intravenous bevacizumab should be considered as a potential "bridge" therapy to LT.

CONCLUSION

Liver involvement is very common in HHT patients and hepatologists should be aware of this condition and the available diagnostic and prognostic tools. Fortunately, clinically significant liver disease is uncommon, but its management could be challenging. Liver transplantation remains the only curative treatment for these patients. Endovascular and surgical approaches should be avoided in patients with liver VMs. Bevacizumab has shown promising results, but it should be used with caution and only in referral centers.

FOOTNOTES

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ORIGINAL ARTICLE

Clinical Trials Study

Respiratory muscle training with electronic devices in the postoperative period of hepatectomy: A randomized study

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Abstract

BACKGROUND

Many studies have been developed with a focus on surgical techniques and drugs, but few that address the importance of rehabilitation in the pre and postoperative period, and the specific benefits for each surgical procedure or type of neoplasm, aiming to minimize respiratory complications in the postoperative period.

AIM

To compare the strength of the respiratory muscles in the pre and postoperative periods of hepatectomy by laparotomy and to verify the incidence of postoperative pulmonary complications among the groups studied.

METHODS

Prospective, randomized, clinical trial study that compared the inspiratory muscle training group (GTMI) with the control group (CG). After the collection of sociodemographic and clinical data, in both groups, preoperatively, on the first and fifth postoperative days, vital signs and pulmonary mechanics were evaluated and recorded. Albumin and bilirubin values were recorded for the albumin-bilirubin (ALBI) score. After randomization and allocation of participants, those in the CG underwent conventional physical therapy and those in the GTMI underwent conventional physical therapy plus inspiratory muscle, in both groups for five postoperative days.

RESULTS

Of 76 subjects met eligibility criteria. The collection of 41 participants was completed: 20 in the CG and 21 in the GTMI. The most frequent diagnosis was 41.5% with liver metastasis, followed by 26.8% with hepatocellular carcinoma. As for respiratory complications in the GTMI, there was no incidence. In the CG, there were three respiratory complications. Patients in the CG classified as ALBI score 3 presented, statistically, a higher energy value compared to patients classified as ALBI score 1 and 2 (P = 0.0187). Respiratory variables, measured preoperatively and on the first postoperative day, had a significant drop in both groups from the preoperative to the first postoperative day ($P \leq 0.0001$). When comparing the preoperative period and the fifth postoperative day between the GTMI and the CG, the maximal inspiratory pressure variable in the GTMI was statistically significant (P = 0.0131).

CONCLUSION

All respiratory measures showed a reduction in the postoperative period. Respiratory muscle training using the Powerbreathe® device increased maximal inspiratory pressure and this may have contributed to a shorter hospital stay and better clinical outcome.

Key Words: Breathing exercises; Physiotherapy; Postoperative care; Hepatectomy; Liver neoplasms

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Core Tip: Randomized study to evaluate the importance of respiratory muscle training in the postoperative period of hapatectomy. All respiratory measures showed a reduction in the postoperative period. Respiratory muscle training using the Powerbreathe® device increased maximal inspiratory pressure and this may have contributed to a shorter hospital stay and better clinical outcome.

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INTRODUCTION

Liver neoplasms can have primary or secondary origins, among them, hepatocellular carcinoma (HCC) is the main and most frequent type of tumor (70% and 85% of cases); cholangiocarcinoma[1] and benign tumors (hemangiomas, adenomas and focal nodular hyperplasia). The most common metastases are secondary liver neoplasms: colorectal, stomach, breast, esophageal, lung, among others[2,3].

To assess the prognosis of these patients, the albumin-bilirubin (ALBI)[4] score is also used, which assesses liver function based on serum albumin and bilirubin values. It is considered an easy score to obtain, through an accessible blood test at patient admission, and has been shown to be useful for the assessment of survival[4,5].

Resection may be required in both benign and malignant liver lesions. Benign lesions are usually asymptomatic and their resection occurs only when they become symptomatic; adenomas, however, are at risk of malignancy and require excision even in the absence of symptoms[6].

Large liver resections should be performed when the lesions are larger, as there is an increase in progression-free survival[6].

With the surgical procedure, there may be postoperative pulmonary complications that are related to preoperative comorbidities (smoking, previous lung or heart disease, malnutrition, obesity) or surgical factors (procedure time, effects of anesthetic and sedative drugs extended)[7].

The reduction in functional residual capacity, forced vital capacity and partial pressure of oxygen in arterial blood[8] are characteristics of upper abdominal surgeries, resulting from inadequate lung insufflation and superficial breathing pattern. Diaphragmatic dysfunction is related to bed restriction and local pain[1,9]. The development of postoperative pulmonary complications is related to increased morbidity, duration of mechanical ventilation, length of hospital stay and, consequently, mortality[8,10].

Physiotherapeutic follow-up in the postoperative period is essential and extremely important[6,10] in order to minimize and/or avoid the incidence of pulmonary complications and consists of performing a kinetic-functional assessment using resources such as ventilometry and manovacuometry. Likewise, intervention, which can be performed with the objective of strengthening the respiratory muscles through electronic devices[10].

The objective of the present study was to evaluate and compare the strength of the respiratory muscles in the pre and postoperative periods of patients undergoing hepatectomy by laparotomy and to verify the incidence of postoperative pulmonary complications among the groups studied.

MATERIALS AND METHODS

The present study was carried out at the Hospital de Clínicas, Faculty of Medical Sciences, State University of Campinas (HC-FCM-UNICAMP), São Paulo, Brazil. Data collection was carried out in the Gastrosurgery Ward and in the Adult Intensive Care Unit for a period of 16 mo, from 2018 to 2019.

This is a prospective, randomized, clinical trial, comparative study between two groups: inspiratory muscle training group (GTMI) and control group (CG).

Approved by the Research Ethics Committee of FCM-UNICAMP, Campinas, SP, having received authorization for data collection with Opinion: 2748781; CAAE: 90806218.7.0000.5404. Randomization was performed after the collection of all data and measured variables and was performed by the researcher through an electronic random draw available on the website https://www.random.org/. It was registered in the Brazilian Registry of Clinical Trials (REBEC), UTN No: U 1111-1236-4194, available at: http://ensaiosclinicos.gov.br/, and the similarity check of the Turnitin system was performed: State University of Campinas, Faculty of Medical Sciences library, October 5, 2020 Opinion No. 223/2020.

Inclusion criteria were patients over 18 years of age, of both genders, who underwent hepatectomy by laparotomy, extubated in the immediate postoperative period and who agreed and signed the Informed Consent Form.

Exclusion criteria were patients undergoing videolaparoscopy, those who did not cooperate with the proposed measures and exercises, patients with hemodynamic instability, and also those who used invasive ventilatory support 24 h after surgery.

The variables collected in the pre, first and fifth postoperative days were: Vital signs, clinical assessment and pulmonary mechanics through respiratory measurements: minute volume (MV in liters/min), tidal volume (CV in mL), Vital Capacity (V_T in liters/min) with the ventilometer device; Energy (joules), inspiratory flow (liters/min), volume (liters), power (watts), and pressure (cmH_2O) with the Powerbreathe® device; Maximum inspiratory pressure (PIM in cmH_2O) and Maximum expiratory pressure (PEM in cmH_2O), with the manovacuometer device.

After the interview, data collection and preoperative respiratory measurements, the sequence of randomization numbers was verified, and their allocation (CG or GTMI) was noted.

In the preoperative period and on the fifth postoperative day in both groups, the value of serum albumin and bilirubin, collected routinely from patients, during hospitalization, was recorded for the ALBI score, classified as Grades 1, 2 and 3 as detailed in Figure 1.

The data obtained during the interview, anamnesis, electronic medical records and respiratory measurements were later entered into an electronic spreadsheet (Excel, Windows, 2013-United States) through double-checking.

For statistical analysis, the computer program used was The SAS System for Windows (Statistical Analysis System), version 9.4. SAS Institute Inc, 2002-2008, Cary, NC, United States, with a significance level adopted for the study of 5% for all tests.

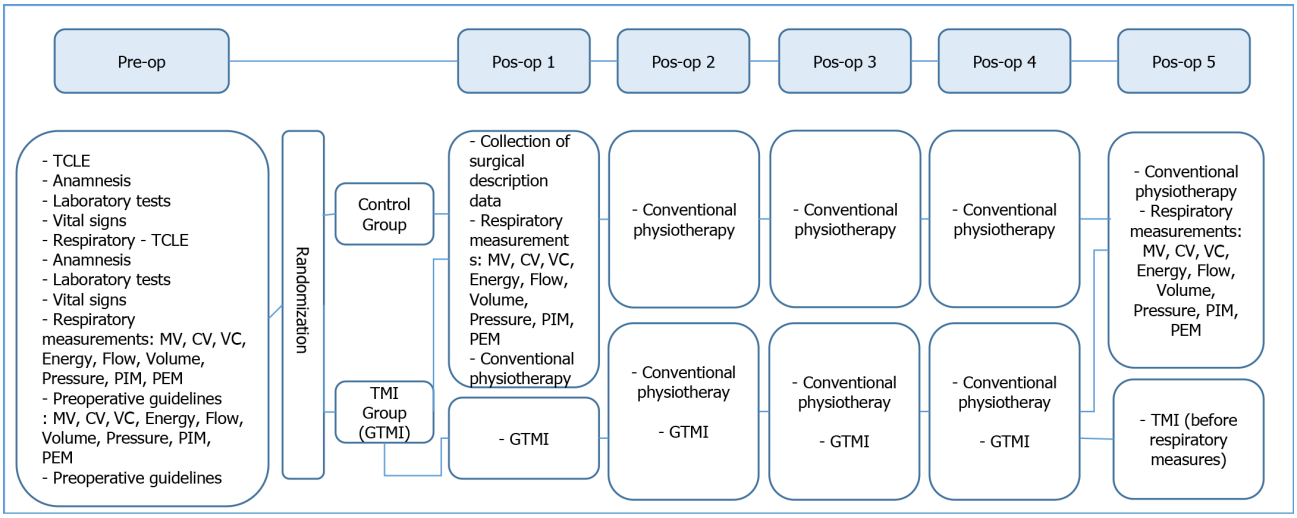
For the comparison between the groups, the chi-square or Fisher's exact tests were used for categorical variables and the Mann-Whitney test for numerical variables. For the comparison between times and groups, analysis of variance for repeated measures was used. Data were transformed into ranks due to the absence of normal distribution.

The relationship between the preoperative variables and the preoperative score, as well as between the variables on the fifth postoperative day and the score on the fifth postoperative day, were verified using Spearman's correlation coefficient; checked in each group. The comparison between preoperative PIM and PEM with ideal PIM and PEM was performed using the Wilcoxon test.

RESULTS

A total of 76 subjects met the eligibility criteria for the study, and 70 participants were randomized and allocated toCCG (n = 36) and GTMI (n = 34). Considering both groups, a total of 20 individuals were excluded from the study (videolaparoscopy, chemoembolization and exploratory laparotomy, or without indication for surgical resection due to tumor extension).

There was loss to follow-up (n = 9) in both groups (six for refusing care - two in each group due to pain complaints in the surgical wound and one in each group due to excessive vomiting, one in each group due to a stay on invasive mechanical ventilation longer than 24 h and one death in the control postoperative period). Of the total sample, 41 individuals were studied, 20 of which were allocated to the CG and 21 to the GTMI.



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Figure 1 Description of data collection. Conventional physiotherapy: Techniques for bronchial hygiene maneuvers, pulmonary re-expansion maneuvers, sitting, standing, and ambulation. CG: Control group; GTMI: Inspiratory muscle training group; TCLE: Free and informed consent form; Pre-op: Pre-operative; Post-op: Post-operative; MV: Minute volume; CV: Tidal volume; VC: Vital capacity; PIM: Maximum inspiratory pressure; PEM: Maximum expiratory pressure; TMI: Inspiratory muscle training.

There was no significant difference between the groups according to gender (P = 0.910), age (P = 0.140) and body mass index (BMI) (P = 0.140) (Table 1).

Table 2 describes the main diagnoses and antecedents presented. There was no statistically significant difference between the groups when intraoperative and postoperative complications were analyzed.

The length of hospital stays and complications in the postoperative period are described in Table 3. Patients in the GTMI had a shorter hospital stay when compared to the CG (5.4 d x 7.1; P = 0.0596) as seen in Table 3.

In the preoperative period, 22 patients (53%) were classified with ALBI 1; 16 (39%) patients with ALBI 2; and 3 (7%) patients with ALBI 3. On the fifth postoperative day, there was a worsening in the ALBI score classification, with 1 (2%) patient classified as ALBI 1; 24 (58%) patients classified as ALBI 2; and 16 (39%) patients classified as ALBI 3.

Patients with an ALBI score of 2 or 3, when compared with respiratory measurements, had higher V_T values (P = 0.0207) and higher MV (P = 0.0310), but with no difference in the "energy" variable between the groups.

On the fifth postoperative day, patients in the CG with ALBI score grade 3 had a higher value in the variable "energy" compared to patients classified as ALBI score grade 1 and 2 (P = 0.0187).

In the CG, when comparing the preoperative pressure variable with the first postoperative day, it was observed that patients who had undergone chemotherapy (n = 7) had a lower value (1.7 ± 4.6) in relation to patients who did not undergo chemotherapy (n = 13; 6.5 ± 4.4) P = 0.0394. The other measures did not show a statistically significant difference.

The respiratory variables (MV, V_T, PIM, PEM, CV, pressure, energy, inspiratory flow and power) had a statistically significant decrease when comparing the preoperative period with the first postoperative day ($P \leq 0, 0001$), as described in Table 4.

The GTMI patients showed a tendency (P = 0.0583) to have a higher volume (liters/min) and a higher PIM value (P = 0.0131) on the fifth postoperative day (Table 4). The other variables evaluated showed no difference in statistics when groups were compared.

DISCUSSION

The present study analyzed two forms of physical therapy intervention: conventional physical therapy and the association with the Powerbreathe® device for training the inspiratory muscles.

From the point of view of sample characterization, the variables were homogeneous, with a predominance of males in both groups and a mean age of 53 years, which corroborates the literature, since liver cancer is more prevalent in men[11]. This fact may be related to the higher consumption of alcohol, and consequently, the presence of cirrhosis, and to hepatitis C virus, as men have lifestyles that allow greater exposure to the virus, such as drug use and sexual intercourse without condoms[12].

When the BMI variable is evaluated, the study indicates an overweight population, with a mean value of 27.3 kg/m^2. Studies show an association of obese non-cirrhotic patients with liver cancer. 17% were recorded for overweight individuals and 89% for obese individuals with a BMI > 30 kg/m^2, with

Table 1 Demographic and clinical data of the studied groups, *n* (%)

Variables	Group			
	GTMI (*n* = 21)	CG (*n* = 20)	Total/average (*n* = 41)	*P* value
Feminine gender	7 (33.3)	7 (35.0)	14 (34.1)	0.9104[2]
Male gender	14 (66.7)	13 (65.0)	27 (65.9)	
Mean age (yr) (SD)	56.7 ± 13.1	49.3 ± 16.1	53.1 ± 14.9	0.1401[1]
Mean BMI (SD)	28.3 ± 4.0	26.2 ± 6.2	27.3 ± 5.2	0.1404[1]

[1]Based on the Mann-Whitney test.
[2]Based on the χ^2 test.
GTMI: Inspiratory muscle training group; CG: Control group; BMI: Body mass index; SD: Standard deviation.

Table 2 Diagnosis and background of the studied groups, *n* (%)

Variables	Group			
	GTMI (*n* = 21)	CG (*n* = 20)	Total (*n* = 41)	*P* value
Diagnostics				
Liver metastasis	9 (42.9)	8 (40.0)	17 (41.5)	
Hepatocellular carcinoma	8 (38.1)	3 (15.0)	11 (26.8)	
Hepatic nodule	3 (14.3)	4 (20.0)	7 (17.1)	
Cholangiocarcinoma	1 (4.8)	4 (20.0)	5 (12.2)	
Hemangioma	0 (0.0)	1 (5.0)	1 (2.4)	-
Background				
Hepatic cirrhosis				
No	15 (71.4)	18 (90.0)	33 (80.5)	
Yes	6 (28.6)	2 (10.0)	8 (19.5)	0.2379[2]
Hepatitis C				
No	17 (81.0)	18 (90.0)	35 (85.4)	
Yes	4 (19.0)	2 (10.0)	6 (14.6)	0.6628[2]
Previous neoplasm				
No	10 (47.6)	8 (40.0)	18 (43.9)	
Yes	11 (52.4)	12 (60.0)	23 (56.1)	0.6232[1]
Chemotherapy				
No	12 (57.1)	13 (65.0)	25 (61.0)	
Yes	9 (42.9)	7 (35.0)	16 (39.0)	0.6062[1]
Smoker/former smoker				
No	15 (71.4)	18 (90.0)	33 (80.5)	
Yes	6 (28.6)	2 (10.0)	8 (19.5)	0.2379[2]

[1]Based on the chi-square test.
[2]Based on Fisher's exact test.
GTMI: Inspiratory muscle training group; CG: Control group.

an average 24% increase in risk for each 5 kg/m^2 increase in BMI. It has also been reported that weight gain in adulthood increases the risk of cancer by up to 2.5 times. Carcinogenesis in this population needs to be further studied, since in the present study the population was also overweight[13].

Table 3 Complications and length of hospital stay in the postoperative period, *n* (%)

Variables	Group			
	GTMI (*n* = 21)	CG (*n* = 20)	Total (*n* = 41)	*P* value
Postoperative complications				
No	15 (71.4)	13 (65.0)	28 (68.3)	
Yes	6 (28.6)	7 (35.0)	13 (31.7)	0.6584[2]
Intraoperative bleeding (*n*)	2 (9.5)	0 (0.0)	2 (4.9)	
TEP	0 (0.0)	1 (5.0)	1 (2.4)	
Paralytic ileus	2 (9.5)	1 (5.0)	3 (7.3)	
Anemia	1 (4.8)	1 (5.0)	2 (4.9)	
PNM	0 (0.0)	1 (5.0)	1 (2.4)	
$SPO_{2\ drop} < 92$	0 (0.0)	2 (10.0)	2 (4.9)	
ARI	1 (4.8)	0 (0.0)	1 (2.4)	
Biliary fistula	0 (0.0)	1 (5.0)	1 (2.4)	-
Postoperative hospital stay (in days)				
Mean (SD)	5.4 ± 2.0	7.1 ± 3.9	6.2 ± 3.2	0.0596[1]

[1]Based on the Mann-Whitney test.
[2]Based on the Chi-square test.
GTMI: Inspiratory muscle training group; CG: Control group; TEP: Pulmonary thromboembolism; PNM: Pneumonia; SpO_2: Peripheral oxygen saturation; ARI: Acute renal failure; SD: Standard deviation.

Table 4 Comparison of respiratory variables measured with the manovacuometer, ventilometer and with the *Powerbreathe*® between the preoperative, the first and fifth postoperative days of the inspiratory muscle training group and control group

Variables	GTMI (*n* = 21)			CG (*n* = 20)			*P* value[2]
	Pre-op	PO first day[1]	PO fifth day	Pre-op	PO first day	PO fifth day	
Manovacuometer							
PIM (cmH_2O)	110.9 ± 41	71.6 ± 42	115.6 ± 51	115.4 ± 73	77.9 ± 56	99.6 ± 68	0.0131
PEM (cmH_2O)	101.0 ± 28	58.1 ± 26	83.1 ± 30	103.5 ± 43	63.0 ± 37	84.8 ± 42	0.6186
Ventilometer							
CV (liters/min)	2789 ± 938	1423 ± 589	1937 ± 585	2705 ± 836	1356 ± 392	1737 ± 541	0.6014
V_T (mL)	483.2 ± 273	425.2 ± 183	543.7 ± 221	473.1 ± 213	354.9 ± 161	474.2 ± 162	0.2866
VM (liters/min)	6905 ± 3449	7060 ± 2753	11012 ± 5381	7732 ± 3849	6573 ± 2746	9117 ± 3325	0.2091
Powerbreathe®							
Energy (joules)	1.24 ± 1.4	0.61 ± 1.2	0.57 ± 0.3	0.87 ± 0.2	0.30 ± 0.1	0.52 ± 0.2	0.3098
Flow (liters/min)	1.78 ± 1.0	0.86 ± 0.8	1.21 ± 0.5	1.52 ± 0.7	0.64 ± 0.3	0.93 ± 0.4	0.4224
Power (watts)	0.99 ± 1.2	0.53 ± 1.0	0.53 ± 0.2	0.67 ± 0.3	0.32 ± 0.2	0.42 ± 0.2	0.3533
Pressure (cmH_2O)	4.86 ± 2.0	4.53 ± 1.7	4.31 ± 0.2	4.32 ± 0.2	4.11 ± 0.1	4.31 ± 0.5	0.1405
Volume (liters)	1.82 ± 0.8	0.84 ± 0.5	1.15 ± 0.4	1.75 ± 0.5	0.61 ± 0.2	0.85 ± 0.2	0.0583

[1]In Po1 all respiratory measurements were statistically lower ($P \leq 0.0001$).
[2]The statistical test used was ANOVA for repeated measures with transformation by rank of the effect of the interaction between groups and times.
GTMI: Inspiratory muscle training group; PO: Post-operative; CG: Control group; Pre-op: Pre-operative; PIM: Maximum inspiratory pressure; PEM: Maximum inspiratory pressure; VC: Vital capacity; V_T: Tidal volume; VM: Minute volume.

The most frequently found diagnosis was liver metastasis (41.5%), followed by HCC (26.8%), liver nodule (17.1%), cholangiocarcinoma (12.2%) and hemangioma (2.4%). As for the antecedents, 56.1% had previous neoplasia, 39% had already undergone chemotherapy at some point, 19.5% had liver cirrhosis, 19.5% were smokers and 14.6% had hepatitis C. Metastases are the most frequent tumors, as presented in the study, followed by HCC as the main cause of primary liver cancer[2,13].

The mortality rate was 2.4%, a percentage considerably lower than that found in the literature of 6.0% and 7.7%. The low mortality in the present study may be related to the performance of the procedure by an experienced and trained multidisciplinary team[14] and the population studied was not composed of elderly people[14].

The presence of intraoperative and postoperative complications in both groups was 31.7%. Previous studies showed a higher percentage of complications (37.5%)[15], being a high-risk surgical procedure and its prognosis may be related to the type of primary tumor. Even though the surgical procedure is performed by highly qualified surgeons, the literature describes high rates of morbidity and mortality [15,16].

A considerable worsening of the parameters studied was observed after the surgical procedure, requiring measures to minimize the deleterious effects of the postoperative period, such as physical therapy, breathing techniques and inspiratory muscle training (TMI).

When the respiratory complications were evaluated, it was observed that the GTMI did not present any incidence, even with smokers and ex-smokers, which may be related to the better performance of the respiratory muscles after inspiratory muscle training. In the CG, however, it can be observed that three patients developed postoperative pulmonary complications, one patient developed pneumonia, one patient had a drop in SpO_2 for more than one day, and one patient developed pulmonary thromboembolism.

It was observed that, even with the performance of conventional physical therapy in both groups, the CG presented postoperative pulmonary complications, and the GTMI did not, which favors the indication of TMI in the postoperative period despite not showing statistical significance in complications.

Several studies describe that the inspiratory muscle training exercise minimizes the chances of complications in the postoperative period, including pleural effusion, atelectasis and pneumonia, as well as a shorter hospital stay[17,18], corroborating the findings of the present study, which demonstrated a shorter hospitalization in patients who underwent training of the inspiratory muscles. There was a statistical trend with a mean of 5.4 d (P = 0.0596) compared to 7.1 d in the CG, since the only differentiated intervention between the groups was TMI, and the CG had respiratory complications while the GTMI, did not[18].

The ALBI score was developed to be a prognostic predictor for HCC. Currently, its application has been studied in non-HCC patients in the postoperative period, including those with gastric cancer, and in patients with acute and chronic liver failure. This score seems to be superior to other scores such as Child-Pugh and model for end-stage liver disease (MELD) score to predict the occurrence of hepatic events[4,5].The score was applied to all study participants, being evaluated before and on the fifth postoperative day, demonstrating a worsening of the score in the postoperative period. Studies in hepatectomy by laparotomy describe a worsening in liver function after resection when compared to preoperatively[19].

A limitation of the present study was that it did not compare the MELD and Child-Pugh scores with the ALBI score, although these classifications are for cirrhotic patients and we had a good number of non-cirrhotic patients.

In the preoperative period, patients with a worse prognosis with an ALBI grade 2 and 3 had statistically higher volume (liters/min) and higher minute volume (liters/min).

Postoperatively, only patients in the CG with ALBI score 3 had a statistically higher energy value (joules) when compared with patients with ALBI score 1 and 2 (P = 0.0187).

The volume (liters/min), minute volume (liters/min) and energy (joules) were significantly higher in the ALBI score 3. Albumin may be associated with malnutrition, inflammation and, consequently, sarcopenia, and with a worse prognosis[20,21]. Sarcopenia is the deterioration of muscle mass, muscle strength and subsequent physical performance[20,22]. In liver disease, patients have fatigue, lower aerobic capacity, and ventilatory restriction, which may be correlated with a higher ventilatory demand described in this study[20,23].

Considering the current literature, we identified that this is the first study that compared the ALBI score before and after the operation, associating them with training of the inspiratory muscles.

It was observed that, in the GTMI, although six patients were classified with ALBI score 3, a level that corresponds to a 75% increase in mortality and postoperative complications[4,5], the same patients showed no statistical difference in any of the variables when comparing loss of strength of the inspiratory muscles and pulmonary mechanics and did not present respiratory complications.

In patients who underwent chemotherapy, there was a statistical difference: in the CG, the variable pressure (cmH_2O) (P = 0.0394); in GTMI, the variable energy (joules). There was a statistical trend (P = 0.0549) when comparing the pre and the first postoperative day in the intergroups.

Some studies describe that fatigue in cancer patients who underwent chemotherapy is associated with possible physiological and metabolic adaptations such as deconditioning due to the continuous

loss of muscle mass, including the diaphragm muscle. It would also be associated with cardiotoxicity and neurotoxicity[24,25], which may explain this result.

The ideal PIM variable is a predictive value of maximal inspiratory pressure, obtained through a formula that uses age and sex to obtain the result, which can be compared with the actual measurement of each individual. The calculation performed showed an average of 103 cmH_2O and, after statistical evaluation, there was no difference between the groups for the preoperative average. The PIM variable measured in all participants in the preoperative period, with the manovacuometer device, obtained an average value of 113 cmH_2O, suggesting that the study participants did not present preoperative weakness of the inspiratory muscles.

With the decrease in respiratory measures in the postoperative period, a worsening of the clinical outcome can be expected and the TMI can result in an increase in PIM. This may reflect on the strength of the inspiratory muscles and may reduce the chance of complications and mortality. In this study, it was observed that there was a significant increase in PIM (P = 0.0131) after inspiratory muscle training, when comparing the CG with the GTMI. Although there is no specific study of TMI in hepatectomy, this result is in line with some published studies, in which it is argued that TMI, or the association with other exercises, can increase PIM. This is in addition to reducing respiratory complications and improving postoperative pulmonary performance[26-28].

A recent study described that this value of PIM, in isolation, proves to be little beneficial. PIM values associated with other variables, such as outcome, mortality, and fewer postoperative complications may show clinical benefits[27].

Some studies describe that the increase in PIM is directly related to the strength of the inspiratory muscles and lower respiratory complications in the postoperative period[27,28].

The Powerbreathe® device, through a linear load and pressure during inspiration, recruits muscle fibers, and in addition to being a light, easy-to-handle equipment, features software, which provides visual feedback of the amount of inspired air volume, speed and strength, which the patient is doing with each breath. This makes it possible to monitor the patient regarding his progress in training, in addition to storing the data and issuing a report on respiratory measurements and how the training was [27,29].

The success of TMI depends on the engagement of the physical therapy team, with scientific data that bring benefits to patients[29].

Despite evidence that TMI increases inspiratory muscle strength and improves patient outcome, it is still not a standardized practice in most hospitals worldwide[29].

There are few studies in the literature on TMI with the Powerbreathe® device in the postoperative period of specific surgeries. Currently, there are studies in athletes[26] and some studies in patients with chronic obstructive pulmonary disease, congestive heart failure[28], cardiac surgery, ventilator weaning [29,30] and esophagectomy[27].

It is noteworthy that, in this study, in the GTMI, there were no respiratory complications.

CONCLUSION

All respiratory measures showed a reduction in the postoperative period. The number of postoperative pulmonary complications was low and there was no difference between the studied groups. In patients who underwent chemotherapy, changes in some respiratory parameters may be associated with the toxic effects of therapy, such as sarcopenia. Respiratory muscle training using the Powerbreathe® device increased maximal inspiratory pressure and this may have contributed to a shorter hospital stay and better clinical outcome.

ARTICLE HIGHLIGHTS

Research background

The study was developed in view of the growing number of surgical procedures and the need for scientific evidence that demonstrates the need for specialized physiotherapeutic evaluation to prevent and/or minimize postoperative complications. Inspiratory muscle training with an electronic device has been shown to be efficient in several pathologies, but limited in surgical patients with an indication to start in the preoperative period in order to assess and recognize respiratory mechanics, aiming to minimize and treat complications in the postoperative period.

Research motivation

Patients who undergo hepatectomy by laparotomy evolve with limited ventilatory mechanics. Respiratory restriction by the surgical incision, postoperative pain, diaphragmatic injury in the surgical procedure, subsequent weakness of the respiratory muscles, can lead to complications in the postoperative period. Inspiratory muscle training may be able to reduce the risk of pulmonary complic-

ations by improving the strength, resistance of respiratory muscles and lung function.

Research objectives

The objective of the present study was to evaluate and compare the strength of the respiratory muscles in the pre and postoperative periods of patients undergoing hepatectomy by laparotomy and to verify the incidence of postoperative pulmonary complications among the groups studied.

Research methods

A prospective, randomized, clinical trial study that compared the inspiratory muscle training group with the control group. Data were collected in both groups, preoperatively, on the first and fifth postoperative days, vital signs and lung mechanics were evaluated and recorded. The value of albumin and bilirubin was noted for the albumin-bilirubin (ALBI) score. After randomization and allocation of participants, one group performed conventional physical therapy and the other group performed conventional physical therapy plus inspiratory muscle training, in both groups for five postoperative days.

Research results

Of the 41 participants included, the most frequent diagnosis was 41.5% with liver metastasis, followed by 26.8% with hepatocellular carcinoma. As for respiratory complications in inspiratory muscle training group (GTMI), there was no incidence. In the control group (CG), there were three respiratory complications. Patients in the CG classified with ALBI score 3 had, statistically, a higher energy value compared to patients classified with ALBI scores 1 and 2 ($P = 0.0187$). The respiratory variables, measured preoperatively and on the first postoperative day, had a significant drop in both groups from the preoperative period to the first postoperative day ($P \leq 0.0001$). When comparing the preoperative period and the fifth postoperative day between the GTMI and the CG, the inspiratory muscle training variable in the GTMI was statistically significant ($P = 0.0131$).

Research conclusions

All respiratory measures showed a reduction in the postoperative period. Respiratory muscle training using the Powerbreathe® device increased maximal inspiratory pressure and this may have contributed to a shorter hospital stay and better clinical outcome.

Research perspectives

Through specific knowledge of the changes presented in the postoperative period, develop individualized protocols for inspiratory muscle training to minimize and avoid possible complications, improve the quality of care and reduce the length of stay of patients undergoing hepatectomy.

FOOTNOTES

Author contributions: Pereira MG designed the research study, participated in data collection, analyzed the data, wrote the manuscript and revised the manuscript; Silva AMO wrote the manuscript and revised the manuscript; Galhardo FDM wrote the manuscript and revised the manuscript; Almeida BDM participated in data collection; Lopes RL participated in data collection; Boin IFSF designed the research study, analyzed the data, revised the manuscript; all authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Liver steatosis in patients with rheumatoid arthritis treated with methotrexate is associated with body mass index

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Abstract

BACKGROUND

Methotrexate (MTX) is the usual first-line treatment for rheumatoid arthritis (RA). Long-term use of MTX has been associated with liver steatosis (LS) and liver fibrosis (LF).

AIM

To determine if LS in patients treated with MTX for RA is associated with MTX cumulative dose (MTX-CD), metabolic syndrome (MtS), body mass index (BMI), the male sex, or LF.

METHODS

A single-center, prospective study of patients receiving MTX for RA was performed from February 2019 to February 2020. The inclusion criteria were patients aged 18 years or older diagnosed with RA by a rheumatologist and being treated with MTX (without limitation on the duration of treatment). The exclusion criteria were previous diagnosis of liver disease (hepatitis B or C virus infection, known nonalcoholic fatty liver disease), alcohol consumption greater than 60 g/d in males or 40 g/d in females, human immunodeficiency virus infection on antiretroviral therapy, diabetes mellitus, chronic renal failure, congestive heart failure, or BMI greater than 30 kg/m^2. Patients receiving leflunomide in the 3 years prior to

the study were also excluded. Transient elastography (FibroScan, Echosens®, Paris, France) was used for fibrosis determination (LF > 7 KpA) and computer attenuation parameter (CAP) for LS (CAP > 248 dB/m). Demographic variables, laboratory data, MTX-CD (> 4000 mg), MtS criteria, BMI (> 25), transient elastography, and CAP scores were collected from all patients.

RESULTS

Fifty-nine patients were included. Forty-three were female (72.88%), and the mean age was 61.52 years (standard deviation: 11.73). When we compared MTX-CD ≤ 4000 mg (26 patients; 14 with LS and 12 without) with > 4000 mg (33 patients; 12 with LS and 21 without), no statistical differences were found (P = 0.179). We compared CAP scores stratified by MtS, BMI, sex, and LF. There were no significant differences in CAP scores based on the presence of MtS [CAP/MtS: 50 no MtS (84.75%); 9 MtS (15.25%); P = 0.138], the male sex (CAP/sex: 8 male/18 female LS; 8 male/25 female no LS; P = 0.576), or LF [CAP/fibrosis: 53 no LF (89.83%); 6 LF (10.17%); P = 0.239]. LS determined by CAP was significantly associated with BMI > 25 (CAP/BMI: 22 BMI ≤ 25 (37.29%); 37 BMI > 25 (62.71%); P = 0.002].

CONCLUSION

LS in patients with RA treated with MTX was not associated with MTX-CD, LF, the male sex, or MtS. However, BMI was significantly related to LS in these patients.

Key Words: Methotrexate; Rheumatoid arthritis; Liver steatosis; Liver fibrosis; Transient elastography; Computed attenuation parameter

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Core Tip: Methotrexate (MTX) is the cornerstone of treatment for rheumatoid arthritis and has been associated with the development of liver fibrosis (LF) and liver steatosis (LS). The objective of this work was to study if LS in patients with rheumatoid arthritis treated with MTX and determine the association with body mass index, MTX cumulative dose, sex, LF, and metabolic syndrome. We concluded that LS in patients with rheumatoid arthritis on MTX treatment was not related to MTX-cumulative dose, LF, the male sex, or metabolic syndrome. In our study, body mass index was significantly associated with LS in these patients.

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INTRODUCTION

Methotrexate (MTX) has been used in the treatment of oncological and chronic inflammatory diseases. It is also the cornerstone of treatment for rheumatoid arthritis (RA). The most concerning long-term adverse effect of this treatment is the development of liver fibrosis (LF)[1-5]. Liver steatosis (LS) has been associated with RA and with MTX treatment[6]. Liver biopsy has been the gold standard for the study of LF and LS, but it has several limitations[3]. There is a disparity of fibrosis values between biopsy samples, and it is an invasive technique accompanied by risks[3].

Recent studies have been carried out on non-invasive measurements of LF. Transient elastography (TE) is a non-invasive method without side effects that also allows the sequential determination of liver fibrosis measurements over time, which makes it of great interest for the follow-up of these patients[2-4].

MTX, as a risk factor for secondary LS, has been studied recently. RA has been associated with moderate to severe LS; predisposing factors such as higher body mass index (BMI), the male sex, and MTX cumulative dose (MTX-CD) have been published[7]. However, there have been conflicting results, and the impact of MTX on nonalcoholic fatty liver disease (NAFLD) is still unclear[6-11].

The computer attenuation parameter (CAP) measures carried out at the time of TE correlates with the histological LS[12]. The CAP algorithm calculates the ultrasound signal attenuation[12]. LS has been evaluated recently using CAP in chronic MTX users and was common with moderate and severe LS predicting moderate to severe LF[13].

The objective of our work was to determine if LS in patients with RA treated with MTX is associated with BMI, MTX-CD, sex, LF, and metabolic syndrome (MtS).

MATERIALS AND METHODS

We performed a single-center, prospective study of patients receiving MTX for RA. The principle objective of this work was to study the presence of LF by TE and aspartate aminotransferase to platelet ratio index (APRI)[14] as well as the detection of LS by ultrasonography and CAP. Fibroscan® (FS) (Fibroscan®402, Echosens, France, www.echosens.com) was used for fibrosis determination (LF > 7 KpA). CAP was used for LS (CAP > 248 dB/m)[11]. Demographic variables, laboratory data, MTX-CD (> 4000 mg), MtS criteria, BMI (> 25), TE, and CAP scores were collected from all patients.

Patients were recruited between February 1, 2019 and January 31, 2020 from the Gastroenterology-Rheumatology clinics of our hospital. The inclusion criteria were patients aged 18 years or older diagnosed with RA by a rheumatologist, and being treatment with MTX (without limitation on the duration of treatment). The exclusion criteria were previous diagnosis of liver disease (hepatitis B or C virus infection, known NAFLD), alcohol consumption greater than 60 g/d for males or 40 g/d for females, HIV infection on antiretroviral therapy, diabetes mellitus, chronic renal failure, congestive heart failure, or BMI greater than 30 kg/m^2. Patients receiving leflunomide in the 3 years prior to the study were also excluded.

Demographic data analysis, treatment history, and MTX-CD were collected through computerized medical records. LF was defined by FS (measurement greater than 7 Kpa) and by APRI score (result greater than 0.7). The FS assessment was performed by a trained nurse. At the time of inclusion in the study, a blood test was performed to calculate the APRI score [aspartate aminotransferase level (upper limit of normal)/platelet level × 100]. High transaminase levels were defined as results above 33 U/L. Finally, disease activity was defined by a rheumatologist using the Disease Activity Score in 28 joints-c-reactive protein score. Data were collected by means of a questionnaire, a review of the computerized clinical history, and a visit to the gastroenterology clinic.

Statistical analysis

Initially, a descriptive analysis was performed by calculating the mean and standard deviation (SD) (or median and interquartile range) for quantitative variables. For qualitative variables, absolute and relative frequencies were calculated as percentages. To compare the distribution of qualitative variables, the χ^2 test or Fisher's exact test was used. Similarly, the Student's *t*-test or the Mann-Whitney *U* test was used to compare quantitative variables. STATA 16.1 software was used for all the analyses. Statistical review of the study was performed by a biomedical statistician (IU).

Ethics

The clinical research ethics committee of the Gipuzkoa health area (Código de Protocolo: ACLFSC-2018-01; Acta 01/2019) approved this study, and participants signed an informed consent form prior to inclusion.

RESULTS

We included 59 patients in the study. There were 43 females (72.88%), and 61.52 years (SD: 11.73) was the mean age. Clinical characteristics are presented in Table 1 and laboratory data in Table 2 (Supplementary materials). The mean duration of the MTX treatment was 82.4 mo (SD: 65.1). The mean MTX-CD of the patients was 5214.5 mg (SD: 4031.9). Twenty-six patients presented an MTX-CD ≤ to 4000 mg. Thirty-three had an MTX-CD > than 4000 mg.

Treatment duration and times of disease progression were longer in the MTX-CD > 4000 mg group. MTX monotherapy was used in 46 patients (77.90%). Only 7 patients (11.80%) were on nonsteroidal anti-inflammatory drug therapy in association with MTX.

Ultrasonography was performed in 56 patients, of whom 39 presented no LS (69.64%), and 17 (30.36%) had LS. CAP was determined in all 59 patients, categorizing 33 patients without LS and 26 patients with LS.

We then compared both methods (56 patients in total). Ultrasonography presented a positive predictive value of 88.2% [95% confidence interval (CI): 63.6%-98.5%] and a negative predictive value of 76.9% (95%CI: 60.7%-88.9%), with a sensitivity of 62.5% (95%CI: 40.6%-81.2%) and a specificity of 93.8% (95%CI: 79.2%-99.2%) compared to CAP. When comparing MTX-CD ≤ 4000 mg (26 patients, 14 with LS and 12 without) with > 4000 mg (33 patients; 12 with LS and 21 without), we found no statistical differences in LS between low and high MTX-CD (P = 0.179) (Figure 1A). CAP scores were compared stratified by BMI, sex, LF, or MtS. No significant differences were observed based on the the male sex (CAP/sex: 8 males/18 females LS; 8 males/25 females no LS; P = 0.576), LF [CAP/Fibrosis: 53 no LF

Table 1 Clinical characteristics

Clinical characteristics	Value
Female/male	43 (73%); 16 (27%)
Age in yr	61.52 (11.73)
Height in cm	162.02 (7.66)
Weight in kg	67.33 (10.52)
Waist circumference in cm	88.81 (10.92)
BMI in kg/m²	25.55 (3.05)
BMI < 25 score	22 (37.29%)
BMI > 25 score	37 (62.71%)
Metabolic syndrome	9 (15.25%)
Type 2 diabetes	2 (3.57%)
DAS28 score	2.36 (1.14)
Treatment duration MTX in mo	82.43 (65.08)
MTX-CD in mg	5214.5 (4031.9)
FibroScan in kPa	5.02 (2.24)
APRI in score	0.32 (0.15)
CAP in dB/m	251.33 (51.13)

Data are *n* (%) or mean ± SD. APRI: Aspartate aminotransferase to platelet ratio index; BMI: Body mass index; CAP: Computed attenuation parameter; DAS28: Disease Activity Score in 28 joints; MTX-CD: Methotrexate cumulative dose.

Table 2 Laboratory data

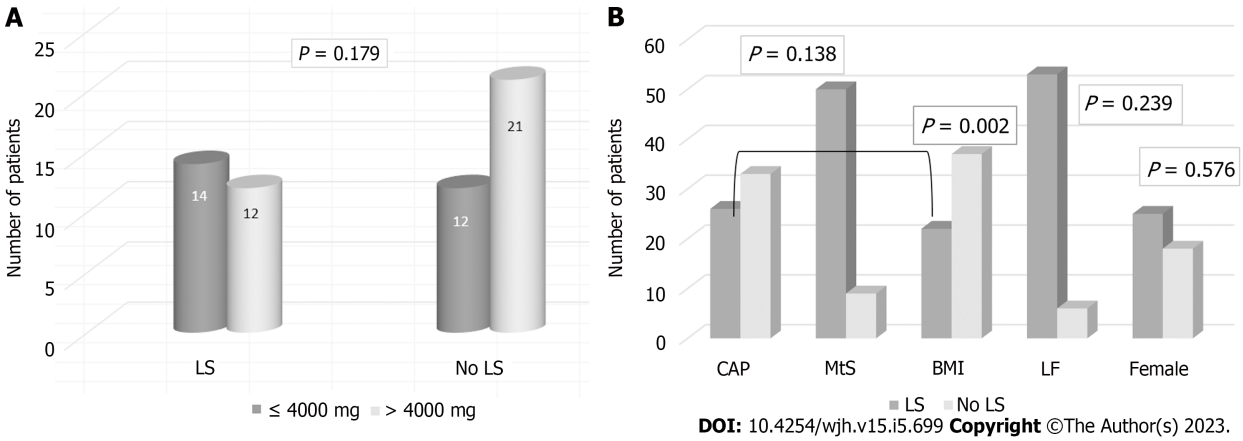
Classification	Value
AST in U/L	24.52 (12.56)
ALT in U/L	22.23 (12.15)
GGTP in U/L	23.42 (13.43)
AP in U/L	76.05 (23.72)
Bilirubin in mg/dL	0.50 (0.25)
Albumin in g/dL	4.39 (0.29)
Glucose in mg/dL	101.50 (16.08)
Triglycerides in mg/dL	99.43 (45.87)
Cholesterol in mg/dL	205.42 (43.57)
HDL-cholesterol in mg/dL	62.85 (14.74)

Data are mean ± SD. ALT: Alanine aminotransferase; AP: Alkaline phosphatase; AST: Aspartate aminotransferase; GGTP: Gamma glutamyl transpeptidase; HDL: High-density lipoprotein.

(89.83%); 6 LF (10.17%); $P = 0.239$], or MtS [CAP/MtS: 50 no MtS (84.75%); 9 MtS (15.25%); $P = 0.138$]. Nonetheless, LS measured by CAP was significantly related with BMI > 25 [CAP/BMI: 22 BMI ≤ 25 (37.29%); 37 BMI > 25 (62.71%); $P = 0.002$] (Figure 1B).

DISCUSSION

MTX is the gold standard of RA treatment, both in monotherapy and associated with biological therapies[15]. LF has been associated with chronic MTX use in this disease. There is increasing evidence



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Figure 1 Liver steatosis. A: Liver steatosis by methotrexate cumulative dose; B: Liver steatosis by computer attenuation parameter compared with metabolic syndrome, body mass index, liver fibrosis, and sex. CAP: Computer attenuation parameter; MtS: Metabolic syndrome; BMI: Body mass index; LF: Liver fibrosis; LS: Liver steatosis.

that LF is broadly affected by other factors: Alcohol, other associated drugs, and MtS are directly related with the development of LF[16-19].

Drugs can affect LS development. The possible effect of MTX in the presence of LS in patients with RA is currently being studied. According to laboratory research, folate deficiency produced by chronic MTX treatment could promote liver fat accumulation[20], but folic acid supplementation has been recommended and is currently being used in treatment regimens. Studies have shown conflicting results, and the impact of MTX on LS is still unclear[6].

Choi *et al*[6] investigated whether MTX-CD in 368 RA patients led to LS determined by ultrasound, but they did not detect a significant association between LS development and MTX administration, suggesting that to adjust for individualized risk factors for NAFLD may be more efficient than MTX discontinuation in LS detection/management. Hypertriglyceridemia and higher BMI were associated with an increased risk of LS.

Erre *et al*[7] recently studied the independent association of LS and RA. In 223 patients with RA, they found that RA is independently associated with LS (moderate to severe), scored by ultrasound, and male sex, higher BMI, and MTX-CD are independent risk factors for the development of LS[7].

Mori *et al*[8] studied the association between NAFLD and liver injury during MTX treatment in 846 patients with RA. They did not observe a significant impact of MTX dose and duration on histological severity. On the other hand, Sakthiswary *et al*[9] concluded, in a retrospective study, that the MTX-CD was the only independent predictor of MTX-associated LS with transaminitis in a cohort of 978 patients with RA.

Recently, detection of LS by CAP in chronic MTX users was published for the first time. Tomaszewski *et al*[13] studied 172 patients on MTX (45 with RA). Diabetes mellitus, hypertension, and BMI ≥ 30 were predictors of LS. LS determined by CAP was frequent. Moderate and severe LS in this study predicted moderate to severe fibrosis of the liver.

Our prospective study was designed to determine in patients with RA treated with MTX if LS, as measured by CAP, was associated with BMI, sex, LF, or MTX-CD. When we compared MTX-CD ≤ 4000 mg with > 4000 mg, no statistical differences were found. There were no significant differences between the presence and absence of MtS, the male sex, or LF, but LS determined by CAP was significatively associated with BMI > 25 ($P = 0.002$).

Our study had limitations. The sample size was relatively small, and we included all the patients with RA on MTX treatment, without a treatment duration limitation. More females than males were included in this study, and given the limited sample size, it is difficult to conclude that there is no relationship between sex and LS. The strengths of the study were that it was a prospective study and that LS was determined as measured by the CAP.

CONCLUSION

We concluded that in our series of patients treated with MTX for RA, LS is not associated with MTX-CD, LF, the male sex, or MtS. In our study, BMI is significantly associated with LS. It seems that other factors, apart from MTX-CD or treatment duration, are more important for the development of LS in these patients.

ARTICLE HIGHLIGHTS

Research background

Methotrexate (MTX) remains the cornerstone of treatment for rheumatoid arthritis (RA), both in monotherapy and in association with other treatments. The most concerning adverse effect of this treatment, in the long term, is liver fibrosis (LF). Liver steatosis (LS) has been associated with RA and with MTX.

Research motivation

MTX, as a risk factor for secondary LS, has been studied recently. RA has been independently associated with moderate to severe LS. Sex, higher body mass index (BMI), and MTX cumulative dose (MTX-CD) are predisposing factors. However, the studies have shown conflicting results, and the impact of MTX on LS is still unclear.

Research objectives

The objective of our work was to study if LS in RA patients treated with MTX was related to BMI, MTX-CD, metabolic syndrome (MtS), sex, or LF.

Research methods

We performed a prospective study of RA patients treated with MTX. The principal objective of this work was to study the presence of LF by transient elastography and aspartate aminotransferase to platelet ratio index as well as the detection of LS by ultrasonography and computer attenuation parameter (CAP).

Research results

Fifty-nine patients were included in the study. When comparing MTX-CD ≤ 4000 mg with > 4000 mg, we found no statistical differences in LS between low and high MTX-CD. We compared CAP scores with MtS, BMI, sex, and LF. There were no significant differences based on the presence or absence of MtS, the male sex, or LF. LS determined by CAP was significantly associated with BMI > 25.

Research conclusions

We concluded that, in our series, LS in RA patients treated with MTX is not related to sex, MTX-CD, MtS, or LF. BMI > 25 is significatively associated with LS in our study. Other factors, apart from MTX-CD or time in treatment, are more important for the development of LS in these patients.

Research perspectives

The routine incorporation of FS for the study of LF and LS in RA patients with MTX treatment is critical and will aid in understanding the real impact of MTX on LS. More studies (larger and multicentric) are recommended to validate these results.

FOOTNOTES

Author contributions: Castiella A and Lopez-Dominguez L were the guarantors and designed the study; Castiella A, Lopez-Dominguez L, Sanchez-Iturri MJ, Urreta I, De Diego A, Belzunegui J, and Zapata E participated in the acquisition, analysis, and interpretation of the data and drafted the initial manuscript; Castiella A, Lopez-Dominguez L, and Zapata E revised the article critically for important intellectual content.

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CASE REPORT

Giant cavernous hemangioma of the liver with satellite nodules: Aspects on tumour/tissue interface: A case report

Anne Kristin Fischer, Karl Tobias Erich Beckurts, Reinhard Büttner, Uta Drebber

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Abstract

BACKGROUND

Giant hepatic cavernous hemangioma with multiple satellite nodules is a rare subtype of hepatic cavernous hemangioma, the most common vascular liver tumor. We report on a tumor with unusual histologic features: (1) Finger-like infiltration pattern; (2) lack of encapsulation; (3) blurred tumor/liver interface; and (4) massive satellitosis-referring to the article "Hepatic cavernous hemangioma: underrecognized associated histologic features".

CASE SUMMARY

A 60-year-old man presented with increasing uncharacteristic abdominal discomfort and mildly elevated blood parameters of acute inflammation. Imaging revealed an unclear, giant liver tumor of the left liver lobe. A massive vascular tumor with extensive satellitosis broadly infiltrating the adjacent liver parenchyma was resected *via* hemihepatectomy of segments II/III. Histopathological diagnosis was giant hepatic cavernous hemangioma with multiple satellite nodules, featuring unusual characteristics hardly portrayed in the literature. Retrospectively, this particular morphology can explain the difficult pre- and perioperative diagnosis of a vascular liver tumor that is usually readily identifiable by modern imaging methods.

CONCLUSION

This case emphasizes the exact histological workup of tumor and tumor-induced parenchyma changes in radiologically unclassifiable liver tumors.

Key Words: Giant hepatic cavernous hemangioma; Satellite nodules; Tumour/liver

interface; Vascular liver tumours; Preoperative imaging; Case report

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Core Tip: This case highlights that attention to tumour/tissue boarders and knowledge about unusual perilesional parenchyma changes is not only of academic pathological interest, but has an important role in unclear preoperative imaging to discriminate between benign and malignant entities in interdisciplinary hepato-oncology and highly precise modern imaging techniques.

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INTRODUCTION

Giant hepatic cavernous hemangioma with multiple satellite nodules is a rare subtype of hepatic cavernous hemangioma, the most common vascular liver tumor. We report on a tumor with unusual histologic features: (1) Finger-like infiltration pattern; (2) lack of encapsulation; (3) blurred tumor/liver interface; and (4) massive satellitosis-referring to the article "Hepatic cavernous hemangioma: underrecognized associated histologic features" by Kim *et al*[1], 2005, in *Liver International*.

CASE PRESENTATION

Chief complaints

We report on a 60-year-old man with an unclear liver tumour of the left liver lobe. Increasing abdominal pressure, finally emanating to the left thorax, indigestion, and night sweat.

History of present illness

Symptoms increased for two months.

History of past illness

No special notes.

Personal and family history

Sixty-year-old man with an unclear liver tumor of the left liver lobe.

Physical examination

Symptoms comprised increasing abdominal pressure, finally emanating to the left thorax, indigestion, and night sweat over two months. Weight loss or exhaustion were not perceived.

Laboratory examinations

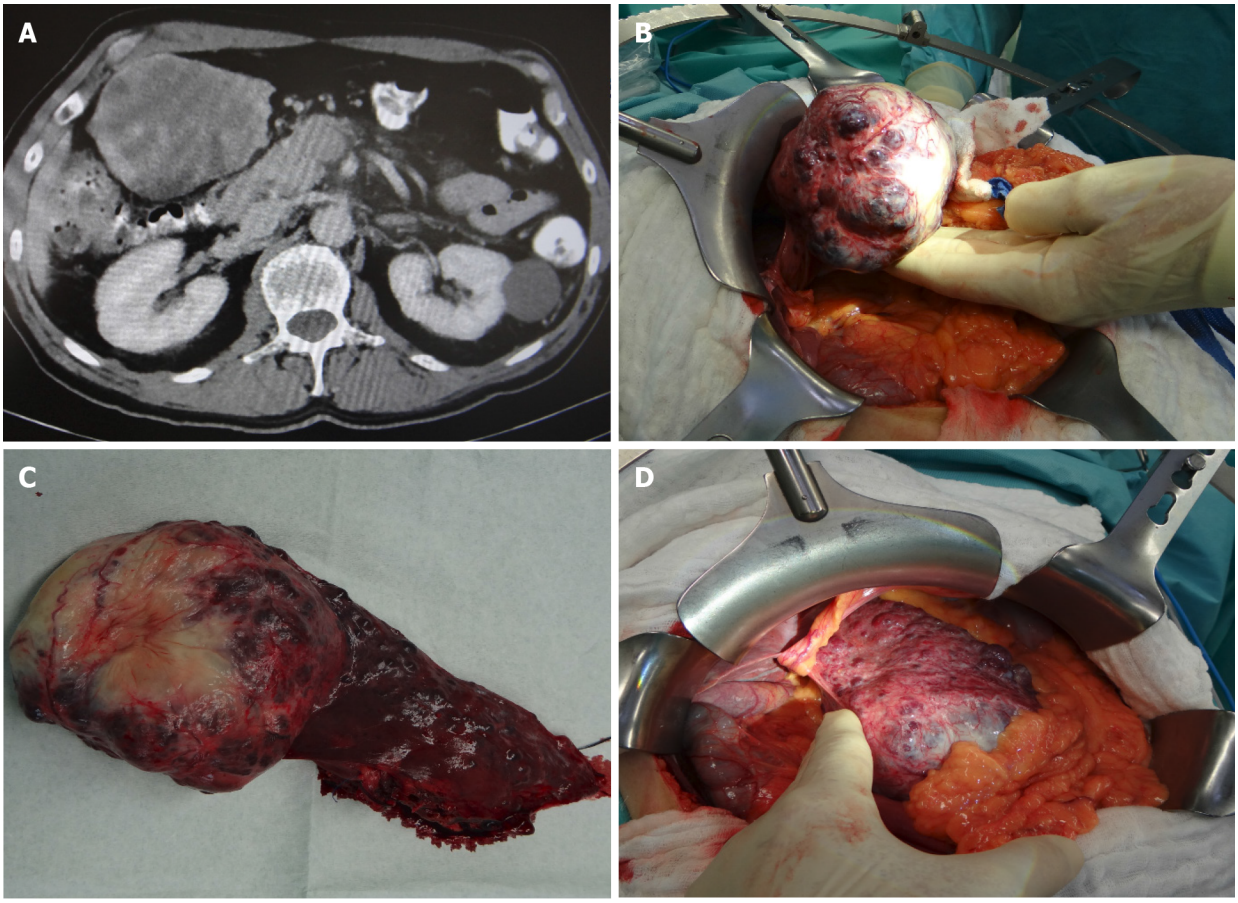
Leukocytes, aspartate transaminase, alanine transaminase, and lactate dehydrogenase were mildly elevated, liver enzymes in the normal range. C reactive protein was mildly elevated at first but increased significantly with aggravation of symptoms.

Imaging examinations

Contrast enhanced computed tomography (CT) showed a voluminous exophytic tumor arising from liver segment III, with atrophy of the left liver lobe, compression of the liver hilus and the left colon flexur (Figure 1A). Radiological diagnosis of hemangioma remained unclear because of blurred tumour boarders and multiple tumour satellites in the adjacent liver parenchyma.

Intraoperative presentation

Intraoperatively a solid and spongy dark red tumour measuring 10.5 cm was detected, with multiple small satellite nodules (0.1-0.9 cm) in the neighbouring liver parenchyma (Figure 1B + hypen + D), reminding of metastasis.



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Figure 1 Computed tomography imaging and intraoperative pictures. A: Computed tomography imaging demonstrating a giant multicystic vascular liver tumor; B and D: Intraoperative presentation; C: Macroscopic tumor. Main vascular tumor on the left side, adjacent liver parenchyma with multiple small tumor nodules on the right side.

Histological Presentation

Histology revealed a main vascular tumour with a fibrous capsule (Figure 2A), with multiple diffusely spreading unencapsulated capillary tumour foci at the periphery (Figure 2C, Figure 3A + hypen + F), readily identifiable through dilatated vessels with intravascular hemocongestion and capillaries in a retiform pattern, with interposed trabecula of liver parenchyma (Figure 3C + hypen + F, Figure 4A). The vessels were lined by flat inconspicious endothelial cells without mitotic activity. Immunohistochemistry (CD34, ERG and Fli1) highlighted the vascular nature (Figure 4B + hypen + D). No pathological nuclear TP53 accumulation or proliferative activity was found (Figure 4E and F). Capsule-like fibrosis was only observed around the main tumour, whereas satellite nodules lacked a fibrous interface with the adjacent liver parenchyma. Sometimes, small bile ducts and sparse lymphoplasmocytic inflammatory infiltration surrounded the main tumour.

FINAL DIAGNOSIS

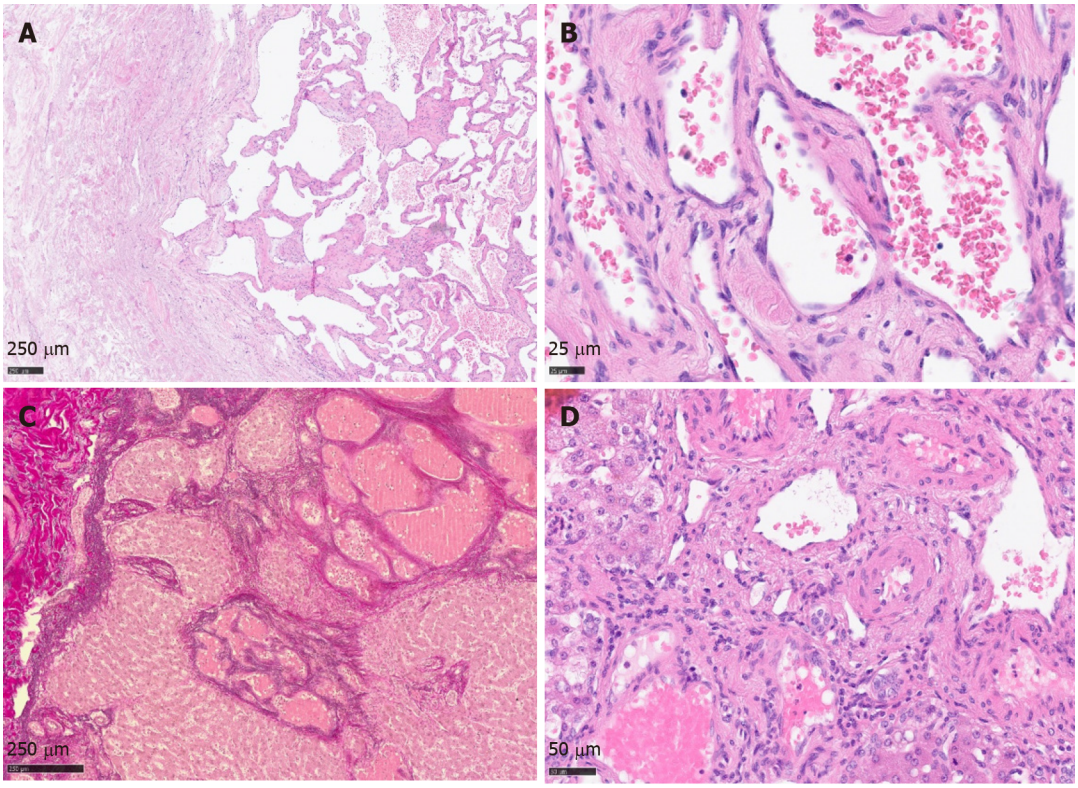
Giant hepatic vascular hemangioma with satellite nodules.

TREATMENT

Left lateral hemihepatectomy of segments II/III with construction of a bladder fistula was performed.

OUTCOME AND FOLLOW-UP

After resection the patient recovered well and was devoid of symptoms.



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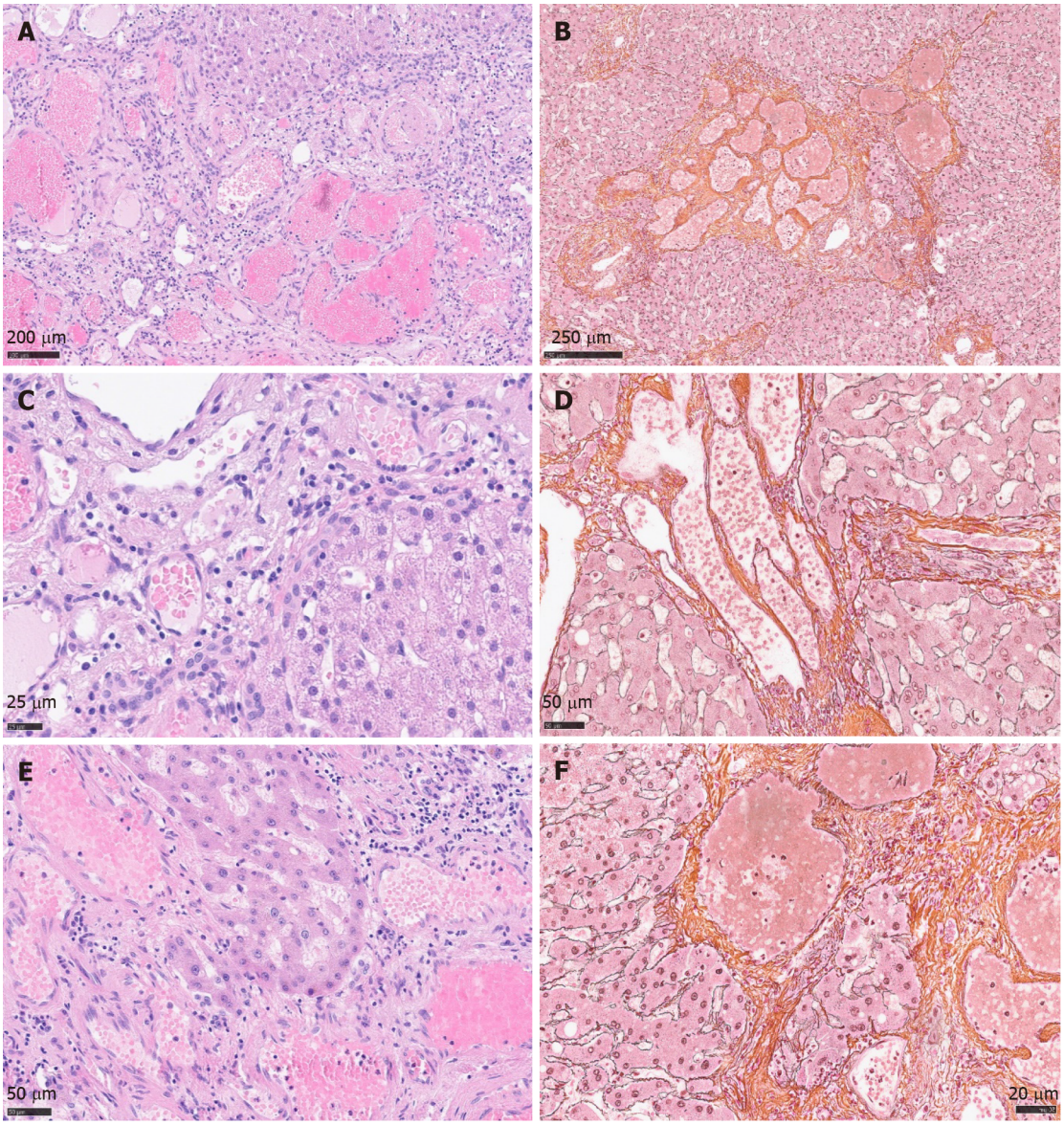
Figure 2 Histology of giant cavernous hemangioma with satellite nodules. A: Tumor overview with dilatated vascular channels on the right side and fibrous regressive tissue on the left side; B and C: Main tumor, high resolution. Vascular channels lined by inconspicuous endothelium; D: EvG staining highlighting tumor nodules in the periphery.

DISCUSSION

Hepatic cavernous hemangioma (CH) is a well-known entity, the most common benign vascular liver tumor with an incidence of 0.4% to 20% in autopsies[1-4]. The term "giant cavernous hemangioma" should be applied for tumors greater than 4 cm[2,5,6], 5 cm[1,7], or even 10 cm[6], depending on the literature. It occurs more often and has greater dimensions in (young) women than in men[1-7]. Etiology and pathogenesis are still unknown, though hormonal influence is discussed as a possible trigger[1,2,7]. Some tumors express estrogen receptors, and growth during puberty, pregnancy, and under medication with oral contraceptives is observed[1,2,7,8-11]. However, single studies also negate a correlation between hormonal influence, sex, and tumor size[5]. A solitary lesion under 3 cm is typical, classically seen in the right posterior liver lobe[2,12], although tumors can occur anywhere in the liver[2,5,12]. In up to 10% of cases, multifocal tumors arise and seldom diffuse hemangiomatosis is found, both much more often in women than in men[1,2,5]. Rarely are hemangiomas associated with focal nodular hyperplasia[2]. They are also observed in hereditary hemorrhagic telangiectasia (HHT; Rendu-Osler-Weber disease)[1,2].

Spontaneous involution by intratumoral thrombosis and vascular obliteration, as well as secondary fibrosis and calcification with phleboliths can occur[1,2,5,7], rarely resulting in a so-called "solitary necrotic nodule" as an end-stage form of completely sclerosed hemangioma[2]. Most tumors are asymptomatic and only detected by incidence. If the hemangioma lies directly under the liver capsule and starts to expand, capsule stretching can cause abdominal pain, and the tumor can even be palpable by clinical examination. Small CH only require surgery if symptomatic, extended tumors should be resected because of the elevated risk of rupture, acute thrombosis and tumor bleeding[1,2,7,13]. Alternatively, transarterial embolization or percutaneous radiofrequency ablation can be an option[2]. Rarely is liver transplantation necessary[1,5]. A rare complication in giant hemangioma in the liver or in extremities is Kasabach Merritt syndrome[2,5], a form of disseminated intravascular coagulopathy in convoluted tumor vessels with coagulopathy, thrombocythemia, and hypofibrinogenemia, triggered by intravascular aggregation of thrombocytes, strong activation of coagulation, and consumption of fibrinogen, with extensive bleeding[14,15].

In most cases highly precise contrast enhanced ultrasound of the liver or contrast-enhanced CT or magnetic resonance imaging does not require histological confirmation of the diagnosis, sparing invasive liver biopsy with the risk of bleeding. Typical imaging reveals peripherical nodular enhancement in the arterial phase, resulting from tumor feeding *via* liver arteries, with progressive

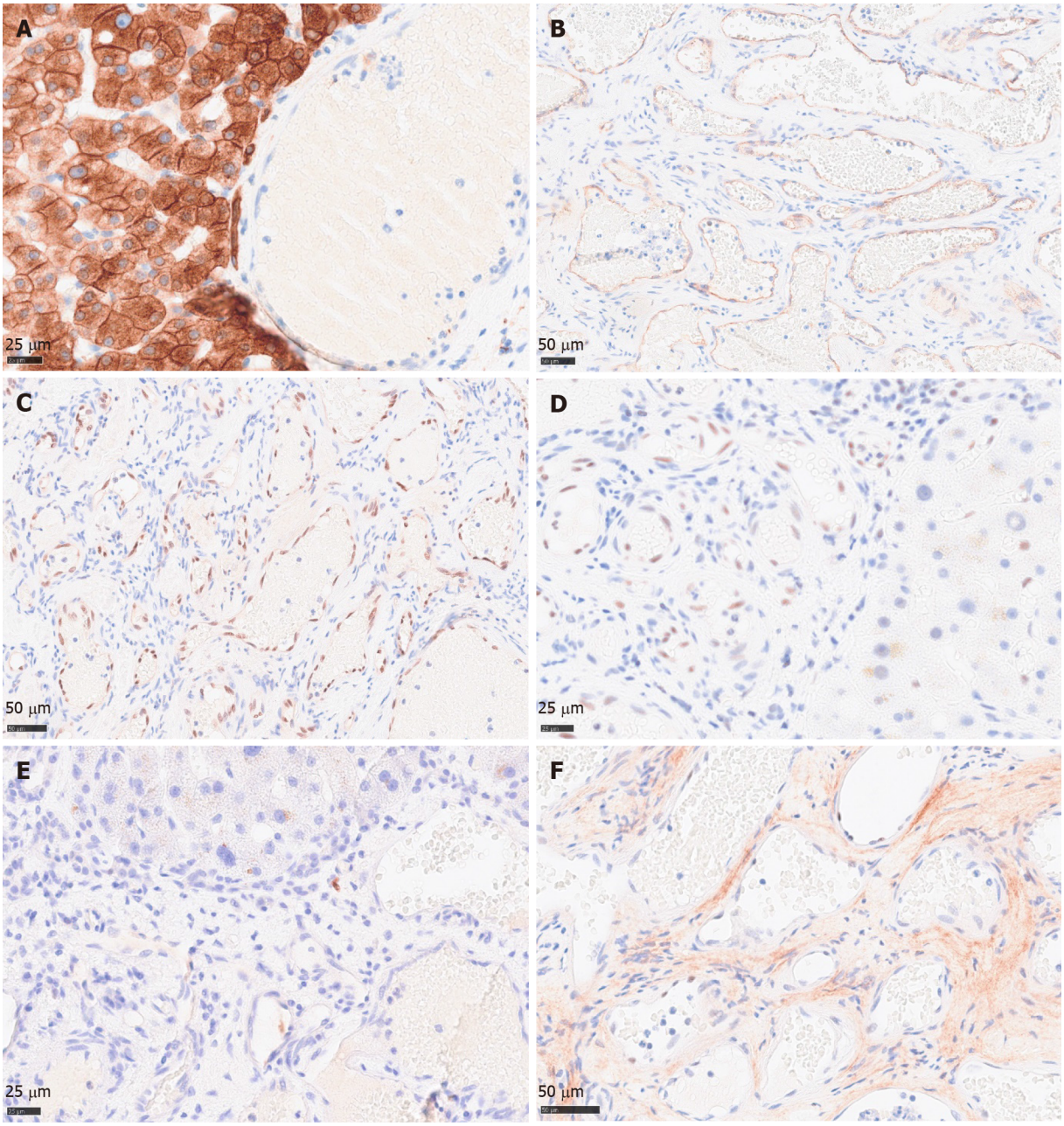


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Figure 3 Histology of giant cavernous hemangioma with satellite nodules. A and B: Satellite nodules of giant hepatic cavernous hemangioma with ill-defined borders, overview in H&E staining and Gomori reticulin fiber staining; C-F: Dilatated vascular channels, partially with hemangioma-like morphology, showing bland endothelial lining. Direct contact with adjacent liver parenchyma without formation of a fibrous capsule. High resolution. H&E, left side; Gomori, right side.

centripetal partial or complete fill-in in the portal venous phase, and washout in the late phase[16-18]. However, classical radio-morphological features can be lost with increasing tumor size and morphological variations like multinodularity[1,2,5] or rarely even liver infiltration, then referred to as diffuse hemangiomatosis[1]. Differential diagnosis of hepatic hemangiosarcoma must be considered. Other (vascular) disorders like peliosis hepatis, Budd Chiari syndrome, or venous occlusive disease/sinus obstruction syndrome can mostly be excluded by anatomic distribution in the liver, lacking zonal growth and filling phenomenon[2].

Cavernous hemangioma endothelial cells, so-called "CHECs" by Zhang *et al*[19], 2006, show an enhanced angiogenic activity compared with normal liver sinusoidal endothelial cells or "LSECs". They express elevated levels of vascular endothelial growth factor (VEGF), metalloproteinases, and angiopoietins[19,20]. The VEGF influence on vascular proliferation of liver hemangioma was also clinically noted. Shrinkage of incidentally detected liver hemangioma was observed during antiangiogenic therapy in patients with colon carcinoma who were treated with bevacicumab[21,22], a recombinant humanized monoclonal anti-VEGF-antibody hampering neoangiogenesis in various tumors or diabetic retinopathy. In hypoxic conditions, (neo) angiogenesis is promoted by autocrine and paracrine secretion of VEGF, which activates the PI3-Kinase/Akt-pathway and the Ras-dependent signaling pathway through Mitogen-activated protein kinases extracellular signal-regulated protein kinase 1 (ERK1) and ERK2. Hypoxia leads to ERK1 and ERK2 activation by phosphorylation, which then hamper degradation of hypoxia inducible factor 1α (HIF1α). This factor consecutively binds the



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Figure 4 Immunohistochemical profile of cavernous hemangioma. A: Broad spectrum keratine CKAE1/AE3 demonstrating pushing growth pattern of satellite tumor nodules against preexistent liver parenchyma; B: CD34 highlighting vascular tumor channels; C and D: ERG and Fli1 nuclear staining of endothelial tumor cells; E: Negative proliferative activity of satellite tumor nodules, protruding into the liver parenchyma; F: Negative or weak nuclear accumulation of TP53.

hypoxia-responsible element of the VEGF promotor in the nucleus, enhancing VEGF expression, and resulting finally in the proliferation of endothelial cells[21,22]. Hu *et al*[23], 2006, found an aberrantly enlarged endoplasmic reticulum (ER) in "CHECs" by electron microscopy and a downregulation of the protein Derlin-1 that plays a role in the transport of misfolded proteins from the ER to the cytosol for degradation. A shrinkage of the ER to normal size again was observed when Derlin-1 was overexpressed[19], implying a possible error in protein degradation in consecutive storage in ER in "CHECs".

In our case, the tumor displayed massive satellitosis, but not the diffuse small cystic infiltration pattern of hemangiomatosis. Apart from the main tumor, we did not find the classical histomorphological criteria for CH ("well demarcated", "fibrous capsule-like border")[1,2,5,7] in the satellite nodules. However, we recognized several atypical features reported by Zimmermann *et al*[24], 1996, and Kim *et al*[5], 2005, in their series of giant cavernous liver hemangiomas with unusual features, like the so-described "interdigitating pattern"[24] where tumor parts have finger-like expansion into the liver parenchyma, without formation of a typical fibrous interface (Figure 2C + hypen + F). Considering these particular features, together with the classical morphology of the main tumor, other differential diagnosis like peliosis hepatis, hereditary hemorrhagic telangiectasia, or hemangiosarcoma could be

readily excluded.

Spreading pattern also evoked the question of primarily multiple solitary hemangiomas in one liver lobe, of which one nodule started to expand massively, perhaps because of benefited localization next to greater arteria, or general arterial supply only sufficient for the expansion of one nodule. However, a review by Bioulac-Sage *et al*[2], 2008, describes a similar extension pattern of dilatated vessels in the close periphery of giant hemangiomas (0.1-2.0 cm beyond tumor borders), so-called hemangioma-like vessels (HLV)[2], discussing the "HLVs" as a process of expansion. In our case, we found satellite nodules infiltrating the whole resected liver lobe, up to 10 cm away from the main tumor (Figure 1C). The extremely low proliferation index and lack of TP53 accumulation in satellite nodules contradicted a rapid tumor expansion.

Blurred tumor borders and satellite nodules were a challenging aspect in preoperative imaging and, together with the untypical clinical setting (age, sex), did not permit a firm preoperative radiological diagnosis or definite exclusion of malignancy.

CONCLUSION

Giant cavernous hemangioma of the liver with unusual features is a challenging preoperative diagnosis. It requires thorough combined radiological and histomorphological workup with special regard to (1) finger-like infiltration pattern; (2) lack of encapsulation; (3) blurred tumor/liver interface; and (4) massive satellitosis. Moreover, attention must be paid to areas with diffuse and dense vascular spreading pattern, so that hemangiomatosis is not overlooked. Considering these rarely described features is essential in preoperative imaging and liver biopsy, to not prematurely drop the diagnosis of cavernous hemangioma, as well as to enlarge the portfolio of (malignant) differential diagnosis. Cases like this enhance the importance of interdisciplinary collaboration of radiology, hepatology, and hepatopathology, and the correlation of rare histomorphological aspects with modern imaging methods.

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FOOTNOTES

Author contributions: Fischer AK designed, wrote and edited the manuscript; Drebber U made the final diagnosis; Beckurts KTE performed the operation and delivered clinical information; all authors discussed the results and contributed to the final manuscript.

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CASE REPORT

Cerebrospinal fluid liver pseudocyst: A bizarre long-term complication of ventriculoperitoneal shunt: A case report

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Abstract

BACKGROUND

Ventriculoperitoneal (VP) shunt placement has become a standard of care procedure in managing hydrocephalus for drainage and absorption of cerebrospinal fluid (CSF) into the peritoneum. Abdominal pseudocysts containing CSF are the common long-term complication of this frequently performed procedure, mainly because VP shunts have significantly prolonged survival. Of these, liver CSF pseudocysts are rare entities that may cause shunt dysfunction, affect normal organ function, and therefore pose therapeutic challenges.

CASE SUMMARY

A 49-year-old man with history of congenital hydrocephalus status post bilateral VP shunt placement presented with progressively worsening dyspnea on

exertion, abdominal discomfort/distention. Abdominal computed tomography (CT) scan revealed a large CSF pseudocyst in the right hepatic lobe with the tip of VP shunt catheter into the hepatic cyst cavity. Patient underwent robotic laparoscopic cyst fenestration with a partial hepatectomy, and repositioning of VP shunt catheter to the right lower quadrant of the abdomen. Follow-up CT demonstrated a significant reduction in hepatic CSF pseudocyst.

CONCLUSION

A high index of clinical suspicion is required for early detection of liver CSF pseudocysts since their presentation is often asymptomatic and cunning early in the course. Late-stage liver CSF pseudocysts could have adverse outcomes on the treatment course of hydrocephalus as well as on hepatobiliary dysfunction. There is paucity of data to define the management of liver CSF pseudocyst in current guidelines due to rare nature of this entity. The reported occurrences have been managed by laparotomy with debridement, paracentesis, radiological imaging guided fluid aspiration and laparoscopic-associated cyst fenestration. Robotic surgery is an additional minimally invasive option in the management of hepatic CSF pseudocyst; however, its use is limited by lack of widespread availability and cost of surgery.

Key Words: Pseudocyst; Cerebrospinal fluid; Liver cysts; Ventriculoperitoneal shunt; Laparoscopy; Cyst fenestration; Case report

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Core Tip: Liver cerebrospinal fluid (CSF) pseudocysts are a rare subtype of abdominal cysts that are a late complication of ventriculoperitoneal (VP) shunt. The majority of patients are asymptomatic in early stages, however, as the size of the liver CSF pseudocyst increases this may result in ineffective drainage of CSF, thereby aggravating hydrocephalus symptoms. Liver CSF pseudocyst may be confused with other cystic lesions of liver. Early diagnosis and repositioning VP shunt catheter may prevent both neurological and hepatic complications.

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INTRODUCTION

Hydrocephalus is a condition of excess cerebrospinal fluid (CSF) accumulation into the ventricles of the brain causing neurologic manifestations due to pressure effects of expended ventricles on surrounding neural tissues. Placement of shunts to re-direct CSF has become a treatment option that has significantly improved outcomes and survival of patients with congenital hydrocephalus. Conventionally, shunts can drain CSF fluid into the abdominal peritoneal cavity or right atrium. Drainage of CSF into the right atrium could present with pulmonary emboli which is a life-threatening complication[1]. Numerous studies have determined the presence of ventriculoperitoneal (VP) shunts leading to pulmonary embolism[1]. The primary hypotheses derived from the presence of a foreign body into the heart leading to thrombus formation and CSF creating a procoagulant environment in the heart and lungs. These reasons lead to VP shunts being preferred over right atrial drainage since the peritoneal cavity can absorb fluid efficiently due to larger surface area and better diffusion property of fluids, thus making it a safe and sustainable options for drainage of CSF in the management of hydrocephalus. Generally, due to the increased lifespan of these individuals, complications of the VP shunt are seen later in adulthood. These are associated with abdominal cysts typically at the location of the tip of shunt catheter. A liver CSF pseudocyst is a bizarre long-term complication of this common procedure (VP shunt) that may be confused with other liver cystic lesions. Early diagnosis and repositioning VP shunt catheter may prevent both neurological and hepatic complications.

CASE PRESENTATION

Chief complaints

Patient presented with progressively worsening dyspnea on exertion, abdominal discomfort/distention.

History of present illness

A 49-year-old man with history of intellectual disability due to congenital hydrocephalus status post (s/p) VP shunt placement on right side at 3 mo and left side at 7 years of age, presented with progressively worsening dyspnea on exertion, abdominal discomfort/distention and bilateral lower extremity swelling associated with pain, erythema, and drainage. Patient denied abdominal pain, nausea, vomiting, diarrhea, or constipation. Patients VP shunt placement at 3 mo and 7 years of age never required exchange due to malfunction.

History of past illness

His medical history was also significant for gallstone pancreatitis s/p cholecystectomy.

Personal and family history

The patient denied family history of congenital abnormalities or cancers.

Physical examination

On physical examination, vitals were significant for tachycardia (115/min) and elevated blood pressure (151/81 mmHg), while he was afebrile (36.7 °C), saturating 96% on room air and respiratory rate of 18 breaths per min. Physical examination revealed mild abdominal distension, hepatomegaly without abdominal tenderness and bilateral lower extremity edema with mild erythema.

Laboratory examinations

Laboratory work up was significant for mildly elevated glucose 157 mg/dL (65-140 mg/mL), D-dimer 2.20 mcg/mL (0-0.5 mcg/mL) and Hemoglobin A1C 8.5% (4.8%-5.6%). The patient had normal level of aspartate transaminase 27 u/L (0-33 u/L), alanine transaminase 38 u/L (10-49 u/L), total bilirubin 0.5 mg/dL (0.3-1.2 mg/dL), slightly elevated alkaline phosphate 126 u/L (46-116 u/L), and low albumin 3.1 g/dL (3.2-4.8 g/dL).

Imaging examinations

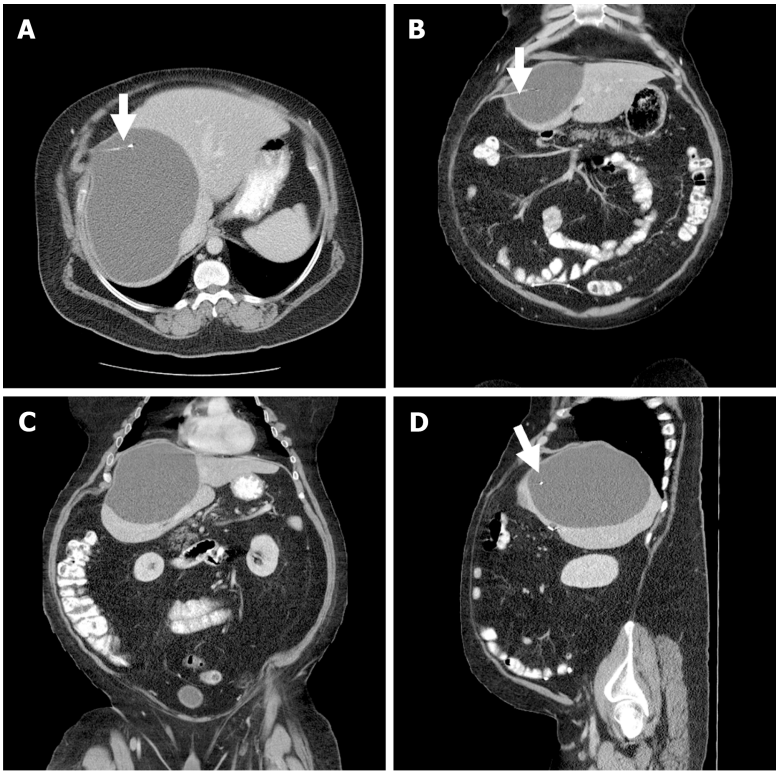
Chest computed tomography (CT) with intravenous contrast was negative for pulmonary embolism however revealed a large 18 cm × 13 cm × 13.5 cm hepatic cyst in the right lobe of liver. A subsequent CT abdomen and pelvis demonstrated a 17.5 cm × 12.6 cm × 12.7 cm cystic lesion in the right hepatic lobe with the tip of VP shunt catheter into the cavity of liver cyst (Figure 1). A head CT was negative for worsening hydrocephalus and confirmed the unchanged position of the intraventricular ends of VP shunt catheters compared to previous radiological imaging of brain (Figure 2A). Shunt series radiographs were also negative for disruption of the VP shunt catheters (Figure 2B-D). A hepatobiliary nuclear scan was unremarkable for biliary leak or sphincter of the Oddi dysfunction (Figure 3).

FINAL DIAGNOSIS

A diagnosis of bilateral lower extremities cellulitis with incidental right hepatic lobe CSF pseudocyst was made.

TREATMENT

Patient underwent robotic laparoscopic liver cyst fenestration with partial hepatectomy and repositioning of VP shunt catheter to the right lower quadrant of the abdomen (Figure 4). During the procedure, intraoperative ultrasound was used to locate the liver cyst and to evaluate the cyst wall thickness. The cyst cavity was opened and clear cyst fluid was drained into the peritoneal cavity. Multiple other fenestrations were done in the thin walls of the cyst to facilitate further drainage. Cyst fluid was not collected for analysis given it was clear cyst fluid, without noticeable pus, blood, or pseudo-membrane in the cyst cavity concerning for infectious etiology. However, cyst wall biopsy was obtained to rule out malignancy. Patient's cellulitis was managed with oral clindamycin 300 mg every 6 h for 7 d. His postoperative recovery was uneventful. There was no evidence of malignancy or parasitic infection on hepatic pseudocyst wall biopsy. The patient was discharged home on postoperative day 2.



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Figure 1 Computed tomography scan of abdomen. A: Transverse view shows a large right hepatic lobe cyst with evidence of tip of right ventriculoperitoneal shunt catheter within the cavity of pseudocyst (arrows); B and C: Axial view of hepatic pseudocyst; D: Lateral view of hepatic pseudocyst with ventriculoperitoneal shunt catheter in cyst cavity (arrow).

OUTCOME AND FOLLOW-UP

Follow up abdominal CT scan done 5 wk after hospital discharge showed a significant reduction of right hepatic pseudocyst size 10 cm × 13 cm (Figure 5). On follow up, patient denied abdominal pain, distention/discomfort, and signs of VP shunt malfunction.

DISCUSSION

Abdominal CSF pseudocyst was first reported in 1954 as a complication of VP shunt[2]. The incidence rate of these pseudocysts ranges from 0.25% to 10%[3,4]. However, hepatic CSF pseudocysts are extremely uncommon and only scant number of cases were reported in the medical literature (Table 1)[2-28]. The etiology of liver CSF pseudocysts associated with VP shunts is unknown, however, inflammation triggered with prolonged irritation of hepatic surface and peritoneum due to shunt catheter is the proposed mechanism resulting in pseudocyst formation[4,9,10]. Infective complications of pseudocysts are observed in very few cases and are typically caused by *Staphylococcus aureus*, followed by *S. epidermidis*, *Escherichia coli*, *Salmonella typhi*, *Propionibacterium acnes*, *Streptococcus faecalis*, *Acinetobacter sp.* and *Pseudomonas aeruginosa*[4,6,10,18]. The proposed pathogenesis of pseudocyst infection with intestinal and dermal bacteria results from systemic and localized inflammation around VP shunt catheter. Several risk factors such as abdominal trauma, abdominal surgeries, repeated shunt revisions, peritoneal adhesions, ascites and peritonitis predispose to CSF-pseudocyst infection[4,6,10,18].

Patients with small abdominal pseudocysts remain asymptomatic however those with larger pseudocysts commonly present with abdominal pain (63%), abdominal distention (37%), abdominal tenderness (31%) and abdominal mass (29%) on initial presentation[18]. The abdominal pain may be severe and diffuse, with a point of maximal intensity located at the terminal site of catheter, usually in the right or left upper quadrant of abdomen. A subset of patients may also have associated symptoms such as constipation, nausea or vomiting[4]. Since majority of the affected population is pediatric, the presenting symptoms tend to be related to increased intracranial pressure such as lethargy, headaches and altered mental status[9]. However, these symptoms are infrequent in adults who typically present with pain/pressure related symptoms depending upon location of the cyst or VP shunt catheter tip. The diagnosis is typically made using radiological modalities, however abdominal ultrasound is the initial diagnostic modality[4,5]. Abdominal CT scan is the preferred radiological imaging because it provides

Table 1 Summary table of reported cases of the liver cerebrospinal pseudocysts in patients with ventriculoperitoneal shunts

Ref.	Year	Age	Sex	Cyst location	Etiology of hydrocephalus	Treatment	Follow-up (wk)	Outcome
Current case	2023	49	M	Intra-axial	Congenital	Robotic laparoscopic pseudocyst fenestration, partial hepatectomy, and a repositioning of VPS	4	Complete resolution of symptoms without any complications
Achufusi *et al* [5]	2020	39	F	N/A	Congenital	Laparotomy with pseudocyst drainage followed by removal of VPS and insertion of new VPS		Complete resolution of pseudocyst; no recurrence
Arsanious and Sribnick[8]	2019	21	F	N/A	Meningitis	Laparotomy and pseudocyst drainage with externalization and repositioning of VPS	6	Complete resolution of pseudocyst; no recurrence
Koide *et al*[6]	2019	12	M	Extra-axial	Myelomeningocele	US-PCA of pseudocyst with externalization of VPS followed by reinsertion of new VPS	3	Complete resolution of pseudocyst; no recurrence
Bettis *et al*[7]	2019	56	M	Intra-axial	Trauma	N/A	N/A	N/A
Canaz *et al*[9]	2017	34	F	Extra-axial	NPH	Laparotomy and pseudocyst drainage followed by repositioning of VPS	36	Spontaneous resolution of hepatic CSF pseudocysts
Canaz *et al*[9]	2017	25	F	Extra-axial	Meningitis	Laparotomy and pseudocyst drainage followed by repositioning of VPS	24	Pseudocysts were spontaneously reabsorbed and post op CSF culture showed cyst fluid was sterile
Tomiyama *et al* [10]	2014	50	F	N/A	SAH	VPS removal and reinsertion and cyst drainage	8	No recurrence of intraperitoneal pseudocyst on postoperative abdominal scans and improvement in hydrocephalus on brain CT
Dabdoub *et al* [4]	2013	40	M	Intra-axial	Trauma	VPS removal and reinsertion and cyst drainage	36	No clinical recurrence of hepatic CSF pseudocysts
Verma *et al*[11]	2012	35	M	Intra- and extra-axial	Tumor	Pseudocyst aspiration and externalization of shunt followed by reinsertion	3	Complete resolution of cyst on abdominal ultrasound
Berkmann *et al* [13]	2011	34	F	N/A	Congenital	Laparoscopic shunt removal and new shunt reinsertion	24	No recurrence and no adverse events
Peltier *et al*[12]	2011	49	M	Extra-axial	Meningitis	Ultrasound guided aspiration and repositioning of shunt	1	Complete resolution of the cyst; no recurrence
Faraj *et al*[3]	2011	18	M	Intra-axial	Bacterial meningitis	Laparotomy and pseudocyst drainage followed by repositioning of shunt	N/A	Patient made a full recovery
Kolić *et al*[14]	2010	30	F	Intra-axial	SAH	Ultrasound guided aspiration and external ventricular drainage	4	Patient died due to sepsis- 1 mo after hepatic CSF pseudocyst drainage
Aparici-Robles and Molina-Fabrega[15]	2008	22-50	F (4), M (2)	Intra-axial	Abscess; tumor; meningitis; and SAH	Laparotomy with pseudocyst drainage and repositioning of shunt		Relapse in 2 out of 6 patients (5-6 mo)
Banka *et al*[17]	2007	7	M	N/A	Myelomeningocele	Repositioning of shunt		
Kaplan *et al*[16]	2007	10	F	Extra-axial	Tumor	Laparotomy and pseudocyst drainage followed by repositioning of shunt		
Hsieh *et al*[18]	2006	39	M	Extra-axial	Trauma	Externalization of shunt followed by reinsertion		Patient made full recovery and cysts were fully resolved (1

							mo)
Koçak *et al*[28]	2004	15	F	Extra-axial	Tuberculosis meningitis	Ultrasound aspiration and distal end removal followed by new shunt reinsertion	Almost complete resolution of the cyst observed after 1 wk
Chitkara *et al*[19]	2004	5	F	Intra-axial	Noncommunicating	Externalization of shunt followed by reinsertion	Follow up done at 3 mo-patient was entirely asymptomatic and cyst had completely resolved
Kumar *et al*[20]	1995	23	M	N/A	Meningitis	Ultrasound aspiration and distal end removal	
Engelhard and Miller[21]	1992	20	M	N/A	Congenital	Removal of shunt and reinsertion	
Wang and Miller[22]	1989	3	M	Extra-axial	Congenital	Pseudocyst resection and distal end revision	N/A
Touho *et al*[24]	1987	66	M	N/A	Trauma	Repositioning of shunt	
Wolbers *et al*[23]	1987	29	F	Extra-axial	Tumor	Repositioning of shunt	Patient made full recovery(2 yrs)
Rana *et al*[25]	1985	12	M	Intra-axial	Tumor	Repositioning of shunt	Complete resolution of cyst in follow up CT scan
Latchaw and Hahn[26]	1981	40	M	Intra-axial	Aqueductal stenosis	Drainage of hepatic pseudocyst and repositioning of the shunt	
Fischer and Shillito[27]	1969	3	F	Extra-axial	Aqueductal stenosis	Drainage of hepatic pseudocyst and lumboperitoneal shunt	Patient did not experience or report any adverse complications (10 mo)

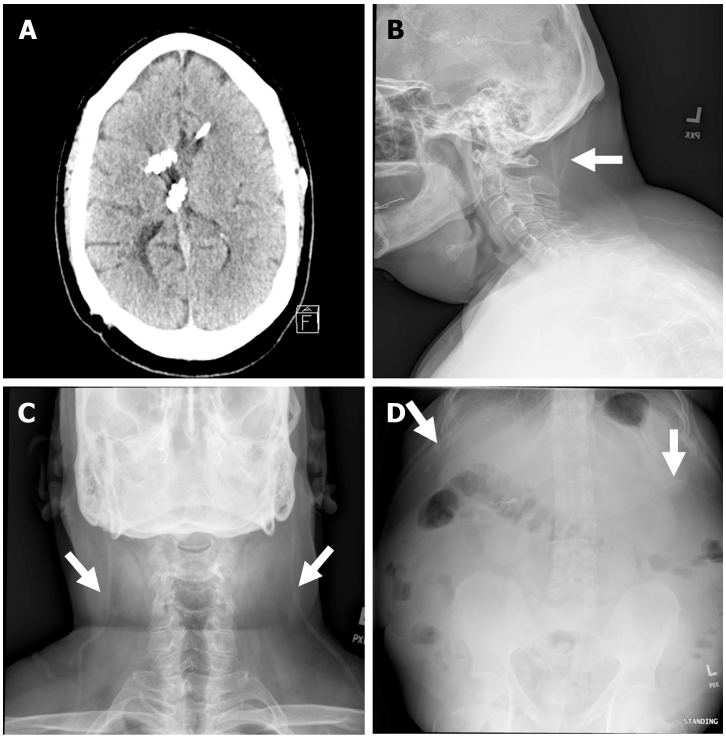
Summary table of reported cases of extra and intra axial hepatic cysts. Data from reports stratified by date published, age of patient, gender, location of cyst, etiology, treatment and follow up outcomes. M: Male; F: Female; VPS: Ventriculoperitoneal shunt; N/A: Not available; US-PCA: Ultrasound guided percutaneous aspiration; CSF: Cerebrospinal fluid; NPH: Normal pressure hydrocephalus; SAH: Subarachnoid hemorrhage; CT: Computed tomography.

information on the characterization of cyst size, location, and position of the VP shunt catheter. It also helps to differentiate other possible etiologies of abdominal pain[4]. Rarely, in adults who present with altered mental status, CT scan of the head may be sought out to evaluate location of proximal end of VP shunt catheter, status of ventricular dilation and to rule out other intracranial abnormalities.

The liver CSF pseudocysts are broadly classified as intra or extra axial[4]. The intra-axial liver pseudocysts are those found within the hepatic parenchyma with the VP shunt tip lodged within the liver. The extra-axial liver pseudocysts are usually located under the hepatic subcapsular space due to penetration of the VP shunt tip in the Glisson's capsule. The symptomatic clinical presentation is seen in the fourth or fifth decade of life[2-28]. The cysts have thus far also shown an equal male/female predominance[2-28]. There was an even distribution of the incidence of intra-axial and extra-axial location[2-28]. The patients in the reported cases had a range of etiologies for their hydrocephalus such as infectious, congenital, traumatic, and mechanical/obstructive leading to the shunt placement[6].

The characteristics and clinical presentation of our case is similar to previously reported cases of intra-axial hepatic CSF pseudocysts. However, duration since VP shunt placement to clinical presentation is relatively longer compared to previously reported cases. This is likely due to lack of predisposing risk factors in our case such as no prior history of abdominal surgery, trauma, peritonitis, ascites and his VP shunt catheter never required revision. Although liver CSF pseudocyst was identified on the CT chest, however, a dedicated CT abdomen pelvis is essential for evaluation of other abdominal cysts/collection and planning for surgical repositioning of VP shunt catheter.

The management of liver CSF pseudocyst is controversial because of rarity of disease[5,7,9]. In asymptomatic individuals wherein the cyst is an incidental finding on abdominal imaging, the consensus is expectant management with routine monitoring with abdominal ultrasound or radiological imaging[9]. The duration of surveillance imaging is unclear in the current literature. It is reasonable to performs ultrasound abdomen in 1-2 year for monitoring of pseudocyst size and planning of further treatment with conservative *vs* surgical approach. In symptomatic patients, management is variable ranging from robotic or laparoscopic repositioning/exchange of VP shunt catheter with or without cyst fenestration and antibiotic therapy[4,10]. Management is patient specific and varies on a case-by-case basis. Typically, a CT-guided biopsy is performed for a diagnostic purpose. If the biopsy reveals an infection, treatment involves antibiotic therapy as well as shunt externalization till the infection clears [4]. The shunt is repositioned during externalization after infection treatment. Depending upon the availability of expertise, the liver CSF pseudocysts are managed with elective laparoscopic excision of



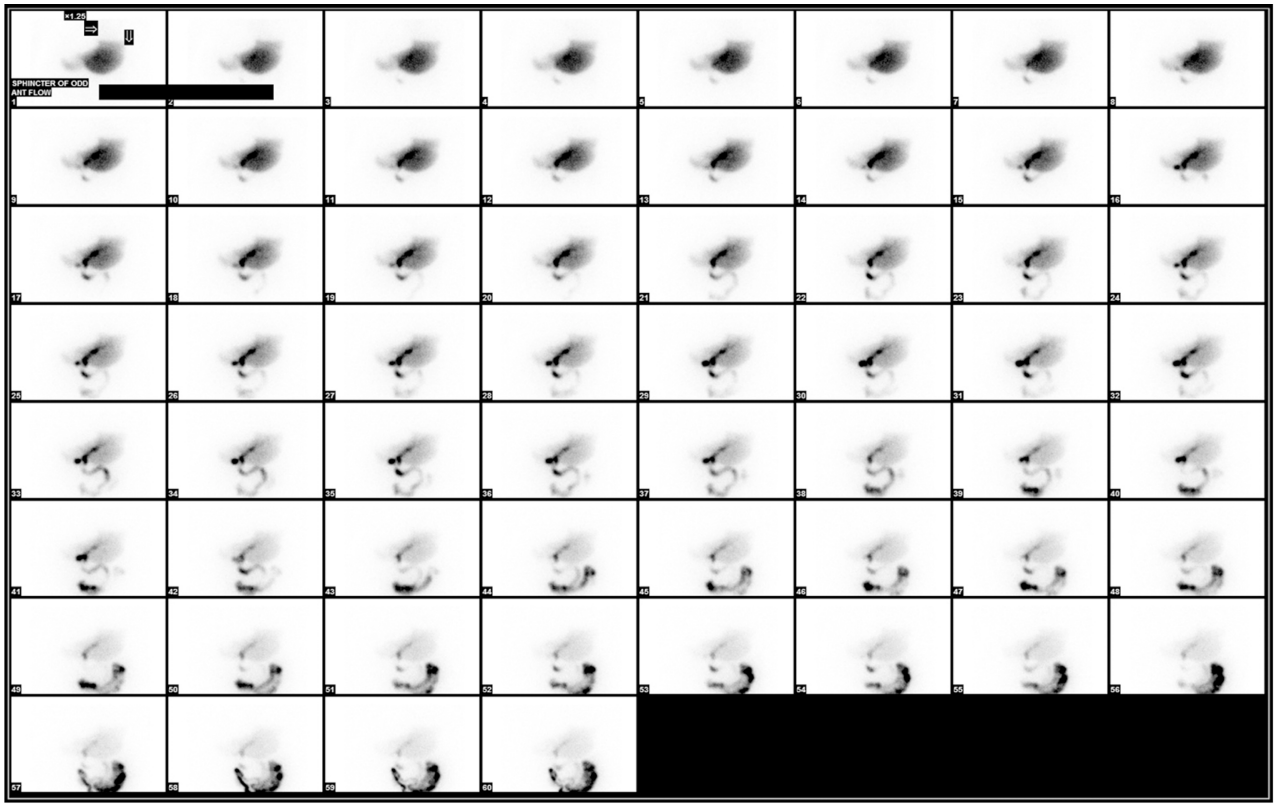
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Figure 2 Computed tomography scan of head and shunt series radiographs. A: Computed tomography scan of head showing ventriculoperitoneal (VP) shunt catheters both in right and left lateral ventricles without evidence of ventricular dilation or VP shunt catheter displacement; B: Lateral view of shunt series radiograph showing right and left VP shunt catheter (arrows) without disruption through neck; C: Anterior-posterior view of shunt series radiograph showing intact VP shunt catheter through chest (arrows); D: Abdominal view of shunt series radiograph showing distal ends of right and left VP shunt catheter in the peritoneal cavity (arrows).

pseudocyst, debridement of cystic wall and/or reposition the VP shunt catheter in the lower quadrant of abdomen. The majority of case studies managed the liver CSF pseudocyst with surgical or ultrasound guided drainage, the VP shunt was removed and/or repositioned along with cyst drainage[4-6,8-10].

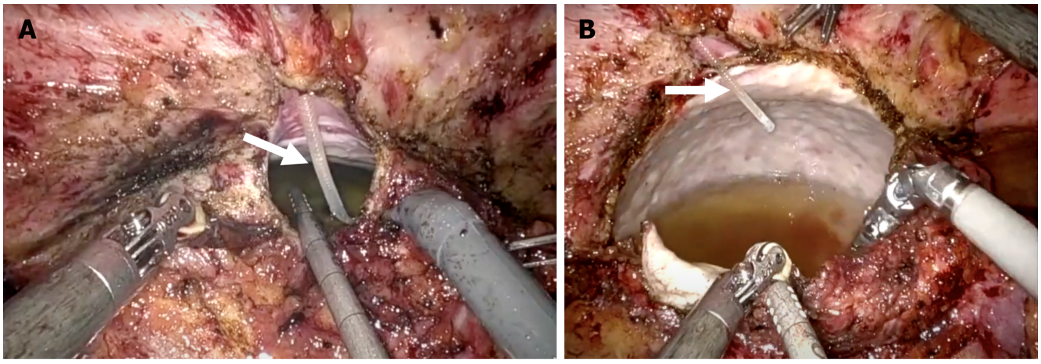
In the present case robotic laparoscopic cyst fenestration with partial hepatectomy and repositioning of VP shunt catheter was performed. Robotic surgery has several advantages over other surgical options such as wide cyst fenestrations could be achieved with robotic ergonomics that provide magnified three-dimensional view and enables access to cysts located in posterosuperior segments of liver[29]. Recurrence rate after robotic fenestration is lower than conventional laparoscopic or other surgical approaches as wristed technology of robotic surgery overcomes limitations of other surgical options [29]. The dual console system of robotic surgery plays an important role in surgical training by enabling direct mentorship to trainee where one click on button swap the control between trainee and mentors. Taken together, robotic surgery assisted cyst fenestration/deroofing is a safe and feasible approach in the management of hepatic CSF pseudocysts. The utility of robotic surgery should be balanced with cost, limited worldwide availability of robotic technology and expertise.

The pseudocysts that have been documented thus far have had a good prognosis, with near complete or complete resolution of symptoms at follow up appointments that were between 3 to 9 wk[2-28]. In our case there was a significant reduction of cyst size on follow up CT scan of abdomen. Most of the cysts (12 out of 15 case studies) showed near complete or complete resolution of hepatic CSF pseudocysts with either surgical or ultrasound guided drainage, however clinical characteristics and etiology of cysts are variable with no specific differences in clinical variables (Table 1)[2-28]. Large prospective studies are required to further investigate clinical characteristic of patients who will benefit surgical *vs* conservative approach for spontaneous resolution of hepatic CSF pseudocysts. Rare nature of hepatic CSF pseudocyst is predominant roadblock for conducting prospective studies. The physician dependent treatment plan had similar outcomes in both groups of patients, except that the self-absorbing cysts took longer to resolve (about 9 mo *vs* immediately after surgical procedure)[9]. The physician dependent treatment plan in managing patients with hepatic CSF pseudocyst is dependent on several factors, including the severity of symptoms, the size and location of the pseudocyst, the patient's overall health, and the physician's expertise to determine, surgical *vs* non-surgical management. Patients with acute illness, larger size pseudocyst and persistent symptoms despite conservative management, may benefit from surgical approach. The recurrence rate of the liver CSF pseudocysts on short term follow up is low. Only one case study has reported recurrent pseudocyst in 2 out of 6 patients. A fatal outcome due to sepsis was reported in on case study[14].



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Figure 3 Hepatobiliary nuclear scan without evidence of biliary leak or dysfunction of sphincter of oddi.

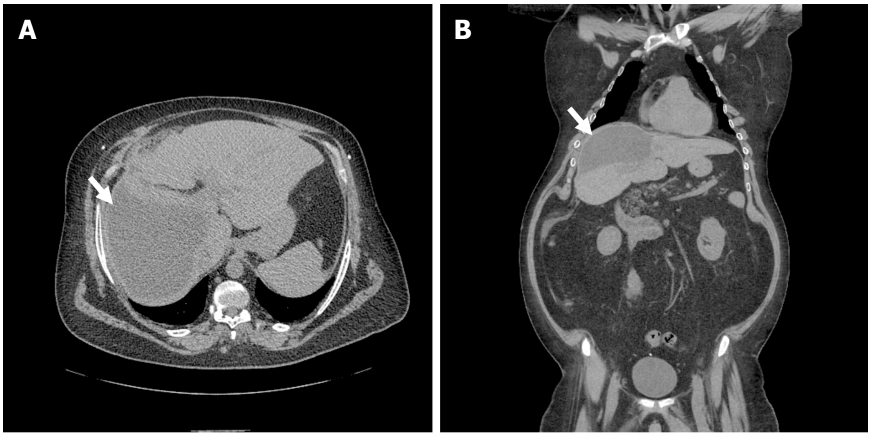


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Figure 4 Intraoperative images of hepatic cerebrospinal fluid pseudocyst. A: A large right hepatic lobe pseudocyst with tip of right ventriculoperitoneal shunt catheter within the cyst cavity (arrow); B: A large volume of cerebrospinal fluid can be seen within the cyst cavity (arrow).

CONCLUSION

Liver CSF pseudocysts are a rare subtype of abdominal cysts that are a late complication of VP shunt. The majority of patients are asymptomatic in early stages, though larger liver CSF pseudocyst may result in ineffective drainage of CSF, thereby aggravating hydrocephalus symptoms. In asymptomatic patients with hepatic CSF pseudocysts, it is reasonable to performs ultrasound abdomen in 1-2 year for monitoring of pseudocyst size and planning of further treatment with conservative vs surgical approach. While there is no current consensus on the optimal treatment modality, however, laparoscopic drainage with repositioning of shunt catheter has favorable outcomes. Robotic surgery is an additional minimally invasive option in the management of hepatic CSF pseudocyst; however, its use is limited by lack of widespread availability and cost of surgery.



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Figure 5 Computed tomography scan of abdomen and pelvis 5 wk after robotic laparoscopic cyst drainage. A: Transverse view of computed tomography (CT) scan shows interval decrease in cyst size (arrow); B: Axial view of CT scan shows interval decrease in cyst size (arrow).

FOOTNOTES

Author contributions: Yousaf MN, Naqvi HA, Kane S, and Chaudhary FS contributed to manuscript writing, proofreads, table and review of data; Faust TW, Hawksworth J, and Nayar VV contributed to overall supervision of manuscript and review of data; all authors read and approved the final manuscript.

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REVIEW

Ductular reaction in non-alcoholic fatty liver disease: When Macbeth is perverted

Yang-Huan He, Jia-Xing Pan, Lei-Ming Xu, Ting Gu, Yuan-Wen Chen

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Abstract

Non-alcoholic fatty liver disease (NAFLD) or metabolic (dysfunction)-associated fatty liver disease is the leading cause of chronic liver diseases defined as a disease spectrum comprising hepatic steatosis, non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatic carcinoma. NASH, characterized by hepatocyte injury, steatosis, inflammation, and fibrosis, is associated with NAFLD prognosis. Ductular reaction (DR) is a common compensatory reaction associated with liver injury, which involves the hepatic progenitor cells (HPCs), hepatic stellate cells, myofibroblasts, inflammatory cells (such as macrophages), and their secreted substances. Recently, several studies have shown that the extent of DR parallels the stage of NASH and fibrosis. This review summarizes previous research on the correlation between DR and NASH, the potential interplay mechanism driving HPC differentiation, and NASH progression.

Key Words: Ductular reaction; Non-alcoholic steatohepatitis; Hepatic progenitor cells; Cell differentiation; Inflammatory cells; Liver fibrosis

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Core Tip: This is the first review focusing on recent advances in the relationship of hepatic cells with ductular reaction (DR), in fatty liver-related steatohepatitis and fibrosis. Recent advances in DR, a common compensatory reaction in liver injury, shed light on the effects of hepatic progenitor cells, hepatic stellate cells, myofibroblasts, inflammatory cells, and their secreted substance. In particular, hepatic progenitor cell differentiation was thoroughly discussed in developing steatohepatitis and fibrosis. This review summarizes the correlation between DR and steatohepatitis and fibrosis, the advanced stages of non-alcoholic fatty liver disease, or metabolic (dysfunction) related fatty liver disease.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), which affects approximately 25% of adults worldwide, is the leading cause of chronic liver diseases[1]. NAFLD refers to a disease spectrum including hepatic steatosis, non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatic carcinoma[2]. In early 2020, an international expert group led a consensus-driven process to develop a more appropriate term for NAFLD, and the term "metabolic (dysfunction) related fatty liver disease (MAFLD)" was recommended[3]. NASH/MASH is characterized by ≥ 5% hepatic steatosis, hepatocyte injury or necrosis, and inflammation[2,4]. NASH is a critical stage in NAFLD development and is associated with NAFLD prognosis; thus, it has become the focus of NAFLD research. NASH is the second most common indication for liver transplantation in the United States[1]. The occurrence and progress of NASH are related to several factors such as glucose and lipid metabolism, immune response, and gut microbiota[5-7]. The diagnosis and severity classification of NASH depends on histopathological examination. The main pathological features of NASH are hepatocyte balloon degeneration, inflammatory infiltration, Mallory-Den K corpuscle, and zone 3 fibrosis[2,8]. Some studies have shown that neutrophil infiltration and portal inflammatory infiltration are also characteristics of NASH[9,10].

Ductular reaction (DR) is a compensatory reaction commonly detected in various liver injuries[11], involving the participation of hepatic progenitor cells (HPCs), hepatic stellate cells (HSCs), myofibroblasts, inflammatory cells (such as macrophages), and their secreted substances. Among them, the proliferation and differentiation of HPCs are the core of DR[12]. DR is commonly found in the livers of NASH patients. Moreover, there is a parallel relationship between DR and the severity of inflammation and fibrosis in NASH patients[13-15], suggesting that DR has an important role in the progression of NASH.

Based on clinical investigations, the present review summarizes the correlation between DR and NASH. It discusses the shaped HPC differentiation fate in the context of NASH and its influence on NASH progression.

OVERVIEW OF DUCTULAR REACTION AND CORRELATION BETWEEN HPC AND DR

DR is a compensatory reaction in the portal area caused by biliary diseases, viral hepatitis, NAFLD, acute fulminant liver failure, *etc*[16]. DR is heterogeneous in both pathology and pathophysiology. Desmet divided DR into four types based on pathology: Type 1, Type 2A, Type 2B, and Type 3[17].

Type 1 is predominant in acute complete bile duct (BD) obstruction, alpha-naphtyl isothiocyanate intoxication, and cytokine (*e.g.*, interleukin 6)-induced ductular increase. It results from the proliferation of preexisting cholangiocytes. Type 1 causes the biliary tubes to elongate, branch out, and widen their lumens, allowing them to adjust to the swelling and inflammation of the portal mesenchyme. Type 2A has been interpreted as "ductular metaplasia of hepatocytes." It is often detected in periportal areas, most characteristically, in chronic cholestatic conditions. In lasting cholestasis, bile acids increase the number of cholangiocytes, which promote the development of pericellular fibrosis, and in this way, it enhances bile ductular metaplasia of hepatocytes. Of note, Type 1 and Type 2A can be reversed when the causative trigger is eliminated; the ductular structures are cleared by apoptosis; and the associated fibrosis is ameliorated to a considerable extent. Prolonged hypoxia induces Type 2B, which manifests in areas of parenchymal hypoxia, specifically in the centrolobular region of liver lobules and the centronodular region of cirrhotic nodules. Although often slower in development, its microscopic pattern is comparable to that of Type 2A in terms of ductular metaplasia or dedifferentiation of mature hepatocytes, which is associated with myofibroblast-induced fibrosis. Type 3 occurs in cases of massive loss of parenchymal cells and is characterized by the activation and proliferation of HPCs located in the

ductules and canals of Hering. As bipotential cells, HPCs can differentiate into hepatocytes and BD cells [17].

There is consensus that the fate of HPC differentiation is the core of DR, determining the pathological type of DR and affecting disease development[18]. Epithelial cell adhesion molecule and the neural cell adhesion molecule/sex-determining region Y-Box 9 (SOX9) have been previously considered markers of HPCs, cytokeratin-7 (CK7) and CK19 have been used to identify cholangiocytes, and albumin and hepatic nuclear factor 4-alpha have been considered markers of hepatocytes[19-21]. HPCs located in the Hering canal typically differentiate into biliary cells in a normal liver[18] but do not lead to DR. HPCs are activated and differentiate into hepatocytes or biliary cells during liver injury. For example, HPCs differentiate into hepatocytes in acute fulminant hepatic failure and contribute to liver regeneration[22, 23]. CK7 immunohistochemistry is also positive in HPCs, which can predict liver injury severity; for instance, HPCs differentiate into CK7+ cells in the portal area in chronic hepatitis C and exacerbate liver injury[13,14,24-26]. Furthermore, a similar phenomenon has been found in hepatitis B virus-injected murine models[27]. In addition, DR is significantly associated with hepatocellular carcinoma peritumoral hepatic inflammation, liver fibrosis, tumor node metastasis classification stage, and poor prognosis[28]. Hepatocyte-derived ductular HPCs can give rise to hepatocellular carcinoma *via* concomitant activation of yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif transcription factors. Autophagy suppresses the formation of hepatocyte-derived cancer-initiating HPCs in the liver[29].

HPCs are activated in the majority of liver diseases[30]. During liver injury, a ubiquitous DR affects the differentiation *vs* dedifferentiation type of HPCs, depending on the severity of the liver injury[31]. Proliferating BDs in DR are misshapen, lack an apparent lumen, and are associated with increased portal inflammation and fibrosis[19,32]. It has been previously demonstrated that HPC activation is sufficient to regenerate a large proportion of the liver parenchyma using targeted deletion of mouse double minute 2 (MDM2) in mouse hepatocytes. This kind of HPC activation may be induced by the tumor necrosis factor-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor-inducible 14 pathway[33]. Interestingly, in the hepatocyte-specific β-catenin knockout model, hepatocytes lose their regenerative capacity, and cholangiocytes still express β-catenin. β-catenin-positive cholangiocytes (differentiated HPCs) differentiate into β-catenin-positive small hepatocytes, which then proliferate and repopulate the liver[34,35]. A previous study reported that YAP levels are increased in NAFLD patients and NAFLD mouse models[36]. A recent study showed that the DR reaction is more intense and hepatocytes trans-differentiate into cholangiocytes protected from cholestatic damage by activating Hippo-YAP in the Tjp2 cKO mouse model (more susceptible to cholic acid-induced liver injury) fed 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)[37]. A murine BD ligation model of liver fibrosis showed that heme oxygenase-1-mediated pro-resolution M2 polarization of macrophages protects the liver from excessive DR and fibrosis with the ligand of numb protein X1 as the key downstream factor[38]. Interestingly, recent studies have shown that HPCs can promote angiogenesis by secreting vascular endothelial growth factor (VEGF) *via* the secretin/secretin receptor/microRNA 125b (miR-125b) axis [39]. However, recent studies have shown that DR cells can promote angiogenesis through slit guidance ligand 2-roundabout 1 signaling channels in various chronic liver diseases (CLDs), contrary to VEGF [40]. Another study showed that the signaling of apelin/APJ (G protein-coupled apelin receptor) can promote intrahepatic angiogenesis[41].

The impact of DR on liver diseases is a double-edged sword. HPCs can be activated and differentiated into hepatocytes to participate in liver regeneration in the case of massive loss of parenchymal cells. Conversely, the activation of HPCs may play a role in the activation of HSCs and the infiltration of inflammatory cells in DR in most CLDs, which can lead to further liver injury, including cirrhosis and tumorigenesis[14,25,42,43].

Correlation between NASH and DR

A state of NAFLD begins with healthy liver parenchyma (steatosis in < 5% of hepatocytes) and then progresses to steatosis in > 5% of hepatocytes with the initiation of DR. The condition progresses to a severe stage with scar tissue accumulation, elevated steatosis, and hepatic ballooning[43]. In recent years, DR has attracted considerable attention in NASH research. It is worth noting that although DR can assist in repairing liver injury by aiding in HPC activation and differentiation, its impact on the progression of chronic liver disease associated with NASH may not always be favorable, especially when liver regeneration capacity is impaired. In fact, in some cases, DR-induced differentiation may even contribute to the occurrence and progression of inflammation and liver fibrosis in NASH. In 2007, Richardson *et al*[14] analyzed data from 118 liver specimens (107 from NAFLD patients and 11 from normal liver) and found that DR commonly existed in NASH, especially in patients with fibrosis. Multivariate analysis demonstrated that the extent of DR was independently associated with hepatocyte replicative arrest [odds ratio (OR) = 6.5] and fibrosis stage (OR = 17.9). Moreover, they further found that the expansion of HPCs was significantly correlated with NASH activity score[14]. In 2013, based on biopsy specimens from 56 adults with NAFLD (10 with steatosis and 46 with NASH) from Austria and the United States, Skoien *et al*[44] found that both centrilobular fibrosis and portal fibrosis stages were positively associated with the extent of DR. In 2018, multicenter observational studies of 90 NAFLD patients showed that DR was identified in 90% of biopsy samples, and its extent was correlated with

fibrosis stage[15]. Similarly, Gadd *et al*[13] also found that DR appeared in almost all NASH patients, and its grade was significantly associated with pathological liver progression. Similar to the results in adult NAFLD, DR can also be found in pediatric NAFLD, and its extent and/or HPC expansion were significantly correlated with fibrosis degree[44-46].

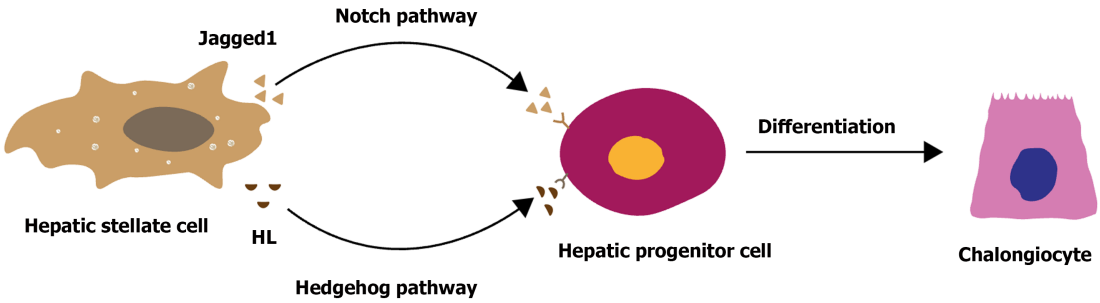
DR also exists in animal NAFLD models. In an 8-wk methionine/choline-deficient (MCD) diet mouse model and a 16-wk western diet mouse model, the number of YAP+, CK19+ reactive-appearing ductular cells, and HPCs were significantly increased with the severity of hepatocyte injury and inflammation[47]. A recent study based on mouse models indicated that during NASH development, YAP activation occurred earlier than DR but they were spatiotemporally correlated. Murine YAP activation may promote hepatocyte dedifferentiation during NASH development[48]. Morell *et al*[49] also established an 8-wk MCD diet mouse model and found that DR extent and HPC number increased steadily over time in the portal and lobular areas. Furthermore, the extent of DR rose significantly in a 12-wk western diet and carbon tetrachloride-treated mouse model, which led to severe NASH-related fibrosis. DR can also occur in other NAFLD animal models, such as rats and monkeys[50,51]. Although some animal models are particularly useful, especially for studying liver regeneration, many features of DR in humans are significantly different from those of animals[18]. The contrasting anatomical features of the two species likely account for this distinction. In humans, cholangiocytes are classified based on the diameter of the biliary tract, which can vary from small to medium to large, resulting in different sizes of the cells. Unlike humans, rodents have small BDs and large BDs, lined by small BDs and large BD cells, respectively, with distinct functional properties[52].

Interestingly, the location of DR varies in different NAFLD patient populations. In pediatric NAFLD patients, DR often appears in the portal/periportal area. In a retrospective study involving 30 children and adolescents with biopsy-proven NAFLD, CK7-positive HPCs localized at the portal-parenchymal interface, *i.e.* the periportal site[45]. Similarly, a cohort study of 32 children and adolescents with biopsy-proven NAFLD showed that DR commonly occurred in the portal area[46]. In another pediatric NAFLD study, the authors gathered 38 biopsy specimens from NASH children in three United Kingdom medical centers. They found DR at the interface between the parenchyma and portal areas in 36 NASH patients[44]. Similarly, portal DR can also occur in adult NAFLD patients[13-15]. However, in adult NAFLD patients, CK7+ cells and/or CK7+ structures can be found in the centrilobular area. Interestingly, CK7+ cells and/or CK7+ structures in centrilobular zones universally occurred in several other CLDs (including chronic viral hepatitis, autoimmune hepatitis, drug-induced liver injury, *etc*), which was termed centrilobular DR[53-55]. Both centrilobular DR and periportal DR were also found in adult NAFLD studies and showed a significant correlation with NASH progression[15,55,56]. Importantly, centrilobular DR was also located, and the correlation of fibrosis stage with centrilobular DR was much stronger than with periportal DR (regression coefficient: 1.856 *vs* 0.646)[15].

The difference in DR localization between pediatric NAFLD and adult NAFLD is plausible. In children, pediatric NASH is characterized by portal inflammation and/or fibrosis[57-59]. Since it is acknowledged that periportal DR is closely related to NASH progression in pediatric NAFLD, the localization of DR in the portal area is reasonable. The concept of centrilobular DR seemingly contradicts the localization characteristic (portal area) in the classic DR definition in adults. However, this phenomenon might be explained from the following two perspectives. From the pathology standpoint, centrilobular fibrosis, *i.e.* zone 3 fibrosis, is one of the typical pathological features of adult NASH[8]. Therefore, DR – a process related to fibrosis – would emerge in the centrilobular area by fibrosis location. Regarding the underlying pathophysiological mechanism, it has been postulated that CK7+ cells/structures in centrilobular DR might stem from hepatocytes through metaplastic response and/or dedifferentiation[55,60]. Hence, the concept of DR in NAFLD should be expanded to cover centrilobular DR[17]. In a cross-sectional analysis, it was found that centrilobular DR was highly correlated with the stage of fibrosis in adult non-alcoholic steatohepatitis[15]. In addition, centrilobular was the dominant injury pattern, presumably due to pressure induced by mechanical injury[53]. Besides, in NASH, the different underlying impact between centrilobular DR and periportal DR on disease development remains to be clarified.

DR microenvironment and HPC differentiation fate in NASH

The DR microenvironment, composed of parenchymal cells, mesenchymal cells, inflammatory cells, and their secreted substances, participates in the activation, proliferation, and differentiation of HPCs[12,61,62]. Different components drive HPC differentiation fate in different directions (Figure 1). Previous studies have indicated that HPCs reside in a specialized microenvironment (niche), which is crucial in determining their cell fate. Laminins, as part of the extracellular matrix (ECM), control the expansion of HPCs in an undifferentiated state, and hence DR, during liver injury. Other studies have demonstrated that HSCs and myofibroblasts might play an essential role in the differentiation of HPCs towards the cholangiocyte cell phenotype, while macrophages may participate in HPC differentiation into hepatocyte phenotypes[12,63]. A previous study showed that estimated glomerular filtration rate (EGFR) ligands were present in the liver microenvironment. In animal models lacking EGFR catalytic activity, the expansion of HPCs can be observed after DDC-induced liver damage, indicating that the lack of EGFR may promote HPC differentiation into hepatocytes, and thus liver regeneration[64]. However, it is noteworthy that the differentiation of HPCs is not modulated by a single factor but by a



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Figure 1 Factors contributing to the differentiation of hepatic progenitor cells in non-alcoholic fatty liver disease and potential pathways associated with hepatic progenitor cells-mediated non-alcoholic fatty liver disease progression.

complicated cellular and molecular network in liver diseases. HPCs tend to differentiate into biliary cell phenotypes in NASH, which may involve the participation of HSCs, myofibroblasts, macrophages, and natural killer T (NKT) cells[13-15,18,44]. At the molecular level, Notch and Hedgehog pathways may be the critical pathways in HPC differentiation into the biliary cell phenotype in NASH patients and mice [16,19,65] (Figure 1).

HSC and HPC differentiation fate in NASH

HSCs, located in the space of Disse, are the critical cells for liver fibrosis development and progression [66,67]. HSCs maintain a quiescent phenotype in normal liver but they can be activated by multiple factors in NAFLD, such as inflammatory cells, damaged hepatocytes, oxidative stress, *etc*[66]. Activated HSCs can acquire a myofibroblast phenotype and increase ECM production, contributing to NASH progression[67].

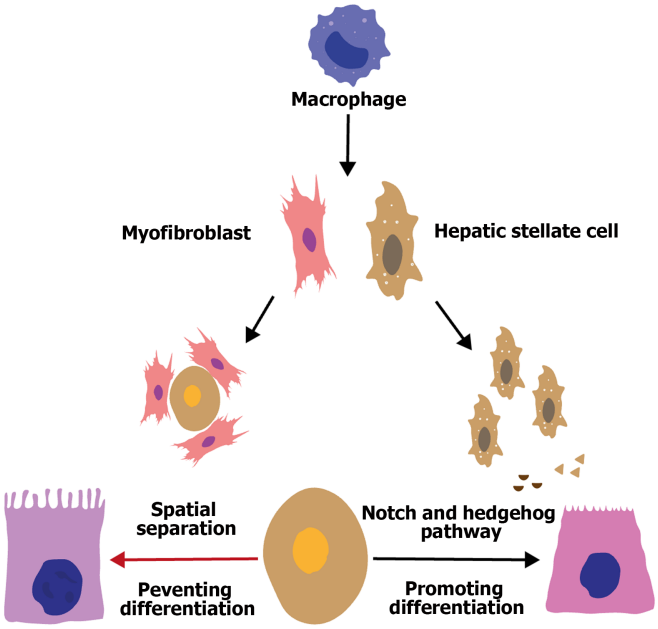
HSC fibrogenic activation promotes HPC differentiation into hepatocytes to restore mass and function[68]. A subfamily of the inhibitor of apoptosis protein family, survivin (also called baculoviral inhibitor of apoptosis repeat containing-5), has minimal expression in differentiated cells and is associated with cell division. Activated HSCs and HPCs can express survivin. Survivin protein is upregulated with increasing fibrogenic activation of HSCs from their quiescent state. Survivin protein can suppress the fibrotic response of HSCs. At this point, the regenerative capacity of hepatocytes is diminished, followed by replenishment with survivin-expressing HPCs, which differentiate into hepatocytes to promote liver regeneration[68].

HSCs also play an essential role in NAFLD-related DR, possibly by inducing HPCs to differentiate into CK7+ and/or CK19+ cells[12,17,69,70]. In NAFLD, the emergence of DR is accompanied by a significant increase in HSCs and ECM in the DR microenvironment, and the number of HSCs is associated with the DR stage and CK7+ HPC expansion[13]. A similar association between HSC and DR can also be found in other liver diseases, such as hepatitis C infection and primary biliary cirrhosis[13,16]. Further studies have partially explained the underlying mechanism of HSC-mediated HPC differentiation[25,69].

Primary studies have shown that HSC-mediated HPC differentiation may involve the Notch and Hedgehog pathways. In the DR microenvironment, activated HSCs can upregulate the Notch pathway in HPCs by expressing Jagged1 (a Notch pathway ligand)[60,63], leading to the expression of Notch pathway target genes such as hes-related family bHLH transcription factor with YRPW motif 1 and hairy and enhancer of split homolog-1[63,71,72]. Increased Notch target gene expression can further increase the expression of hepatic nuclear factor 1β (HNF1β) and HNF6, consequently contributing to HPC differentiation into biliary cells and BD formation[73-75]. Similarly, activated HSCs can upregulate the Hedgehog pathway in HPCs by expressing HL (a ligand of the Hedgehog pathway), leading to an increase in the Gli transcription factor family (Gli1, Gli2, and Gli3)[76]. Furthermore, Gli2 can translocate to the nucleus and promote target gene transcription[77,78], whose activation can promote the proliferation and differentiation of HPCs into CK7+ cells[79-83]. Elevated activity of Notch and Hedgehog pathways was analogous to disease severity in studies of both mouse models of NASH and patients with NASH[48,79,84], indicating the potential role of Notch and Hedgehog pathways in HSC-mediated HPC differentiation (Figure 2).

Macrophages and HPC differentiation fate in NASH

Emerging evidence suggests that macrophages are a heterogeneous population of cells. There are two types of macrophages: Resident macrophages, *i.e.* Kupffer cells, originating from yolk sac-derived erythroid, myeloid progenitors in the fetal liver; and infiltrating macrophages originating from bone marrow-derived circulating monocytes[7]. In NAFLD, macrophages can be activated and differentiated into two types of macrophages: M1 and M2 macrophages[7]. M1 macrophages secrete pro-inflammatory cytokines and have high phagocytic activity, whereas M2 macrophages secrete immune-suppressive but



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Figure 2 Hepatic progenitor cell-mediated hepatic progenitor cell differentiation may involve the Notch and Hedgehog pathways.

pro-fibrogenic cytokines[85,86].

Although it is universally acknowledged that macrophages play a critical role in NAFLD progression, the relationship between macrophages and HPC differentiation in NAFLD-related DR remains elusive. Macrophages were found to promote HPC differentiation into hepatocytes in the DDC diet mouse model, and the Wnt/β-catenin pathway was the key mechanism in this process[69,83,87]. After phagocytosis of the hepatocyte debris, macrophages increase the expression and secretion of Wnt3a (a ligand of the Wnt/β-catenin pathway), activating the Wnt/β-catenin pathway in HPCs[12,63]. Therefore, β-catenin can translocate to the nucleus and bind its co-activators (*e.g.*, CREB-binding protein), promoting the expression of target genes such as SOX9, MYC, and Twist-related protein 1, all of which are associated with HPC differentiation into hepatocytes[63,88]. Studies have shown that HPCs activate during chronic liver injury when hepatocyte proliferation is insufficient to reach homeostasis. During transforming growth factor (TGF)-induced apoptosis in a fibrogenic environment, HPC expands due to a balance between proliferation and apoptosis, which is favorable in a fibrogenic climate. Mitogens that trigger HPC expansion overlap significantly with pro-inflammatory cytokines released by hepatic macrophages including tumor necrosis factor, interferon gamma (IFN-γ), interleukin 6 (IL-6), and TWEAK. Human amnion epithelial cell-treated NASH mice showed a reduction in both HPC and macrophage numbers and expression levels of HPC mitogens and macrophage-released cytokines[89]. In NAFLD patients, macrophages increased significantly in the DR area, and macrophage infiltration was mainly related to the expansion of CK7+ HPCs and fibrosis stage, indicating the potential role of the macrophage in the HPC differentiation fate[13,46]. However, in the context of liver diseases, the role of macrophages in determining HPC differentiation fate is still unclear. Deduced from the aforementioned basic studies, the increased macrophage infiltration in the DR area of NAFLD patients may promote the differentiation of HPCs into hepatocytes. Nonetheless, according to pathological findings, the actual characteristic of NAFLD-related DR is HPC differentiation into cholangiocytes. Therefore, this seemingly contradictory phenomenon might be explained from the following two perspectives.

The regulation of macrophage-mediated HPC differentiation fate may vary across different disease contexts, which is one potential explanation. Disease pathogenesis in the DDC diet mouse model is highly distinct from NAFLD pathogenesis. Therefore, the functional state of macrophages in NAFLD might be correspondingly specific to that in the DDC diet mouse model. Second, the crosstalk between macrophages and HSCs in NAFLD may predominantly contribute to the differentiation of HPCs into cholangiocytes. It has been well established in NAFLD that macrophages can express multiple pro-fibrotic factors (such as platelet-derived growth factors subunit B and TGF-β), contributing to the proliferation and activation of HSCs and myofibroblasts[7,66,90-92]. Notably, macrophages were near HSCs in the DR area in NAFLD patients, indicating a potential promotive effect of macrophages in driving HPC differentiation into cholangiocytes by activating HSCs[13,46].

Conversely, HSCs might hinder macrophage-mediated HPC differentiation into hepatocytes by interrupting the interaction between macrophages and HPCs in spatial separation. In a biliary regeneration model, HPCs were surrounded by a thick sheath-like layer of myofibroblasts and collagen I, which excluded macrophages from forming a close association with HPCs[63]. Similar sheath-like

structures might also exist in NAFLD; however, further studies in NAFLD patients are needed to validate the potential existence of this structure in the DR area. In summary, macrophages may participate in NAFLD-related DR onset and development through crosstalk with cells such as HPCs and HSCs. However, its specific role and related mechanisms warrant further investigation (Figure 3).

Mast cells and HPC differentiation fate in NASH

According to recent studies, NAFLD/NASH development is primarily influenced by the interaction between DR and mast cells (MCs)[93,94]. MCs may promote NAFLD/NASH progression by activating Kupffer cells and HSCs with histamine[94]. Recruitment of MCs is a characteristic of BD injury. It has been proven that knocking down or inhibiting the expression of MCs can effectively reduce DR[95,96]. MC-derived TGF-β1 is a critical regulator of hepatobiliary damage, and blockage of TGF-β1 can ameliorate DR and other features of cholestatic liver injury[97]. MCs were found to promote microvesicular steatosis development *via* the miR-144-3p/aldehyde dehydrogenase 1 family, member A3 (ALDH1A3) signaling pathway in a Western diet mouse model with NASH[98]. Reduced ALDH1A3 expression promotes lipid peroxidation associated with liver fibrosis and steatosis and a reduction in β-oxidation of free fatty acids[99].

Moreover, miR-144-3p showed increased expression in insulin resistance in NASH. Meanwhile, DR expansion in mouse models of Western diet with NASH is more sensitive. The phenotypic changes are associated with the secretion of insulin-like growth factor 1 by cholangiocytes, driving peribiliary infiltration and MC activation. Consistent with this finding, MCs from NASH patients accumulate in the portal area, directly correlating with fibrosis stage[93]. A more relevant study discovered that inhibiting MCs reduced DR, inflammation, fibrosis, and recovery from liver injury after MC injection[94].

Previous studies have demonstrated that elevated farnesoid X receptor (FXR) expressed by MCs can be detected in primary sclerosing cholangitis, primary biliary cholangitis, and NAFLD[100-102]. MC-FXR plays a critical role in liver injury and DR in a cholestasis model, where MCs express FXR and infiltrate the liver promoting liver fibrosis during cholestasis and triggering biliary injury. After migration and activation, MCs induce DR and senescence through paracrine interactions with cholangiocytes. Moreover, the MC-FXR signaling pathway modulates the biliary senescence/senescence-associated secretory phenotype and histamine H1- and H2-receptor signaling pathways to regulate total bile acid and then affects DR and liver injury[103]. According to these studies, MCs are corrected with DR in various liver diseases and may affect the differentiation of HPCs through macrophages, HSCs, and fibroblasts. However, the mechanism by which MCs influence HPC differentiation remains obscure.

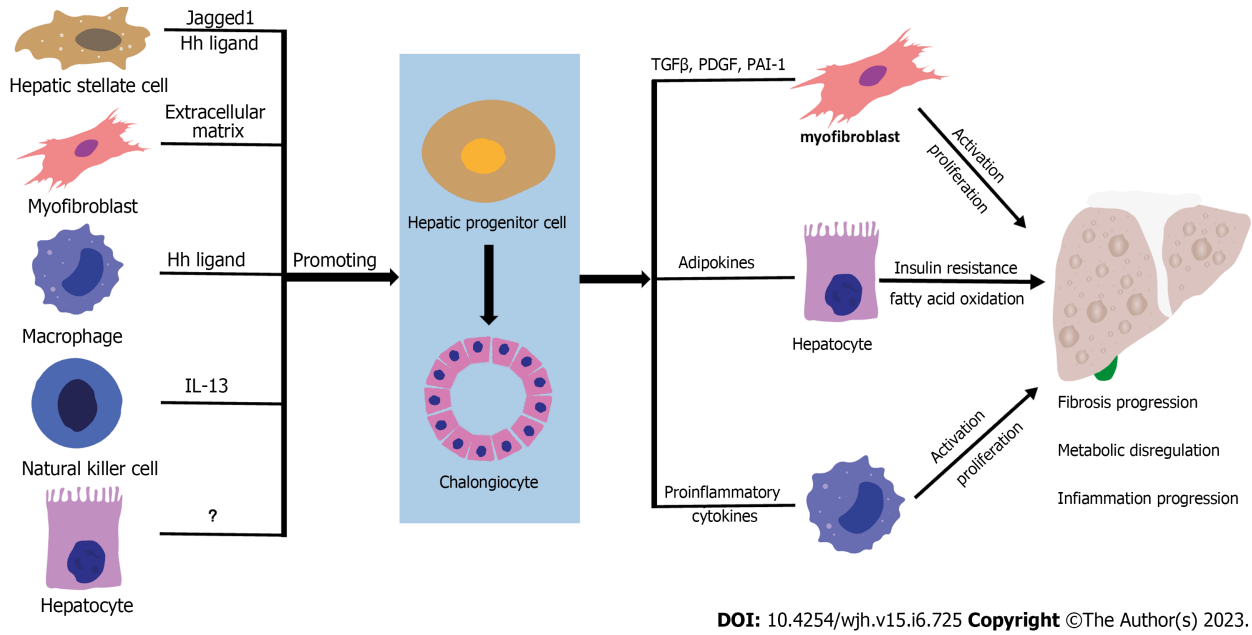
ECM and HPC differentiation fate in NASH

ECM – a supporting structure for organs, tissues, and cells-represents a complex protein network including fibrillar and non-fibrillar collagen, laminin, fibronectin, *etc*[104]. ECM proteins can play a vital role in HPC differentiation fate. For example, loss of the basement membrane, a cell-supporting structure, is correlated with the increased level of HNF4 in HPCs, indicating the differentiation of HPCs into hepatocytes[105]. In addition, laminin can upregulate the expression of the biliary marker gene and downregulate hepatocyte transcription factor C/EBPa in HPCs, driving HPC differentiation into cholangiocytes[106]. A recent study based on mouse models of chronic parenchymal damage showed that iloprost reduces laminin deposition and enhances the differentiation of HPCs into hepatocytes [107]. The disruption of integrin β6, an adhesion receptor that interacts with fibronectin and TGF-β1, inhibits the response of HPCs to tissue damage. Significant ECM deposition, such as collagen deposition, is commonly found in NAFLD-related fibrosis[67,108]. Therefore, the accumulation of ECM during the development of NAFLD may contribute to HPC differentiation and the formation of DR.

Hepatocyte senescence and HPC differentiation fate in NASH

Cellular senescence, a cell cycle arrest response, is mediated by the induction of cyclin-dependent kinase inhibitors p21 and p16[109,110]. In NAFLD, hepatocyte senescence involves multiple factors, such as oxidative stress and inflammation, and is characterized by increased p21 levels[111,112]. Interestingly, hepatocyte senescence, *i.e*. replicative arrest, may activate HPC proliferation and differentiation. Oxidative stress induces hepatocyte senescence with consequent cell cycle arrest and impaired regeneration[113]. A recent study demonstrated that oxidative stress can affect HPC differentiation, and the redox is regulated by various transcription factors, of which nuclear factor (erythroid-derived 2)-like 2 (NRF2) plays a crucial role in HPC differentiation, and its activation can inhibit oxidative stress. As stemness is maintained in HPCs through constitutive NRF2 activation, it is inhibited when HPCs are activated during liver injury, *e.g.*, NASH.

Interestingly, NRF2 inhibition increases the transplantation efficiency of human HPCs[114]. In an MDM2-deleted mouse model, server hepatocyte senescence was characterized by a high p21 level and resulted in significant HPC proliferation and differentiation into hepatocytes[33]. However, in NAFLD patients and the choline-deficient and ethionine-supplemented (CDE) diet mouse model, mild hepatocyte senescence was also identified by a lower p21 level and was positively correlated with DR stage and CK7+ HPC expansion, conversely indicating a potential role of hepatocyte senescence in HPC differentiation into cholangiocytes[14,33]. To reconcile these apparently conflicting findings, some



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Figure 3 Potential role of macrophages in hepatic progenitor cell differentiation fate in non-alcoholic fatty liver disease.

experts have suggested that the absence of hepatocyte senescence may enable hepatocytes to undergo self-regeneration without relying on HPC-mediated regeneration[33]. In addition, hepatocytes are the primary source of liver regeneration in a healthy liver, while HPCs do not participate in normal liver regeneration. Therefore, it might be further speculated that aging and healthy hepatocytes may regulate HPC differentiation. Nevertheless, the mechanism by which aging hepatocytes and/or healthy hepatocytes regulate HPC differentiation fate is yet to be elucidated.

NKT cells and HPC differentiation fate in NASH

NKT cells – a type of innate immune cell in the liver – can participate in the development of liver inflammation and fibrosis[115]. In NAFLD, NKT cells significantly increase in the DR area, and their infiltration extent correlates with both NASH severity and DR stage[80,116]. Conversely, liver biopsies of HBV patients often reveal a pronounced DR and diminished expression of IFN-γ, which is caused by NKT cells. Nevertheless, treatment with IFN-γ has been shown to ameliorate DR in these patients[117]. However, the role of NKT cells in HPC differentiation fate is unclear in NAFLD-related DR. There is evidence suggesting a promotive role of NKT cells in HPC differentiation into cholangiocytes in liver injury models. In these studies, NKT cells increased the expression of IL-13 and the production of Hedgehog ligands, which may drive HPC differentiation into cholangiocytes[80,118-121]. Nevertheless, it is unclear whether NKT cells are required for HPC differentiation into biliary cells in NASH.

Potential role of HPC differentiation in aggravating NASH

In addition to the impact of the NASH-related DR microenvironment on HPC differentiation fate, differentiated HPCs can aggravate inflammation and fibrosis progression in NASH. As aforementioned, there is a close correlation between HPC expansion and NASH progression, indicating the potential role of differentiated HPCs in aggravating NASH. Moreover, the promotive role of differentiated HPCs in NASH inflammation and fibrosis progression has been proven in NASH-related animal models. Although the underlying mechanism has yet to be fully understood, it may involve the participation of HSCs, macrophages, adipokines, and the epithelial-mesenchymal transition (EMT) (Figure 1).

Differentiated HPCs may participate in HSC-mediated NASH-related fibrosis by promoting HSC activation and proliferation. Increased hepatic levels of several factors, such as PDGF, connective tissue growth factor (CTGF), and Hedgehog ligands, have been found in NAFLD animal models[60,122,123]. In basic studies, HPCs are one of the sources of PDGF, CTGF, and Hedgehog ligands[81,122]. The promotive role of these molecules in enhancing HSC proliferation, accumulation, and ECM production has been well established[81,124-126]. Therefore, these pathways may be involved in HPC-mediated HSCs activation in NASH aggravation.

In addition to directly promoting HSC and myofibroblast activation, HPCs may undergo the EMT towards myofibroblasts, consequently leading to hepatic fibrosis progression. EMT is a cell reprogramming process from the epithelial to mesenchymal phenotype[76,77,127]. EMT in hepatocytes, cholangiocytes, and HSCs can be found in various liver diseases and is related to hepatic fibrosis[76,128,129]. A proportion of HPCs can go through the EMT, which is characterized by the upregulation of mesenchymal cell markers [such as alpha-smooth muscle actin (α-SMA) and S100 calcium-binding

protein A4) and downregulation of epithelial cell markers (such as CK7 and CK19)[130-133]. Differentiated HPCs (CK7+] that highly express α-SMA can be found in NAFLD, indicating the presence of HPC-originated EMT and its potential contribution to fibrosis pathogenesis[79]. The onset of EMT in HPCs may involve the Hedgehog pathway activity and TGF-β[79]. Notably, whether high expression of a-SMA or collagen in HPCs can be regarded as the EMT remains controversial. This is because a recent lineage tracing study, using an α-fetoprotein Cre mouse model, provided strong evidence against the existence of HPC-myofibroblast transition[134]. Therefore, further basic studies regarding the origination of α-SMA and CK7 double-positive cells are warranted.

Differentiated HPCs can promote macrophage-mediated inflammation in NASH. Studies have shown that macrophages play an essential role in NASH aggravation[7]. As previously mentioned, significant macrophage infiltration was detected in the NAFLD-related DR area. The number of macrophages is significantly associated with the extent of DR and HPC expansion, indicating that HPCs have a potential role in macrophage recruitment[13]. Primary studies have proven that multiple factors, such as chemokines and pro-inflammatory cytokines, are involved in HPC-mediated macrophage recruitment [7,135-137]. For example, HPCs can contribute to macrophage recruitment by increasing C-C motif chemokine ligand 2 and C-X3-C motif chemokine ligand 1 expression and promote macrophage polarization into M1-type by secreting IL-1, IL-6, and IFN-γ, consequently exacerbating hepatic inflammation[7,135-137]. Therefore, these cytokines may participate in HPC-mediated macrophage infiltration and activation in NASH.

Metabolic dysregulation is a major hallmark in the pathophysiological process of NAFLD, and differentiated HPCs exacerbate by causing dysregulation of the secretion of adipokines, leading to an increase in NASH progression. Adipokines, including adiponectin, leptin, and resistin, contribute to NAFLD development by modulating glycolipid metabolism, inflammatory response, and HSC activation[138]. Although adipokines are mainly produced by adipose tissues, they have also been found to secrete adiponectin and resistin[45,139]. Notably, in NASH, differentiated HPCs increase resistin expression and downregulate adiponectin expression. Moreover, resistin expression in HPCs is positively correlated with the severity of NAFLD.

By contrast, adiponectin expression in HPCs was found to be negatively correlated with the severity of NAFLD, indicating that adipokines play a role in HPC-mediated NASH progression[45]. Adiponectin can suppress hepatic lipogenesis and the production of proinflammatory cytokines but can stimulate insulin secretion and fatty acid oxidation in the liver[140,141]. By contrast, resistin reduces peripheral insulin sensitivity and promotes the expression of proinflammatory cytokines[138,142]. In NASH, adipokine dysregulation aggravates insulin resistance, worsening liver inflammation and injury, which also increases HSC activation, thereby aggravating NASH[45,143-145]. Therefore, the NAFLD-related microenvironment can cause the dysregulation of adipokine expression in HPCs, leading to NAFLD-related metabolic dysregulation.

CONCLUSION

Studies conducted in the past 100 years have shown that DR may be a compensatory reaction to liver injury, but the correlation between DR and NAFLD needs to be sufficiently studied. The expected prevalence of DR in NAFLD patients, and more importantly, the close relationship between DR and the progression of inflammation and fibrosis in NASH, remain to be clarified. Although DR promotes liver regeneration[54,146], it remodels the NASH microenvironment, which aggravates rather than alleviates NASH severity, similar to the initially upright "Macbeth" getting perverted under a corruptive lure. In NAFLD, HPC proliferation and differentiation, the core processes in DR pathogenesis, might be triggered by NAFLD-related liver injury. The cells (such as HSCs and macrophages) and their secreted substances may drive the differentiation of HPCs into cholangiocytes. Conversely, differentiated HPCs may, in turn, aggravate NASH through multiple pathways, which may involve the participation of HSCs, macrophages, adipokines, and the EMT. The involvement of these cells in the interaction between DR and NASH pathogenesis may form a 'vicious circle,' presumably leading to further progression of hepatic inflammation and fibrosis.

However, the bilateral interaction between DR and NAFLD remains to be further verified. For the DR caused by NAFLD, the majority of previous findings about NAFLD-related DR were primarily obtained through observational studies. Several signaling pathways are involved in DR (*e.g.*, Notch, Hedgehog, TWEAK), and it was recently discovered that long non-coding RNA/p300 could influence DR progression[147]. However, how these pathways promote the pathogenesis of DR in the context of NAFLD remains unclear. We are still determining whether the pathways mentioned above are involved in DR-related NAFLD. The key factors driving HPC differentiation in NAFLD need to be further investigated. In addition, in terms of the impact of DR on the pathogenesis of NAFLD, considering our limited understanding of the core molecular mechanism driving DR, it is difficult to provide a direct and exact intervention towards the DR onset, which hinders establishment of a causal effect of DR on NAFLD progression. Therefore, we need further investigations to deepen our understanding of the core and characteristic pathways of DR, to achieve the development of DR-targeted intervention in NAFLD-

related studies. More importantly, the underlying mechanisms of both NAFLD-caused DR and HPC-mediated NAFLD progression may be important targets for treating NAFLD.

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FOOTNOTES

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REVIEW

Recent advances in pathophysiology, diagnosis and management of hepatorenal syndrome: A review

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Abstract

Hepatorenal syndrome with acute kidney injury (HRS-AKI) is a form of rapidly progressive kidney dysfunction in patients with decompensated cirrhosis and/or acute severe liver injury such as acute liver failure. Current data suggest that HRS-AKI occurs secondary to circulatory dysfunction characterized by marked splanchnic vasodilation, leading to reduction of effective arterial blood volume and glomerular filtration rate. Thus, volume expansion and splanchnic vasoconstriction constitute the mainstay of medical therapy. However, a significant proportion of patients do not respond to medical management. These patients often require renal replacement therapy and may be eligible for liver or combined liver-kidney transplantation. Although there have been advances in the management of patients with HRS-AKI including novel biomarkers and medications, better-calibrated studies, more widely available biomarkers, and improved prognostic models are sorely needed to further improve diagnosis and treatment of HRS-AKI.

Key Words: Hepatorenal syndrome; Pathophysiology; Diagnosis; Management; Review

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Core Tip: Hepatorenal syndrome (HRS) is a specific form of acute kidney injury that occurs in the presence of severe acute liver injury (*e.g.*, acute liver failure or severe alcoholic hepatitis), decompensated cirrhosis, or acute on chronic liver failure and is particularly associated with poor prognosis. Here, we reviewed some of the recent advancements in the diagnosis and treatment of HRS.

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INTRODUCTION

Acute kidney injury (AKI) is a common complication in patients with cirrhosis and has been reported in 20%-50% of the hospitalized patients with cirrhosis[1,2]. Within the spectrum of AKI in cirrhosis, hepatorenal syndrome (HRS) with AKI (HRS-AKI) has by far the worst prognosis[3]. HRS-AKI is a rapidly progressive type of AKI with a median survival of few weeks[3,4]. Although most commonly seen in the setting of cirrhosis, HRS-AKI can occur in patients with acute liver injury such as acute liver failure or severe alcoholic hepatitis[3,5]. Observations of acute progressive kidney injury without significant preexisting kidney dysfunction, minimal histological abnormalities, and reversible angiographic changes in renal vasculature led to the hypothesis that HRS-AKI is a functional and potentially reversible phenomenon caused by hemodynamic instability, splanchnic vasodilation and renal vasoconstriction[6-10]. However, more recent data have shown the pathophysiology is more complex[10-14].

In this review, we examine the current understanding of the mechanisms of kidney injury in patients with cirrhosis, discuss the contemporary classification of AKI in patients with cirrhosis, and review the recent advances in diagnosis and management of HRS-AKI.

PATHOPHYSIOLOGY

HRS-AKI is primarily due to an unbalanced but potentially reversible cirrhosis-induced circulatory dysfunction without structural kidney damage[11-14]. This understanding of HRS pathophysiology is supported by clinical findings including the return to normal kidney function occurring commonly after liver transplantation, successful kidney transplant using donors with HRS, and normal postmortem histology and angiography[9,10,15,16]. Direct experimental evaluation of the pathophysiology of HRS remains lacking due to lack of a fitting animal model. The primary mechanism of renal injury, splanchnic hypoperfusion, triggers a physiologic response including sodium retention and renal vasoconstriction. These mechanisms generally cannot compensate to maintain perfusion and as the disease progresses, eventually contribute to circulatory dysfunction and thus worsen renal function. The compensatory mechanisms, maladaptive responses, and their role in disease progression will be detailed in the following sections.

Cirrhosis-induced circulatory dysfunction

Cirrhosis causes elevated intrahepatic vascular resistance and a paradoxical splanchnic vasodilatation due to increased production of mediators such as nitric oxide and prostacyclins[17-20]. Compensatory hyperdynamic circulation initially preserves the effective intravascular volume in early stages of cirrhosis. As the cirrhosis progresses or during times of acute stress, the hyperdynamic cardiac circulation cannot compensate for the splanchnic vasodilation resulting in activation of other compensatory mechanisms. The renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system (SNS), and at later stages, the non-osmotic secretion of arginine vasopressin are subsequently activated to maintain effective intravascular volume[21-24]. These responses cause vasoconstriction as well as water and sodium retention in an attempt to counteract vasodilation and maintain adequate intravascular volume. However, with further disease progression and in the absence of effective treatment, these compensatory mechanisms not only will fail to adequately counterbalance the vasodilation but begin to contribute to renal dysfunction. Renal vasoconstriction begins to impair renal blood flow and therefore worsens renal function. Volume retention contributes to worsening portal hypertension, which in turn worsens the underlying physiology which initiates and perpetuates HRS.

An interesting and specific finding in HRS-AKI is the sequence and distribution of microvascular changes in the kidney itself. In the early stages of cirrhosis and in the presence of mild portal hypertension, as renal blood flow decreases, resistive indices (RIs) measured by Doppler ultrasound show a gradual increase starting from the main renal artery (hilum) toward the cortical arteries, sparing the outer cortex parenchyma[25-27]. By contrast, in later stages and in the presence of severe portal hypertension, the RI gap between hilum and cortex disappears and cortical ischemia occurs[26,27]. Therefore, cortical ischemia is considered to be a hallmark feature of HRS-AKI. Another potential contributing factor to HRS-AKI in cirrhosis is abnormal renal vascular response to stimuli and altered autoregulation of kidney blood flow. Despite decreased renal blood flow, the vasoconstrictor effect of angiotensin II on the efferent arterioles and vasodilator effect of nitric oxide on the afferent arterioles

preserve adequate pressure in the glomeruli to keep glomerular filtration rate (GFR) within normal limits[26]. However, as cirrhosis advances, GFR starts to decline presumably due to the disruption of nitric oxide production and progressive cortical ischemia caused by the very same compensatory mechanisms explained above. Animal models have also shown a blunted vasodilatory response to bradykinin and an augmented vasoconstrictive response to noradrenaline[28,29]. If the decreased renal blood flow is not reversed quickly, then the persistent vasoconstriction and ischemia could lead to acute tubular necrosis (ATN), which may not improve even after the adequate renal blood flow has been restored. The potential for permeant dysfunction as a result of an acute insult from HRS-AKI illustrates the importance of early diagnosis and treatment.

Cirrhotic cardiomyopathy

Cirrhosis-induced cardiac dysfunction or cirrhotic cardiomyopathy is cardiac dysfunction and abnormal response to stimuli in patients with advanced cirrhosis in the absence of structural cardiac disease. This phenomenon is observed in up to 50% of cirrhotic patients with varying degree of severity[30-32]. As mentioned earlier, the compensatory response to the splanchnic vasodilation includes both increase in cardiac output and activation of RAAS and SNS. However, chronic activation of RAAS and SNS may result in impaired cardiac response to stress, diastolic dysfunction, electrophysiological abnormalities (*e.g.,* prolonged QT interval), and eventually decreased cardiac output[30-33]. Although cardiac compensatory mechanisms may be able to maintain adequate profusion under normal circumstances, they can collapse under physiologic and pathologic stressors such as infections (particularly spontaneous bacterial peritonitis), bleeding, or inappropriate use of medications such as β-blockers, diuretics and angiotensin-converting enzyme inhibitors[31,33,34]. Because increased cardiac output is an essential compensatory mechanism to maintain renal profusion in the setting of vasodilation, reduced cardiac output can have significant negative effects on renal profusion and has been associated with development of HRS and poor outcomes in patients with HRS[33].

Inflammation

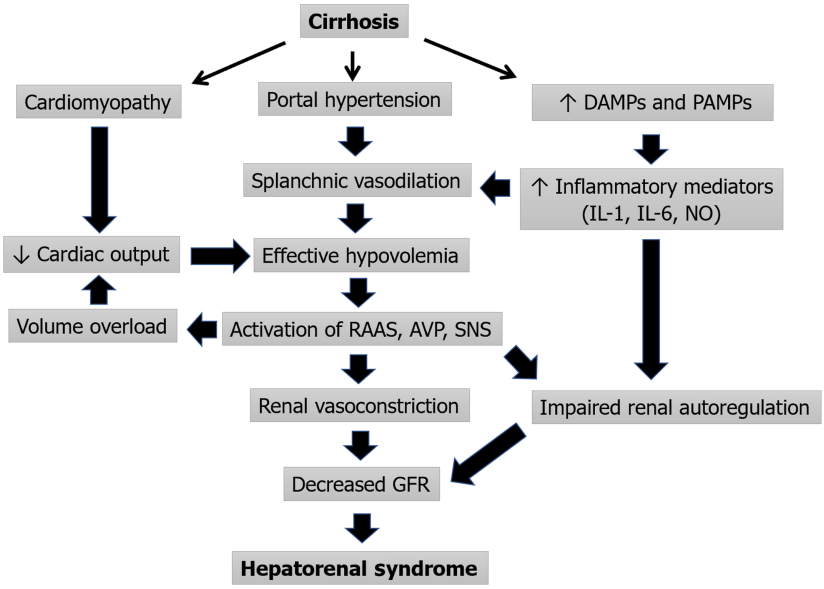
In recent years, the notion that decompensated cirrhosis is a constant inflammatory state has emerged and a growing body of evidence shows pro-inflammatory cytokines and chemokines such as interleukin 6 (IL-6), IL-8 and tumor necrosis factor alpha (TNF-α) may play a central role in the organ dysfunction in patients with cirrhosis[35-38]. The levels of pro-inflammatory cytokines increase with disease progression as a response to sterile (non-infectious) inflammation or infectious inflammation[37,39]. Sterile inflammation typically manifests with systemic inflammatory response syndrome and is mainly driven by danger-associated molecular patterns such as high-mobility group protein B1. In patients with cirrhosis infectious, inflammation is mainly driven by pathogen-associated molecular patterns from gut-derived bacterial translocation but can also be associated with other sources of infection[38-40]. The inflammatory response may have prognostic value in predicting progression of AKI and mortality[40, 41]. Furthermore, levels of certain inflammatory mediators in the serum (IL-6, TNF-α, vascular adhesion protein-1) and urine (monocyte chemoattractant protein-1, neutrophil gelatinase–associated lipocalin) may help to differentiate AKI-HRS from other causes of AKI such as prerenal azotemia. In addition to changes in the systemic inflammatory environment, patients with decompensated cirrhosis have increased expression of inflammatory receptors such as toll-like receptor 4 (TLR-4) in the kidneys[38, 42]. These subtle structural changes can lead to exaggerated tubulointerstitial, glomerular, and vascular injuries in response to relatively minor hemodynamic changes or occult infections[43,44].

Other factors

In addition to circulatory dysfunction and inflammation, there is evidence that other factors contribute to the development of HRS. Bile cast nephropathy or cholemic nephropathy in decompensated cirrhosis may contribute to renal dysfunction as patients with higher bilirubin have lower response to therapy in HRS[45,46]. However, the causative relationship between bile cast nephropathy and renal dysfunction in HRS has not been clearly established. Relative adrenal insufficiency in cirrhosis, formerly called hepatoadrenal syndrome, is a relatively common phenomenon occurring in 24%-47% of patients with decompensated cirrhosis. The lack of normal adrenal function impairs the compensatory response to hypoperfusion and increases the risk of AKI-HRS[47]. Although glucocorticoid supplementation may improve outcomes in patients with relative adrenal insufficiency and septic shock, the effect of supplementation on AKI-HRS outcomes has not been evaluated rigorously. Figure 1 summarizes the pathophysiology of HRS-AKI

DIAGNOSIS

In recent years, the definition and diagnostic criteria of AKI-HRS have been adjusted and novel diagnostic biomarkers have been proposed[48-50]. Two forms of HRS are currently recognized: HRS-AKI (formerly known as HRS type 1) and HRS-non-AKI (HRS-NAKI) (formerly known as HRS type 2). HRS-NAKI is further divided into two subtypes: HRS - acute kidney disease (HRS-AKD) defined as



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Figure 1 Pathophysiology of hepatorenal syndrome. AVP: Arginine vasopressin; DAMPs: Danger-associated molecular patterns; GFR: Glomerular filtration rate; PAMPs: Pathogen-associated molecular patterns; RAAS: Renin angiotensin aldosterone system; SNS: Sympathetic nervous system.

estimated GFR (eGFR) < 60 mL/min per 1.73 m^2 for less than 3 months and HRS-chronic kidney disease (HRS-CKD) defined as eGFR < 60 mL/min per 1.73 m^2 for more than 3 months. Currently, the American Association for the Study of Liver Diseases (AASLD) defines AKI-HRS according to the International Club of Ascites (ICA) criteria as an increase in serum creatinine (SCr) ≥ 0.3 mg/dL within 48 h or increase ≥ 1.5 times from baseline SCr that is known or presumed to have occurred within the preceding 7 d. The other diagnostic criteria for HRS-AKI have remained largely unchanged from HRS type 1 including cirrhosis with ascites, no response after 2 consecutive days of diuretic withdrawal and plasma volume expansion with albumin infusion (1 g/kg body weight per day), absence of shock, no current or recent use of nephrotoxic drugs (non-steroidal anti-inflammatory drugs, aminoglycosides, or iodinated contrast media), and no signs of structural kidney injury. Evidence for structural kidney disease includes proteinuria (> 500 mg per day), microhematuria (> 50 red blood cells per high-power field), and/or abnormal renal ultrasonography. The updated criteria have adopted lower thresholds of creatinine increase and no absolute minimum creatinine necessary for diagnosis primarily to facilitate earlier identification of patients at risk for poor outcomes. Despite improvement in the HRS-AKI criteria, they do not differ for patients with underlying CKD, which can make diagnosis more challenging for these patients.

Novel diagnostic biomarkers

Despite improved understanding of the pathogenesis and diagnostic criteria of HRS-AKI, it remains a diagnosis of exclusion and requires a period of observation after diuretic/nephrotoxic medication withdrawal. Establishing the diagnosis can be difficult due to the similar presentations of other causes of AKI such as prerenal AKI and ATN. In addition, AKI-HRS can result in ATN as the disease progresses, which further complicates distinguishing the two entities[51]. Although the ICA criteria and its proposed treatment algorithm try to address this issue, accurate differentiation may not be feasible in a timely fashion especially when patients' condition is rapidly changing. Thus, there is an unmet need for biomarkers to quickly and accurately differentiate HRS-AKI from other causes of AKI and stratify risk. Accurately predicting renal function in patients with cirrhosis is also essential as this would allow for earlier identification of patients with renal dysfunction. However, accurately estimating GFR is challenging as standard SCr and Cr-based equations are unreliable in patients with cirrhosis because sarcopenia, impaired production of Cr (the precursor of SCr), and increased Cr filtration are common and result in the underestimation of renal dysfunction[52,53]. A new model, the royal free hospital cirrhosis GFR, which includes sodium, presence of ascites, blood urea nitrogen, and international normalized ratio in the equation, has been suggested to be more accurate for estimating renal function in this population. However external validation in large cohorts has not been completed[54]. There are several novel biomarkers under investigation to improve diagnosis and prognostication in AKI-HRS including plasma cystatin C, urinary neutrophil gelatinase-associated lipocalin (uNGAL), interleukin-18 (IL-8), kidney injury molecule-1, and liver-type fatty acid-binding protein and albumin[55-60]. Among these biomarkers, uNGAL, IL-18 and cystatin C appear to be the most promising biomarkers. IL-18 and uNGAL can differentiate ATN from other types of AKI and predict mortality in cirrhosis[59,60]. Specifically, uNGAL is identified in the most recent 2021 AASLD guidelines as the most promising

biomarker in distinguishing HRS-AKI from ATN and suggests measuring it on day 3 from onset of renal dysfunction for greatest accuracy[49]. Plasma cystatin C (the most commonly used marker besides SCr) predicts HRS and mortality in patients with cirrhosis[59,60]. An additional advantage to cystatin C is that it has become more widely performed, can yield results relatively quickly, and can predict GFR more accurately in patients with sarcopenia[59]. Using biomarkers including uNGAL, IL-18, liver fatty acid-binding protein (L-FABP), and albumin in a combination panel may improve their ability to differentiate between HRS and ATN as well as predict AKI progression and death[55]. Specifically, the biomarker combination of cystatin C and uNGAL and predictive models MELD-cystatin C and MELD-NGAL have shown the potential for improving diagnosis and risk stratification, making them attractive topics for future research[58,60]. MicroRNAs (*e.g.*, microRNA-122) and metabolomics signature associated with hepatorenal dysfunction (4-acetamidobutanoate, trans-aconitate, 1-methylhistidine, glucuronate, N4-acetylcytidine, 3-ureidopropionate, 3-methoxytyramine sulfate, cytidine, S-adenosylhomocysteine, and myo-inositol) have shown promising results predicting mortality and kidney dysfunction in small studies but need validation in large prospective cohorts[61,62]. Tables 1 and 2 summarize commonly used methods of estimating GFR and the novel biomarkers and equations for diagnosis of AKI in cirrhosis. Although these novel markers are promising, they are not all readily available, often do not have standard cut-off values, have values that do not correlate with specific stages of AKI, and have cut-off values that vary by type of AKI. Therefore, standardization and validation are needed in prospective studies. In addition, given the importance of early diagnosis and intervention, biomarkers need to have a rapid turn-around to be clinically useful, which is often not the case in many, especially smaller, institutions. However, they have the potential to allow for earlier more specific diagnosis, which facilitates more aggressive intervention, and when appropriate, evaluation for liver transplant.

TREATMENT

Despite the growth of knowledge in pathogenesis and shifts in definition and prognostication, HRS-AKI is still associated with high morbidity and mortality. The mainstay of therapy includes volume expansion and vasoconstrictors. However, there have been changes in the availability and data supporting the use of terlipressin recently. If medical management fails, renal replacement therapy and eventually organ transplant should be considered.

Medical management

In patients with cirrhosis and AKI, when precipitating factors are excluded first patients are generally treated with diuretic withdrawal and a 48-hour volume expansion with albumin (1 g/Kg, 100 g maximum). Historically subsequent treatment has been variable depending on the availability of terlipressin. In places where terlipressin is not available, a combination of midodrine (α_1-receptor agonist), octreotide (splanchnic vasoconstrictor), and albumin are typically used outside the intensive care unit (ICU) and low-dose noradrenaline with albumin are used in the ICU. Terlipressin (vasopressin agonist), which can be administered peripherally, has been used for many years outside the United States and has demonstrated a higher response rate than albumin alone or midodrine, octreotide, and albumin combination regimen and comparable to noradrenaline plus albumin[63-73]. When co-administered with albumin, terlipressin has better outcomes than terlipressin alone, which may be due to some of the anti-inflammatory and immunomodulatory properties of albumin besides oncotic volume expansion[74,75]. Terlipressin has been studied extensively in prospective studies, randomized trials, and meta-analyses and its efficacy in reversal of HRS-AKI (Tables 1 and 2) and as a result has been approved for the treatment of HRS outside the United States for several years[76-80]. However, despite mounting evidence for the benefit of terlipressin, including initial data from the United States-based CONFRIM trial, the United States Food and Drug Administration (FDA) rejected terlipressin due to safety concerns as recently as 2020. This was controversial at the time not only because of terlipressin's wide approval outside the United States but also because the FDA subcommittee on Cardiovascular and Renal Drugs Advisory Committee voted 8-7 in favor of approval. After post-hoc analyses of the CONFIRM trial with proposed changes to mitigate the risk of safety events, terlipressin was ultimately approved in the United States for the treatment of HRS-AKI in 2022, although with several warnings. The CONFIRM trial showed terlipressin was more effective than placebo in reversal of HRS (32% *vs* 17%), although there was no statistical difference in death at 90 d. However, the trial also highlighted safety considerations when using terlipressin. Respiratory failure (14% *vs* 5%) and death within 90 d due to respiratory disorders (11% *vs* 2%) was more common in the terlipressin group compared to in the placebo group. As a result, terlipressin is contraindicated in patients with ongoing ischemia (coronary, peripheral, or mesenteric) and hypoxia or worsening respiratory symptoms. For all patients, continuous pulse oximetry is recommended to monitor the development of respiratory failure. The FDA also recommends paying close attention to volume status as this may predispose patients to respiratory failure and consider discontinuing terlipressin in patients who develop fluid overload. Liver transplantation was performed in 29% of the placebo group compared to 23% in the terlipressin group.

Table 1 Methods of estimating glomerular filtration rate and the novel equations for diagnosis of acute kidney injury in cirrhosis

Ref.	Equation		Variables	Advantage
	Cr-based			
Cockcroft *et al*[93]	Cockcroft-Gault	1976	Age, SCr, sex, weight	
Levey *et al*[94]	MDRD-4	2006	Age, SCr, sex, ethnicity	
Levey *et al*[95]	MDRD-6	2007	Age, SCr, sex, ethnicity, BUN, albumin	
Levey *et al*[96]	CKD-Epi	2009	Age, SCr, sex, ethnicity	
Kalafateli *et al*[54]	The royal free hospital	2017	Age, SCr, sex, ascites, BUN, Na, INR	
	Cystatin C-based		Age, sex, cystatin C	Equations including cystatin C are more accurate in patients with sarcopenia and advanced liver disease
Hoek *et al*[97]		2003		
Larsson *et al*[98]	CKD Epi-Cystatin C	2004		
Inker *et al*[99]		2012		
	Cr-Cystatin C-based		Age, sex, cystatin C, SCr, ethnicity	
Stevens *et al*[100]	CKD EPI -Cr Cystatin C	2008		
Inker *et al*[99]		2012		
Mindikoglu *et al*[101]		2016		

ACLF: Acute on chronic liver failure; AKI: Acute kidney injury; BUN: Blood urea nitrogen; CKD: Chronic kidney disease; INR: International normalized ratio; MELD: Model for end-stage liver disease; Na: Sodium; SCr: Serum creatinine; UTI: Urinary tract infection.

Because of the possibility that terlipressin treatment resulted in clinical change, which precluded patients from transplant, the FDA added a warning label indicating that terlipressin-induced adverse events may make a patient ineligible for liver transplant and risks of using terlipressin may outweigh benefits in patients with MELD ≥ 35. An additional warning was issued for patients with severe acute on chronic liver failure (ACLF grade 3), because the likelihood of adverse events was higher and the response to treatment diminished[72,81]. These findings suggest that renal replacement therapy and liver transplant evaluation should be considered early in patients with high baseline SCr and ACLF grade. Tables 3 and 4 summarize clinical trials and meta-analyses on terlipressin effects on HRS-AKI, although it should be noted that no meta-analyses include the CONFIRM trial.

Norepinephrine, although not FDA-approved for the treatment of HRS-AKI, has shown efficacy and is frequently used off label for the treatment of HRS, especially in the United States where terlipressin was not available until recently. The need for central venous administration and close hemodynamic monitoring generally limits its use to the ICU. Comparison of terlipressin and norepinephrine has been limited to single-center open-label studies, but has not shown a clear difference in the reversal of HRS or mortality[81,82]. Given the lack of clear benefit of terlipressin over norepinephrine, higher cost of terlipressin, warnings issued by the FDA, and established practice patterns using norepinephrine in the United States, it is unclear how quickly and widely terlipressin will be adopted.

Transjugular intrahepatic portosystemic shunt

There is interest in using transjugular intrahepatic portosystemic shunt (TIPS) treatment of HRS-AKI because it can improve portal hypertension and cardiac output, two of the central causes of HRS-AKI. Although data regarding the role of TIPS in HRS generally involves small numbers of patients, a meta-analysis of 128 patients treated with TIPS for HRS showed improvement in renal function in 93% of patients with HRS-AKI. The significance of this finding is difficult to assess as there was no comparison group, significant heterogeneity, and high mortality[83]. There is also significant risk associated with TIPS insertion in patients with HRS-AKI including 90-d mortality of 25%-80%[84]. However, it is difficult to determine to what degree the high mortality was the result of TIPS. Given the lack of prospective or larger well-conducted retrospective analysis of TIPS for HRS as well as high procedural risks and complications associated with TIPS insertion in patients with HRS-AKI, it remains difficult to accurately identify patients who will benefit. Perhaps the clearest benefit of TIPS in the management of HRS-AKI lies in prevention by ameliorating portal hypertension. This is supported by a lower incidence of HRS in patients with diuretic resistant ascites treated with TIPS compared to those treated with serial paracentesis (9% *vs* 31%)[85].

Table 2 Methods of estimating glomerular filtration rate and the novel biomarkers for diagnosis of acute kidney injury in cirrhosis

Ref.	Biomarker	Year Published-Patient population	Advantage(s)	Limitation(s)
Fagundes *et al*[102]	NGAL and/or IL-18	2012-Cirrhosis	Best supporting data; can differentiate HRS-AKI and ATN; predicts AKI progression; predicts mortality; NGAL has good performance in patients with ACLF	Increased in inflammation and infections (UTI); lack of standard cut-offs
Verna *et al*[103]		2012-Cirrhosis		
Tsai *et al*[104]		2013-Cirrhosis		
Gungor *et al*[105]		2014-Cirrhosis		
Belcher *et al*[55]		2014-Cirrhosis		
Barreto *et al*[106]		2014-Cirrhosis		
Qasem *et al*[56]		2014-Cirrhosis		
Treeprasertsuk *et al*[107]		2015-Cirrhosis		
Ariza *et al*[57]		2015-Cirrhosis		
Markwardt *et al*[59]	Cystatin C	2017-Cirrhosis	Predicts AKI progression; predicts short-term mortality; Used in combination with MELD score (MELD-cystatin score)	Increases in CKD
Maiwall *et al*[60]		2017-Cirrhosis		
Jaques *et al*[108]		2019-Cirrhosis		
Belcher *et al*[55]	KIM-1	2014-Cirrhosis	Predicts AKI progression; Predicts short-term mortality	Low sensitivity and specificity for differentiating causes of AKI
Ariza *et al*[57]		2015-Cirrhosis		
Belcher *et al*[55]	L-FABP	2014-Cirhrosis	Predicts AKI progression; predicts short-term mortality	Increased in CKD; poor performance in differentiating causes of AKI
Jiang *et al*[109]		2018-Cirrhosis		
Belcher *et al*[55]	Albumin	2014-Cirrhosis	Can differentiate HRS-AKI and ATN; good performance in ACLF; predicts short-term mortality; readily available	Decreased level in advanced cirrhosis

ACLF: Acute on chronic liver failure; AKI: Acute kidney injury; ATN: Acute tubular necrosis; CKD: Chronic kidney disease; HRS-AKI: Hepatorenal syndrome with acute kidney injury; IL-18: Interleukin 18; KIM-1: Kidney injury molecule 1; L-FABP: Liver-type fatty acid-binding protein; MELD: Model for end-stage liver disease; NGAL: Neutrophil gelatinase-associated lipocalin; SCr: Serum creatinine; UTI: Urinary tract infection.

Renal replacement therapy

Renal replacement therapy (RRT), usually in the form of continuous hemodialysis, is the second-line treatment in patients with HRS-AKI who fail medical management and often regarded as a bridge to organ transplant since it does not address the underlying physiology of HRS. Additionally, RRT does not improve survival in patients with HRS-AKI after failure of medical management[86]. Based on current evidence, RRT is best reserved for potential liver transplant candidates or if HRS-AKI is due to a potentially reversible condition such as infection or bleeding. In patients who are not transplant candidates or if the inciting cause is unclear or unlikely to be reversed, palliative care should be considered prior to initiation of RRT.

Artificial liver support systems

Liver support systems, including molecular adsorbent recirculating system and extracorporeal liver assist device, are forms of albumin dialysis where albumin recirculate as a scavenger of bacterial products and inflammatory cytokines have been considered for HRS-AKI. Thus far, there are no clear benefits in AKI-HRS and studies have shown mixed results regarding improving renal blood flow and survival[48]. Thus, further studies are needed before its use can be officially recommended in HRS-AKI but it may be considered as a bridge to transplant in selected patients.

Organ transplant

Liver transplant is considered definitive treatment for HRS-AKI because it reverses the underlying pathophysiology causing renal impairment. This is evidenced by renal recovery in up to 75% of patients with HRS after liver transplant alone (LTA)[87,88]. The strongest predictor of non-recovery of HRS-AKI is the duration of pretransplant dialysis, with each additional day of pretransplant dialysis increasing the risk of non-recovery by 6%[89]. Other pre-transplant factors associated with lack of renal recovery after LTA are older age, higher baseline SCr, prolonged ischemia during transplant, exposure to nephrotoxic agents, diabetes, and development of ATN[87-91]. Unfortunately, 6%-10% of patients with HRS who have LTA will develop end-stage renal disease by 1-year post-transplant[87-89]. Therefore, it

Table 3 Results of studies using vasoconstrictor therapy in patients with hepatorenal syndrome with acute kidney injury

Ref.	Study design	Treatment	Alb	HRS reversal (%)	Mortality (%)
Uriz *et al*[110], 2000	Prospective	Terlipressin	Yes	77	Not defined
Halimi *et al*[111], 2002	Retrospective	Terlipressin	No	72	Not defined
Moreau *et al*[112], 2002	Retrospective	Terlipressin	Yes	58	Not defined
Ortega *et al*[75], 2002	Prospective	Terlipressin	Yes	77	Not defined
Duvoux *et al*[113], 2002	Prospective	NE	Yes	83	Not defined
Solanki *et al*[60], 2003	Randomized	Terlipressin *vs* placebo	Yes	42 *vs* 0	58 *vs* 100
Alessandria *et al*[64], 2007	Randomized	Terlipressin *vs* NE	Yes	83 *vs* 70	25 *vs* 20
Neri *et al*[65], 2008	Randomized	Terlipressin *vs* placebo	Yes	81 *vs* 19	27 *vs* 58
Sharma *et al*[66], 2008	Randomized	Terlipressin *vs* NE	Yes	50 *vs* 50	45 *vs* 45
Sanyal *et al*[67], 2008	Randomized	Terlipressin *vs* placebo	Yes	34 *vs* 13	57 *vs* 62
Martin-Llahi *et al*[68], 2008	Randomized	Terlipressin *vs* placebo	Yes	44 *vs* 9	74 *vs* 83
Singh *et al*[69], 2012	Randomized	Terlipressin *vs* NE	Yes	39 *vs* 43	70 *vs* 65
Cavallin *et al*[70], 2015	Randomized	Terlipressin *vs* MID plus OCT	Yes	70 *vs* 29	30 *vs* 32
Cavallin *et al*[71], 2016	Randomized	Terlipressin infusion *vs* terlipressin bolus	Yes	56 *vs* 46	59 *vs* 43
Boyer *et al*[72], 2016	Randomized	Terlipressin *vs* placebo	Yes	24 *vs* 15	33 *vs* 35
Wong *et al*[73], 2019	Randomized	Terlipressin *vs* placebo	Yes	29 *vs* 16	73 *vs* 71
Wong *et al*[81], 2021	Randomized	Terlipressin *vs* placebo	Yes	32 *vs* 16	51 *vs* 45

Alb: Albumin; HRS: Hepatorenal syndrome; HRS-AKI: Hepatorenal syndrome with acute kidney injury; MID: Midodrine; NE: Norepinephrine; OCT: Octreotide.

Table 4 Results of recent meta-analyses comparing terlipressin to other vasoconstrictor therapies in hepatorenal syndrome with acute kidney injury

Ref.	Study design	Number of studies	HRS reversal	Mortality benefit	Data quality
Facciorusso *et al*[76], 2017	Meta-analysis	13	Same as NE; better than Alb+OCT; better than Alb+MID+OCT	Possible short-term benefits	Very low to low
Israelsen *et al*[77], 2017	Meta-analysis	10	Same as NE; better than Alb+OCT; better than Alb+MID+OCT	No difference	Very low to low
Nanda *et al*[78], 2018	Meta-analysis	13	Same as NE; better than Alb+OCT; better than Alb+MID+OCT	No difference	Poor to good
Wang *et al*[79], 2018	Meta-analysis	18	Same as NE; better than Alb+OCT; better than Alb+MID+OCT	Confers short-term benefits	Low to high
Best *et al*[80], 2019	Meta-analysis	25	Same as NE; better than Alb+OCT; better than Alb+MID+OCT	No difference	Very low to low

Alb: Albumin; HRS: Hepatorenal syndrome; HRS-AKI: Hepatorenal syndrome with acute kidney injury; MID: Midodrine; NE: Norepinephrine; OCT: Octreotide.

is essential to consider the likelihood of renal recovery after LTA and if the patient would benefit more from simultaneous liver-kidney transplant (SLKT). Currently, the main indications for SLKT are AKI requiring RRT or GFR < 25 mg/dL for more than 4-6 wk (guidelines vary) and CKD, commonly defined as GFR < 30 mg/dL at the time of listing with a GFR persistently < 60 mg/dL for at least 90 d[48,89]. The decision regarding LTA or SLKT must also weigh the potential benefit for the individual patient with consideration of the principles of just and equitable organ allocation. In an attempt to balance these factors agencies responsible for organ allocation have set specific guidelines in their respective countries [92].

CONCLUSION

Although the primary cause of HRS remains circulatory dysfunction resulting in impaired renal profusion, there is now an improved understanding of the role of other factors including the inflammatory environment and cardiac dysfunction. This has contributed to the development of better biomarkers for earlier and more accurate diagnosis of HRS. Despite not being widely available the offer promise that effective treatment can be applied during the critical early stages of the disease where there is the greatest potential for benefit. Medical treatment remains primarily vasoactive medications and albumin, and have not yet been able to exploit the improved understanding of pathophysiology. However, the approval of terlipressin in the United States and clearer delineation of patients most likely to benefit from this therapy offers hope for improved medical management in the future. Despite advances in medical treatment, liver transplantation remains the most definitive treatment and should be considered early in the disease course as delay can increase the risk of incomplete renal recovery after transplant.

FOOTNOTES

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REVIEW

Treatment of liver fibrosis: Past, current, and future

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Abstract

Liver fibrosis accompanies the progression of chronic liver diseases independent of etiologies, such as hepatitis viral infection, alcohol consumption, and metabolic-associated fatty liver disease. It is commonly associated with liver injury, inflammation, and cell death. Liver fibrosis is characterized by abnormal accumulation of extracellular matrix components that are expressed by liver myofibroblasts such as collagens and alpha-smooth actin proteins. Activated hepatic stellate cells contribute to the major population of myofibroblasts. Many treatments for liver fibrosis have been investigated in clinical trials, including dietary supplementation (*e.g.*, vitamin C), biological treatment (*e.g.*, simtuzumab), drug (*e.g.*, pegbelfermin and natural herbs), genetic regulation (*e.g.*, non-coding RNAs), and transplantation of stem cells (*e.g.*, hematopoietic stem cells). However, none of these treatments has been approved by Food and Drug Administration. The treatment efficacy can be evaluated by histological staining methods, imaging methods, and serum biomarkers, as well as fibrosis scoring systems, such as fibrosis-4 index, aspartate aminotransferase to platelet ratio, and non-alcoholic fatty liver disease fibrosis score. Furthermore, the reverse of liver fibrosis is slowly and frequently impossible for advanced fibrosis or cirrhosis. To avoid the life-threatening stage of liver fibrosis, anti-fibrotic treatments, especially for combined behavior prevention, biological treatment, drugs or herb medicines, and dietary regulation are needed. This review summarizes the past studies and current and future treatments for liver fibrosis.

Key Words: Liver fibrosis; Molecular mechanism; Therapeutic targets; Treatments; Clinical trials

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Core Tip: Liver fibrosis accompanies the progression of chronic liver diseases independent of their etiologies. The initiation and progression of liver fibrosis are mainly driven by liver inflammation, cell death, and metabolic dysregulation, which cause the activation of hepatic stellate cells and excessive accumulation of extracellular matrix proteins. Without effective treatments, liver fibrosis can lead to cirrhosis and primary liver cancer. To date, current therapeutic options for liver fibrosis are limited to prevent the initial causing factors for liver inflammation, hepatocyte cell death, and oxidative stress. However, the reverse of liver fibrosis is slowly and frequently impossible for advanced fibrosis or cirrhosis. To avoid the life-threatening stage of liver fibrosis, anti-fibrotic treatments including biological, medicines, dietary change, and behavior prevention are needed, especially for combined therapy.

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INTRODUCTION

Liver fibrosis accompanies the progression of chronic liver diseases independent of etiologies[1], such as hepatitis viral infection, alcohol abuse, and metabolic-associated fatty liver disease (MAFLD). It is commonly associated with liver injury, inflammation, and cell death. Abnormal accumulation of extracellular matrix (ECM) components expressed by liver myofibroblasts, such as collagens and alpha-smooth actin proteins, are the markers of hepatic fibrogenesis[2]. Activated hepatic stellate cells (HSCs) contribute to the major population of myofibroblasts in liver fibrosis[3]. Although many drugs have been investigated in clinical trials, there are no Food and Drug Administration (FDA)-approved treatments for liver fibrosis.

The activation of HSC is a complex pathogenesis in liver fibrosis[4]. Many factors including intrahepatic and extrahepatic factors can drive HSC activation to induce liver fibrosis. A variety of molecular signaling pathways are involved in the regulation of HSC activation[1,5], such as transforming growth factor-β (TGF-β), Toll-like receptors (TLRs), and epigenetic signals (*e.g.*, microRNAs, or miRNAs). The activation of HSCs can be divided into two phases, the initiation and perpetuation phases. RNA sequencing results have shown that fibrogenic transcriptional programs in the initiation phase are also active in the perpetuation phase; therefore, targeting the initial activation of HSC is also critically important for live fibrosis treatment[6].

In this review, the cellular and molecular mechanisms of liver fibrosis are reviewed. Importantly, the currently available treatments for liver fibrosis are summarized and discussed. Some pros and cons of available treatments are discussed. In addition, the future direction for liver fibrosis therapy is predicted.

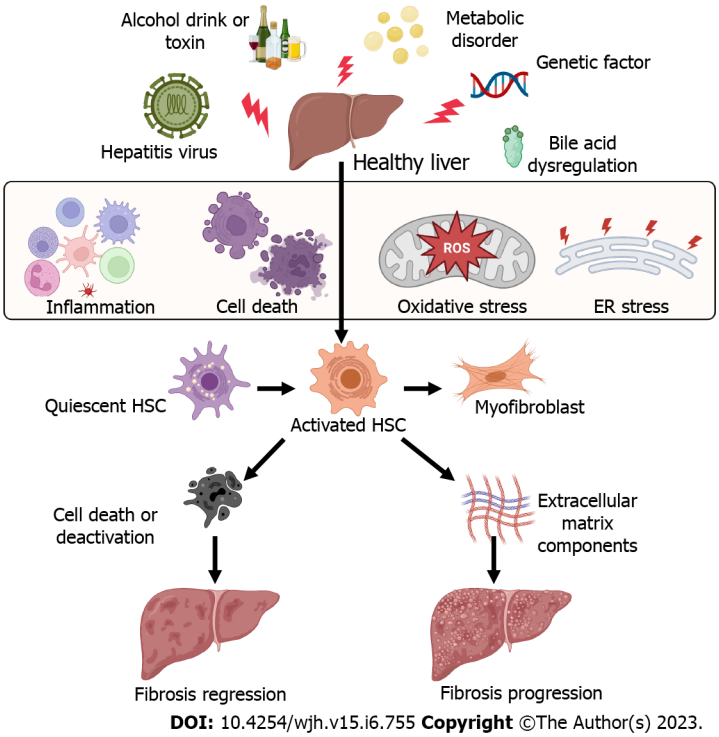
INITIATION OF LIVER FIBROSIS: CELLULAR AND MOLECULAR MECHANISMS

Hepatic cell death

Liver cell death and inflammation are the initial events in chronic liver disease independent of etiologies. Many factors can cause liver injury and hepatic cell death and inflammation[7,8], including hepatitis viral infection, alcohol consumption, metabolic liver disease, abnormal bile acid products, and genetic factors. These pathogenic factors cause immune cell inflammation, hepatocyte death, mitochondrial dysfunction, and endoplasmic reticulum stress (Figure 1), resulting in HSC activation and differentiation to myofibroblasts to lead to liver fibrosis[5,9].

In non-alcoholic steatohepatitis (NASH), hepatocyte death results in the infiltration of monocyte-derived macrophages and upregulation of the expression of inflammatory cytokines[9], such as tumor necrosis factor α (TNF-α), TGF-β1, and interleukin-1β (IL-1β), Hepatocyte death can be classified into programed cell death including pyroptosis, apoptosis, necroptosis, ferroptosis, and autophagy-mediated cell death (Figure 2), as well as non-programed cell death (necrosis). In chronic liver disease, different types of cell death may be associated with the progression of liver fibrosis and end-stage of liver disease, such as hepatocellular carcinoma (HCC). Single-cell RNA sequencing coupled with spatial mapping approaches has been started to dissect the key cellular and molecular functions in liver disease [10].

Pyroptosis is an inflammatory cell death, associated with cell membrane rupture by cleaved gasdermin D[11]. The cleave of gasdermin D is induced by the activation of caspase-1 or caspase-11/4/5 [12]. For example, in mice with non-alcoholic fatty liver disease (NAFLD), feeding a high-fat diet can



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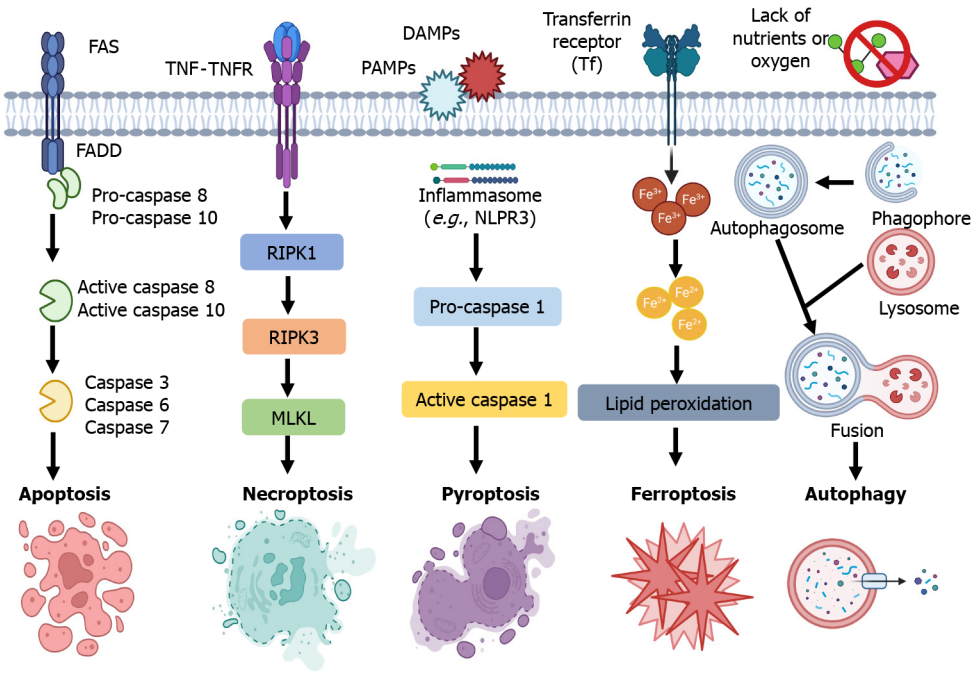
Figure 1 Factors causing the activation of hepatic stellate cells and liver fibrosis. Many factors can cause liver injury and hepatic cell death and inflammation, including hepatitis viral infection, alcohol consumption, metabolic liver disease, abnormal bile acid products, and genetic factors. These pathogenic factors cause immune cell inflammation, hepatocyte death, oxidative stress, and endoplasmic reticulum stress, resulting in hepatic stellate cell activation and differentiation to myofibroblasts to lead to liver fibrosis. All cartoons in this figure were prepared using Biorender (https://biorender.com). ER: Endoplasmic reticulum; HSC: Hepatic stellate cell; ROS: Reactive oxygen species.

increase the expression of caspase-11 to cause pyroptosis of bone marrow monocyte-derived macrophages by cleave gasdermin D[13]. The expression of pyroptosis-related indicators including gasdermin D, IL-1β, and IL-18 has been shown to be increased in human patients with liver fibrosis and mice with CCl_4-induced fibrosis[14]. In addition, S100 calcium-binding protein A8 plays an essential role in macrophage pyroptosis in liver fibrosis, by inducing the expression of nucleotide-binding domain leucine-rich repeat-receptor, pyrin domain-containing-3 (NLRP3) inflammasome, pro-IL-1β, and pro-IL-18 *via* the activation of TLR4/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway[14].

Apoptosis is a form of programmed cell death that occurs in all liver cell types. In hepatocytes, kindlin-2 deficiency can increase the apoptosis of hepatocytes, resulting in liver fibrosis and accumulation of ECM components by activating the TNF signaling pathway[15]. In NAFLD, microRNAs such as miR-22 from adipocyte-derived exosomes can cause hepatocyte apoptosis to increase hepatic inflammation, lipid accumulation, and fibrosis by regulating sirtuin 1 expression[16]. In contrast, promoting the apoptosis of HSCs can inhibit and reverse liver fibrosis. For example, treatment with gomisin D can inhibit CCl_4-induced HSC proliferation and activation in mice and increase HSC apoptosis to reduce liver fibrosis by regulating the platelet-derived growth factor receptor β signaling pathway[17].

Necroptosis is a regulated cell death that has the features of apoptosis and necrosis. Necroptosis can be induced by TLRs, interferons, and death receptors[18], which is mediated by receptor-interacting protein kinase-3 and its substrate mixed lineage kinase-like[19]. In addition, receptor-interacting serine/threonine-protein kinase 1 has an important role in this process (Figure 2). It contributes to hepatocyte death in NASH. Necroptotic hepatocytes cannot be removed by liver macrophages due to the activation of "don't-eat-me" signaling pathway, the CD47/signal regulatory protein alpha axis[20].

Ferroptosis is an intracellular iron-dependent lytic cell death which is different from apoptosis and necrosis[21,22]. Excessive iron accumulation and the inhibition of glutathione peroxidase 4 trigger ferroptosis, which causes cell plasma membrane rupture[23]. Accumulating research studies have demonstrated that ferroptosis is involved in different liver diseases, including alcoholic liver disease, NASH, cirrhosis, and cancer[24,25]. Several molecular signaling pathways are involved in ferroptosis in liver diseases, such as the nuclear factor erythroid 2-related factor 2[26], heat shock protein family A member 8[27], and nuclear receptor coactivator 4[28]. Pharmacological regulation of HSC ferroptosis is a therapeutic strategy for liver fibrosis[29]. For example, curcumol can induce ferroptosis of activated HSCs to inhibit liver fibrosis by inducing autophagy[28].



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Figure 2 Programmed cell death subtypes of hepatic cells, including apoptosis, necroptosis, pyroptosis, ferroptosis, and autophagy-mediated cell death. Apoptosis, necroptosis, pyroptosis, and ferroptosis are programmed forms of cell death, while necrosis is unprogrammed cell death. Autophagy-mediated cell death should be defined when autophagic flux is raised without the involvement of other types of programmed cell death, and pharmacological or genetic inhibition of autophagy blocks cell death. DAMPs: Danger-associated molecular patterns; FADD: Fas-associated protein with a death domain; FAS: Fas cell surface death receptor; MLKL: Mixed lineage kinase domain-like; NLRP3: Nod-like receptor family, pyrin domain containing 3; PAMPs: Pathogen-associated molecular patterns; RIPK1/3: receptor-interacting protein kinase 1/3; TNF: Tumor necrosis factor; TNFR: Tumor necrosis factor receptor. All cartoons in this figure were prepared using Biorender (https://biorender.com).

Furthermore, some dying cells develop autophagosomes that trigger apoptosis and necroptosis, namely autophagy-mediated cell death (ACD). However, ACD should be defined when some criteria are met[30], including: (1) Cell death happens without the involvement of other types of programmed cell death; (2) autophagic flux is raised; and (3) pharmacological or genetic inhibition of autophagy blocks cell death. Overall, the molecules implicated in the process and signaling pathways of hepatic cell death are potential targets for liver fibrosis.

Hepatic innate and adaptive immunity

Liver resident immune cells and infiltration of myeloid cells or circulating immune cells in the liver during chronic liver injury play key roles in the activation of HSCs. For example, in NASH liver, gut microbiota-diet interplay-resulted metabolite can activate liver macrophages to produce profibrotic factors[31], such as TGF-β1. Treatments such as astaxanthin can suppress the infiltration of monocyte-derived macrophages to suppress HSC activation, liver oxidative stress response, and hepatocyte death by decreasing the expression of proinflammatory cytokines[32], such as TNF-α, TGF-β1, and IL-1β. Another study also during NAFLD progression, mast cells can increase Western diet-induced biliary and liver damage to the development of microvesicular steatosis through microRNA (miR-144-3p)-targeted signaling pathway[33].

Adaptive immune cells including T and B cells have various roles in liver fibrosis. The imbalance of liver regulatory T cells and T helper (Th) cells, such as Th1 cells and Th17 cells plays an essential role in liver fibrosis, cirrhosis, and cancer[34]. The populations of multicytokine-producing CD4 T cells were significantly increased in the livers of patients with NASH compared with patients with NAFLD[35]. The cytokines produced by these CD4 T cells include TNF-α, IFN-γ, IL-17A, and IL-10. The phenotype of T cells is also important for their functions. One study showed that the reduction of tissue-resident memory $CD69^{+}CD103^{-}CD8$ T cells significantly decreased the resolution of fibrosis in NASH liver. These CD8 T cells can induce FasL/Fas-mediated apoptosis of HSCs[36]. Therefore, targeting immune cells or their secreted inflammatory cytokines is an optional treatment for liver fibrosis[37].

Furthermore, HSC activation is also associated with vascular aging[38], ischemia/reperfusion injury [39], and angiogenesis[40].

SIGNALING PATHWAYS AND MOLECULAR TARGETS FOR LIVER FIBROSIS TREATMENTS

Bile acid receptors

Farnesoid-X-receptor (FXR) and the G protein-coupled bile acid receptors are two widely studied bile acids regulating receptors, which play important roles in lipid and glucose metabolism, inflammation, fibrosis, and immune responses. Many FXR ligands have been investigated in clinical trials for NASH and liver fibrosis treatments, such as EDP-305[41,42], cilofexor[43,44], and MET409[45,46]. Hepatic concentrations of conjugated 12α-hydroxylated bile acids, such as taurodeoxycholate and glycodeoxycholate, were significantly increased in patients with NASH and mouse liver fibrosis models[47]. These bile acids contribute to HSC activation and liver fibrosis by regulating the signaling of G protein-coupled bile acid receptor 1, also known as TGR5.

Caspases

Caspases are involved in liver cell inflammation and hepatocyte cell death. Meanwhile, they play an essential role in liver fibrogenesis. For example, caspase 3-deficient mice on a methionine- and choline-deficient diet had reduced liver collagen production compared to wild-type mice[48]. Lipotoxicity can induce caspase-mediated apoptosis of hepatic cells and liver inflammation and injury in NAFLD. Treatment of caspase inhibitors such as emricasan (IDN-6556, a pan-caspase inhibitor) can reduce liver injury in patients with NAFLD[49].

Chemokine receptors

Chemokine receptors are commonly expressed by immune cells or inflammatory cells, which can be recruited during liver inflammation and fibrosis. For example, C-C chemokine receptor 2 (CCR2) and CCR5 are highly expressed by monocytes and subtypes of liver macrophages, which can be targeted to ameliorate liver fibrosis[50]. A recent study showed that the roles of CCR2 and CCR5 in liver macrophages are different during liver disease progression in mice with a hepatocyte-specific knock-out of NF-κB essential modulator. CCR2 oversees the recruitment of monocytes during liver injury, whereas CCR5 is needed to promote HSC activation[51].

In addition, many other chemokines and their receptors are involved in the pathogenesis of liver fibrosis, including C-X-C motif ligand 12 (CXCL12) /C-X-C receptor 4[52], chemokine (C-X3-C motif) ligand 1/C-X3-C receptor 1 (CX3CR1)[53], CCL19/CCR7[54], and CXCL12/atypical chemokine receptor 3[55].

Fibroblast growth factors

Fibroblast growth factor 15 (FGF15) is an important endocrine regulator for hepatic bile acid and lipid metabolism, which regulates gut-liver crosstalk in mice[56]. A combined treatment using an inhibitor of apical sodium-bile acid transporter (GSK233072) and adeno-associated virus 8-mediated hepatic FGF15 overexpression significantly can improve the therapeutic efficacy against NASH and fibrosis compared to either single treatment[57]. Bile acid nuclear receptor FXR plays an important role in the regulation of the expression of FGF15/19, bile acid homeostasis, and lipid metabolism, which is the target for NASH and liver fibrosis[58]. The expression of hepatic FXR and plasma FGF19 (the ortholog of mouse FGF15) was decreased in children with NASH compared to their expression in healthy subjects[59].

Galectins

Galectins are carbohydrate-binding proteins and play important roles in liver inflammation, immune response, and fibrosis. Galectin-1 (Gal-1) was shown to be highly expressed in the stroma of HCC by cancer-associated fibroblasts. Silencing Gal-1 in these fibroblasts can suppress inflammation and tumor progression[60]. The serum level of Gal-3 was increased in patients with advanced cirrhosis, and liver expression of Gal-3 was also correlated with liver disease severity and inflammation[61].

Lysyl oxidase family members

Lysyl oxidase (LOX) family members are extracellular copper-dependent enzymes, including LOX, lysyl oxidase-like 1 to 4 (LOXL1 to 4) members, which play important roles in the cross-linking of ECM proteins in fibrosis and carcinogenesis. Inhibition of pan-LOX family, LOX, LOXL1, or LOXL2 has been shown to prevent fibrogenesis and accelerate the reversal of liver fibrosis, as well as fibrosis in other organs. However, the roles of LOX family members as therapeutic targets for liver fibrosis need further to be evaluated[62].

NLRP3 inflammasome

Nucleotide-binding oligomerization (NLR) family pyrin domain-containing 3 (NLRP3) plays a pivotal role in liver fibrosis. Activation of NLRP3 can lead to the inflammatory response through the secretion of IL-1β and IL-18 and activation of caspase-1[63], which is involved in liver cell pyroptosis[64]. Activation of NLRP3 inflammasome can induce hepatocyte pyroptosis and liver fibrosis, while

inhibiting the activation of NLRP3 inflammasome can inhibit the development of NAFLD and NASH in animal models[65,66]. Activation of NLRP3 inflammasome in pyroptosis is mediated by canonical caspase-1-mediated signaling pathway and noncanonical caspase-11-mediated signaling pathway[67].

Peroxisome proliferator-activated receptors (PPARs)

PPARs, comprised of three subtypes PPARα, β/δ, and γ, play important roles in liver lipid metabolism, inflammation, and fibrosis[68-70]. The expression of liver PPARα was shown to be negatively correlated with NASH severity, visceral fat accumulation, and insulin resistance in human patients[71]. Treatment with PPARα/γ dual agonists decreased the concentrations of total cholesterol, triglyceride (TG), and inflammatory cytokine levels in serum, reduced hepatic steatosis, infiltration of inflammatory cells, and decreased the expression of lipogenic gene and NF-κB protein[72]. Another study showed that the levels of very low-density lipoprotein receptors (VLDLR) were increased in PPARβ/δ-deficient mice. In patients with hepatic steatosis, the mRNA levels of PPARβ/δ were suppressed and associated with an increase in VLDLR levels[73]. A pre-clinical study showed that treatment with pan-PPAR agonist lanifibranor can significantly decrease portal pressure and liver inflammation and induce fibrosis regression[74].

TGF-β/Smad

TGF-β/Smad is the most well-studied signaling pathway in fibrosis. SMAD proteins are essential intracellular effectors of TGF-β and show different roles in liver fibrosis[75], including pro-fibrotic functions (*e.g.*, SMAD3 and SMAD4) and protective functions (*e.g.*, SMAD2 and SMAD7). In addition, many studies have demonstrated that regulating the signaling pathway of TGF-β/Smad can prevent liver fibrosis[76], as well as the protein kinase B (PKB, or AKT)/Forkhead box O3 (FOXO3) signaling pathway.

Wnt/β-catenin

Proteins-derived from human amniotic mesenchymal stem cells, including insulin-like growth factor binding protein-3, Dickkopf-1, and DKK-3, can inhibit HSC activation by suppressing Wnt/β-catenin signaling pathway *in vitro*[77]. In vivo study also showed that treatment of niclosamide in rats can prevent CCl_4-induced liver fibrosis by inhibiting the Wnt/β-catenin pathway and glutaminolysis[78]. Another study also showed that Wnt3a can upregulate the expression of protein regulator of cytokinesis 1 to active β-catenin signaling to promote liver fibrosis[79]. The interaction of β-catenin/transcription factor 4 (TCF4) has been shown to increase during liver fibrosis in mice with bile duct ligation (BDL) [80]. Treatment with ICG-001, an inhibitor of the interaction between cyclic adenosine monophosphate response element binding protein binding protein and β-catenin, together with LF3, a small molecule antagonist that inhibits β-catenin/TCF4 transcriptional activity, can reduce liver fibrosis[80].

Yes-associated protein (YAP)

YAP plays a pivotal role in the sensitivity of HSCs to ferroptosis, apoptosis, and senescence in fibrotic livers. Selective depletion of YAP in myofibroblastic HSCs or activated HSCs can promote their senescence or apoptosis to reduce liver injury and fibrosis[81]. Taurocholic acid can induce the activation of HSCs through the sphingosine-1-phosphate receptor 2/YAP/p38 mitogen-activated protein kinase (p38 MAPK)[82].

CURRENT DIAGNOSIS FOR LIVER FIBROSIS

The golden standard method for liver fibrosis diagnosis is liver biopsy. Histological or histochemical staining can be used to stain the cells or the extracellular matrix proteins to identify liver fibrosis. Common histological staining methods for liver fibrosis evaluation are hematoxylin-eosin staining with Masson's trichrome or Sirius Red staining[83]. Due to the pain and the risk of potential complications of liver biopsy, non-invasive techniques (*e.g.*, elastography scanning) and biomarkers (*e.g.*, aminotransferase to platelet ratio (APRI): The aminotransferase/platelet ratio index) can be applied for diagnosing liver fibrosis[84]. Many available scoring systems can be applied for liver fibrosis diagnosis and evaluation, including fibrosis-4 index (FIB-4), APRI, and NAFLD fibrosis score (NFS)[85].

Imaging methods are commonly applied in the clinic to evaluate the progression of liver fibrosis. For example, ultrasound elastography techniques can be applied to characterize liver fibrosis and its stage in adult patients, such as vibration-controlled transient elastography, the most utilized and validated elastography method[86]. A meta-analysis study also showed that magnetic resonance elastography (MRE) and point-shear wave elastography (pSWE) can be applied for liver fibrosis diagnostic, and MRE is a more accurate imaging technique than pSWE[87]. The pooled sensitivities and specificities for MRE and pSWE were 0.94 (95% confidence level/CI: 0.89-0.97) and 0.95 (95%CI: 0.89-0.98), and 0.86 (95%CI: 0.80-0.90) and 0.88 (95%CI: 0.85-0.91), respectively. Their pooled summary receiver operating characteristic curves showed that the area under the curve (AUC) for MRE was 0.98 (95%CI: 0.96-0.99), whereas the AUC for pSWE was 0.93 (95%CI: 0.90-0.95). Another review paper has updated the conven-

tional and molecular imaging diagnostic methods for liver fibrosis[88]. In addition, artificial intelligence models have been applied for the diagnosis of liver fibrosis[89-91]. For example, the clinical features and imaging data collected from a patient can be analyzed for liver fibrosis diagnosis using a machine learning model.

Recently, studies also have shown that miRNAs, the single-stranded, non-coding RNAs containing 21 to 23 nucleotides, are involved in the pathogenesis of liver fibrosis, which are potential biomarkers for diagnosing liver fibrosis and therapeutic targets for liver fibrosis treatment[92]. The methods for liver fibrosis diagnosis have been reviewed in some recent publications[93-96]. Here, we will not discuss more details and will focus on the treatment options for liver fibrosis.

CURRENT TREATMENT OPTIONS FOR LIVER FIBROSIS

In this section, we review some different treatment options for liver fibrosis, such as biological intervention, anti-fibrotic drugs, and other treatment strategies. These treatments either target causing factors of liver fibrosis to accelerate the recovery of liver injury, or induce the balance of liver metabolism, such as anti-hepatitis viral infection, anti-cell death treatment, and regulators of lipid metabolism.

Biological intervention

Inhibition of LOXL2 in the fibrotic tumor microenvironment can synergistically increase the efficacy of sorafenib and 5-fluorouracil for liver cancer cells[97]. However, some treatments in clinical trials did not show promising results. For example, simtuzumab is a monoclonal antibody against LOXL2. In two phase 2b clinical trials, intravenous infusions of simtuzumab (200 or 700 mg) every other day for 48 wk and 96 wk did not show promising effects to decrease liver fibrosis and the progression of cirrhosis in patients with bridging fibrosis[98]. In a pilot clinical trial, intravenous treatment of simtuzumab (700 mg) every 2 wk for 22 wk did not improve liver biopsy fibrosis score for patients with advanced liver fibrosis[99].

Drug treatment

Aramchol, a partial inhibitor of hepatic stearoyl-CoA desaturase, has been shown to improve NASH and liver fibrosis in rodents and decrease liver triglycerides and fibrosis clinical trials[100].

Anti-hepatitis viral infection drugs: Inhibition of hepatitis viral infection can suppress liver inflammation and hepatocyte death to decrease liver injury, resulting in suppression of liver fibrosis. Drugs such as faldaprevir (also known as BI 201335)[101], ribavirin (HCV treatment)[102], and peginterferon alfa-2a (HBV treatment)[103,104], have been tested in clinical trials for the treatment of liver fibrosis. In addition, many other drugs have been evaluated or are under clinical trial evaluation against hepatitis viral infection[105-107], such as simeprevir, daclatasvir, and sofosbuvir.

Cenicriviroc: C-C chemokine receptors 2 and 5 dual antagonist, has been shown to improve liver fibrosis without worsening NASH compared to the placebo in phase 2 clinical trial (Clinicaltrials.gov, NCT02217475)[108]. A phase 3 clinical trial has been designed to confirm the efficacy and safety of cenicriviroc for liver fibrosis treatment in adults with NASH[109].

Cholangitis treatment: Obeticholic acid and ursodeoxycholic acid are the only two FDA-approved medicines for the treatment of primary biliary cholangitis[110], which have the potential to cholangitis-induced liver fibrosis.

Cyclophilin inhibitors: CRV431, a pan-cyclophilin inhibitor, can decrease liver fibrosis in mice treated with CCl_4 for 6 wk and mice with diet-induced NASH[111]. Another cyclophilin inhibitor NV556 also displays an antifibrotic effect in two mouse NASH models, the STAM model (streptozotocin plus a high-fat diet) and methionine- and choline-deficient diet-induced NASH model[112]. In addition, NV556 can also inhibit TGF-β1-induced activation of HSCs *in vitro*.

FGF regulators or analogues: Treatment of pegbelfermin (BMS-986036, 10 mg or 20 mg daily), a PEGylated human FGF21 analogue, can significantly decrease liver fat accumulation in patients with NASH without treatment-related severe adverse effects, and it can also improve liver fibrosis in patients with obesity and type 2 diabetes[113].

FXR agonists: Treatment of obeticholic acid (INT-747), a potent and orally active FXR agonist, can significantly ameliorate liver fibrosis and the histological and biological markers of NASH in patients with NASH[114].

Gal-3 inhibitors: GB1211, an inhibitor of Gal-3, can inhibit the differentiation of epithelial cells into myofibroblasts and macrophage or myofibroblast-induced fibrosis in the liver[115]. GR-MD-02 (belapectin), a galectin-3 inhibitor, has been shown to inhibit liver fibrosis and portal hypertension in rat

fibrosis mode, which is safe and well-tolerated in a phase 1 clinical trial. However, a phase 2b clinical trial showed that treatment of GR-MD-02 did not significantly improve liver fibrosis and reduce portal hypertension (hepatic venous pressure gradient) in patients with NASH[116]. Further studies are required to evaluate these treatments for liver fibrosis.

Glucagon-like peptide-1 (GLP-1) receptor agonist: GLP-1 analogues have been shown to have the effects to reduce liver fat accumulation, liver injury, and insulin resistance in mice with fatty liver disease. Clinical trial (ClinicalTrials.gov, NCT01237119) showed that treatment of GLP-1 analogue liraglutide was well tolerated and suppressed liver fibrosis progression in patients with NASH[117]. Another trial also showed that treatment of liraglutide markedly reduced liver fat content and body weight in patients with uncontrolled type 2 diabetes[118].

Pan-caspase inhibitor: Emricasan (IDN-6556), a pan-caspase inhibitor, can decrease liver cell apoptosis and inflammation and improve portal pressure in rats with CCl_4-induced cirrhosis[119]. However, a clinical trial (Clinicaltrials.gov, NCT03205345) did not show the efficacy of emaricasan against liver fibrosis, but it was safe and well-tolerated.

PPAR agonists: In rats with BDL-induced liver fibrosis, treatment of PPAR-γ agonist thiazolidinedione inhibited HSC activation and liver fibrosis by regulating fibrogenic factors, such as TGF-β1, platelet-derived growth factor, and connective tissue growth factor[120]. Farglitazar (GI262570), an agonist of peroxisome proliferator-activated receptor-gamma (PPARγ), can inhibit HSC activation.

Tropifexor: A non-bile acid FXR agonist, can potently inhibit cholestatic liver injury and fibrosis by enhancing the expression of FGF19 in the ileum and the expression of small heterodimer partner in the livers of piglets but inhibit cholesterol 7α-hydroxylase. In addition, tropifexor can increase the abundance of bile acid-biotransforming bacteria and later the amino acid composition in the intestine and decrease intestinal barrier injury in piglets with BLD[121]. Clinical trial (Clinicaltrials.gov, NCT02855164) also showed that treatment of tropifexor (10-90 μg) once daily for 12 weeks was safe and decreased the levels of alanine aminotransferase (ALT) and hepatic fat fraction (HFF) compared to baseline in a dose-dependent manner. The decrease of ALT and HFF can be sustained for up to 48 wk at high doses of tropifexor (140 μg and 200 μg once daily)[122].

Natural products or herbal medicines

Natural products or herbal medicines display diverse roles in the treatment of liver fibrosis. For example, a classical Traditional Chinese Medicine formula Yinchenhao decoction has been shown to ameliorate dimethylnitrosamine-induced liver fibrosis in rats and suppress liver cell apoptosis[123]. Another study showed that Xiaoyaosan decoction significantly reduced CCl_4-induced liver fibrosis in rats by regulating both TGF-β1/Smad and AKT/FOXO3 signaling pathways[76]. The major components of these herbal medicines such as Tanshinone IIA extracted from the traditional herbal medicine Salvia miltiorrhiza display broad biological activities, such as anti-inflammatory, antioxidant, antiangiogenic, and anticancer functions[124]. Furthermore, clinical trials also illustrate that these traditional medicine formula such as Fuzheng Huayu display therapeutic effects against hepatitis-B-caused cirrhosis in patients[125].

Dietary regulation or supplementation

Consumption of polyunsaturated fatty acids: The endogenous metabolites of n-3 polyunsaturated fatty acids such as 19,20-epoxy docosapentaenoic acid show a protective effect against liver fibrosis in mouse NASH models[126]. G protein-coupled receptors can be regulated by polyunsaturated fatty acids to reduce liver inflammation and fibrosis[127]. For example, supplementation of docosahexaenoic acid, an omega-3 fatty acid, can reduce liver inflammation and prevent liver fibrosis in diet-induced liver fibrosis model *via* G protein-coupled receptor 120 (GPR120) signaling, also known as free fatty acid receptor 4[128].

Probiotics: Treatment with probiotic *Lactobacillus rhamnosus* GG can significantly decrease liver inflammation and fibrosis by reducing the production of hepatic bile acids in mice with BLD[129].

Vitamins: The serum levels of vitamin C have been shown to be negatively associated with the odds of liver fibrosis in patients with NAFLD in United States adults[130]. Another study showed that a decreased serum level of vitamin B12 is associated with an increased risk of liver fibrosis in patients with NAFLD[131]. Treatment of Vitamin D_3 can alleviate liver injury and the expression of ECM proteins such as TGF-β and α-SMA in thioacetamide-induced hepatic fibrosis rat model[132]. The data from the National Health and Nutrition Examination Survey (2017-2018) also showed that levels of 25-Hydroxyvitamin D were inversely associated with liver fibrosis during NAFLD development and progression[133].

Antioxidant and anti-inflammatory agents: Supplementation of natural products with antioxidant and anti-inflammatory components can also ameliorate chronic liver disease to improve liver fibrosis and

inhibit cancer development[9], such as β-sitosterol and silymarin.

Bariatric surgery

Studies have shown that bariatric surgery (BS) can provide long-term benefits for the resolution of liver fibrosis. The two most common procedures of BS are laparoscopic Roux-en-Y-gastric and laparoscopic sleeve gastrectomy. For example, one study showed that NASH was resolved in 84% of patients (95%CI: 73.1%-92.2%) at year 5 post-BS treatment, while fibrosis was decreased in samples from 70.2% of patients (95%CI: 56.6%-81.6%) compared with baseline and fibrosis was disappeared in samples from 56% of all patients (95%CI: 42.4%-69.3%)[134]. BS has been shown to induce NASH disappearance in nearly 85% (95%CI: 75.8%-92.2%) of patients and to decrease fibrosis in 33.8% of patients (95%CI: 23.6%-45.2%) with NASH at 1 year after surgery[135]. Another clinical study showed that excessive weight loss shown in patients with cirrhosis with 73% (33%–167%), 85% (33%–190%), and 73% (29%–107%) after 1, 2, and 3 years of BS[136], respectively. Among 27 patients with cirrhosis, 3 patients had significant improvement in liver function and did not need liver transplantation, whereas 2 out of 27 patients had deleterious liver function post-BS treatment[136].

Genetic intervention

Gene therapy is a critical tool for disease treatment, including liver fibrosis and cancer. Noncoding RNAs, such as miRNAs and long noncoding RNAs, small interference RNAs, and circular RNAs are important. For example, the treatment of siRNA silencing CCR2 can regulate liver immune to inhibit the infiltration of profibrotic macrophages and neutrophils in murine fibrotic livers[137]. Another study showed that circRNA ASPH regulated liver fibrosis by binding miR-139-5p by regulating neurogenic locus notch homolog protein 1 (Notch 1) expression[138].

Transplantation of stem cells

Transplantation of umbilical cord Wharton's Jelly-derived mesenchymal stem cells to rats with CCl_4-induced hepatic fibrosis improved liver function, inflammation, and fibrosis *via* a paracrine mechanism possibly by targeting TGF-β1 signaling pathway[139]. Another study showed that transplantation of human umbilical cord blood mesenchymal stem cells substantially improved liver fibrosis in histopathological evaluation compared to that in the untreated group[140]. Infusions of hematopoietic stem cells into mice with methionine-choline-deficient diet- or CCl_4-induced liver fibrosis can reduce hepatic collagen production and the expression of α-smooth muscle actin[141,142].

Overall, there are several potent preventive and therapeutic treatments for liver fibrosis, including physical activity (*e.g.*, running), dietary change (*e.g.*, avoid of high-fat and high-sugar diet), dietary supplementation (*e.g.*, vitamin C), biological treatment (*e.g.*, simtuzumab), bariatric surgery (*e.g.*, Roux-en-Y-gastric procedure), drug (*e.g.*, pegbelfermin), change of gut microbiota (*e.g.*, probiotics), nanoparticles (*e.g.*, BMS-986263), genetic regulation (*e.g.*, non-coding RNAs), and transplantation of stem cells (*e.g.*, hematopoietic stem cells) (Figure 3).

CLINICAL TRIALS

In this section, we first review some completed clinical trials (Clinicaltrials.gov, Table 1). These treatments including biological treatment, drugs, dietary supplementation, and infusion of stem cells.

Future treatments

There is an unmet need for treatments for liver fibrosis due to the efficacy of available treatments. Some drugs with potent anti-fibrotic effects in pre-clinical models are now waiting to be further evaluated in clinical trials (Table 2). The promising preventive and therapeutic treatments for liver fibrosis, including treatment of hepatitis viral infection (*e.g.*, Peginterferon Alfa 2a), transplantation of mesenchymal stem cells, bariatric surgery for patients with obesity and NAFLD, dietary modification (*e.g.*, Mediterranean diet or Calorie-restricted diet).

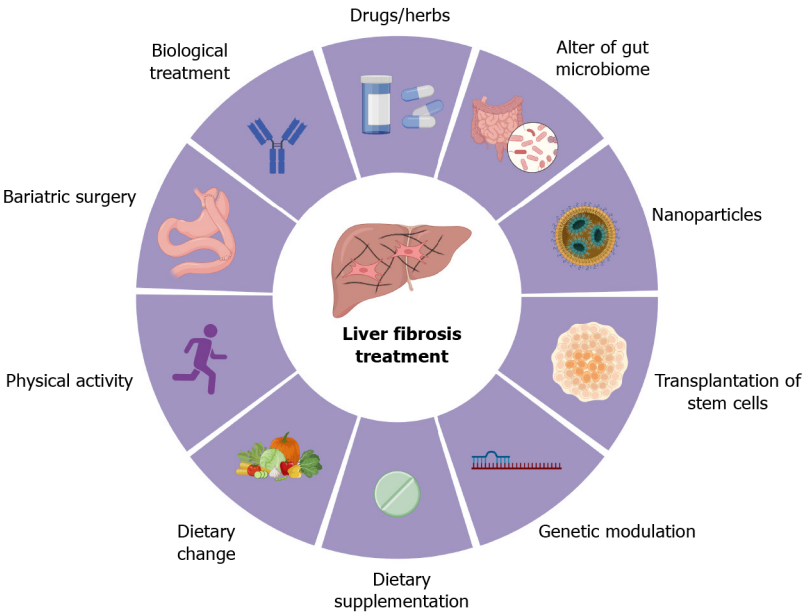
Furthermore, deliver system can be applied to increase the efficiency of anti-fibrotic treatments. For example, BMS-986263, a lipid nanoparticle, has been applied to deliver small interfering RNA to degrade mRNA of heat shock protein 47, a key collagen chaperone involved in the pathogenesis of fibrosis. Treatment of MS-986263 in patients with HCV infection and sustained virologic response improved the Ishak score, the histology activity index score for levels of liver fibrosis[143]. Many other types of nanoparticles have been applied to treat liver fibrosis or its causing chronic liver disease, such as Fibroblast growth factor 2 conjugated superparamagnetic iron oxide nanoparticles[144], cerium oxide nanoparticles[145], and silymarin-conjugated gold nanoparticles[146].

Table 1 Completed clinical trials (Clinicaltrials.gov, accession date: March 1, 2023)

Method	Intervention	Trial number	Phase	Title	Condition
Biological	Peginterferon alfa-2b (SCH 54031)	NCT00049842	3	Prevention of Disease Progress in Chronic Hepatitis C Patients with Liver Fibrosis (Study P02570AM2)	Chronic HCV; Liver fibrosis
	PegIntron (peginterferon alfa-2b; SCH 54031) REBETOL (ribavirin; SCH 18908)	NCT00039871	3	PEG-Intron Plus Rebetol Treatment of Chronic Hepatitis C Subjects Who Failed Response to Alpha-Interferon Plus Ribavirin (Study P02370)	Hepatitis; HCV; Fibrosis; Liver cirrhosis
	Simtuzumab	NCT01707472	2	Study of Simtuzumab in HIV and/or Hepatitis C-Infected Adults with Liver Fibrosis	Liver fibrosis; HCV infection; HIV
	Simtuzumab	NCT01672853	2	Simtuzumab (GS-6624) in the Prevention of Progression of Liver Fibrosis in Adults with PSC	PSC
Dietary regulation	GK#10; Placebo	NCT01598064	N/A	Probiotics for Liver Cirrhosis with Portal Hypertension	Liver cirrhosis; Portal hypertension
Drug	Aramchol	NCT02279524	2	A Clinical Trial to Evaluate the Efficacy and Safety of Two Aramchol Doses Versus Placebo in Patients With NASH	Fatty liver; NASH; Liver fibrosis
	BI 201335	NCT01909778	1	Open Label Single Dose Phase I Trial of BI 201335 to Study Pharmacokinetics and Safety in Patients with Compensated Liver Cirrhosis	HCV; Liver cirrhosis
	BMS-986036; Placebo	NCT03486912	2	A Study of Experimental Medication BMS-986036 in Adults with NASH and Liver Cirrhosis	Hepatic cirrhosis; Liver fibrosis; NAFLD; NASH
	BMS-98603; Other: Placebo	NCT03486899	2	A Study of Experimental Medication BMS-986036 in Adults with NASH and Stage 3 Liver Fibrosis	Liver fibrosis; NAFLD; NASH
	BMS-986263 placebo	NCT03420768	2	A Study of Experimental Medication BMS-986263 in Adults with Advanced Hepatic Fibrosis After Cure of Hepatitis C	Hepatic cirrhosis; Liver fibrosis
	Cenicriviroc placebo	NCT02217475	2	Efficacy and Safety Study of Cenicriviroc for the Treatment of NASH in Adult Participants with Liver Fibrosis	NASH
	CRV431 placebo	NCT04480710	2	A Study of CRV431 Dosed Once Daily in NASH-induced F2 and F3 Subjects	NASH; Fibrosis; NAFLD
	Fuzheng Huayu placebo	NCT00854087	2	Assess the Antifibrotic Activity of Fuzheng Huayu in Chronic Hepatitis C Patients with Hepatic Fibrosis	Chronic HCV
	GI262570 placebo	NCT00244751	2	Antifibrotic Activity of GI262570 In Chronic Hepatitis C Subjects	Cirrhosis, liver
	GR-MD-02 placebo	NCT02462967	2	Clinical Trial to Evaluation the Safety and Efficacy of GR-MD-02 for the Treatment of Liver Fibrosis and Resultant Portal Hypertension in Patients with Nash Cirrhosis	Hypertension, portal
	GR-MD-02; Placebo	NCT02421094	2	Clinical Trial to Evaluate Efficacy of GR-MD-02 for Treatment of Liver Fibrosis in Patients with NASH With Advanced Fibrosis	NASH
	IDN-6556; Placebo	NCT02230670	2	A Study of IDN-6556 in Subjects with Liver Cirrhosis	Liver cirrhosis; Hepatic cirrhosis
	IDN-6556; Placebo	NCT02138253	2	A Trial of IDN-6556 in Post Orthotopic Liver Transplant for Chronic HCV	Liver fibrosis; Liver cirrhosis
	INT-747; Ursodeoxycholic acid; Placebo	NCT00550862	2	Study of INT 747 in Combination with URSO in Patients with PBC	Liver cirrhosis; Biliary injury
	Nitazoxanide; BID	NCT03656068	2	An Evaluation of the Safety and Efficacy of Nitazoxanide on Collagen Turnover in NASH Patients with Fibrosis	NASH; Fatty liver; Fibrosis; Compensated cirrhosis
	Placebo obeticholic acid	NCT00570765	2	Study of INT-747 as Monotherapy in Participants with PBC	Liver cirrhosis, biliary injury
	SEL; Simtuzumab	NCT02466516	2	Safety, Tolerability, and Efficacy of GS-4997 Alone or in Combination with Simtuzumab in Adults with NASH and Fibrosis Stages F2-F3	NASH
	Simeprevir; Daclatasvir;	NCT02349048	2	Study to Assess Efficacy, Safety, Tolerability and	HCV

	Sofosbuvir			Pharmacokinetics of Simeprevir, Daclatasvir and Sofosbuvir in Treatment-naive Participants with Chronic HCV Genotype 1 Infection	
	Tropifexor (LJN452) CVC	NCT03517540	2	Study of Safety, Tolerability, and Efficacy of a Combination Treatment of LJN452 and CVC in Adult Patients with NASH and Liver Fibrosis	NASH
	Peginterferon alfa-2a + Ribavirin; Peginterferon alfa-2a	NCT00006164	3	Long Term Interferon for Patients Who Did Not Clear HCV with Standard Treatment	Chronic HCV; Cirrhosis; Fibrosis; Hepatic cirrhosis
	OMACOR placebo oral capsule	NCT00760513	4	Treatment of non-Alcoholic Fatty Liver Disease With n-3 Fatty Acids	NAFLD
	Ceftriaxone normal saline	NCT04218695	4	Prophylactic Antibiotics in Admitted Cirrhotics	Cirrhosis, LIVER
	Proton pump inhibitors placebo	NCT03175731	4	PPIs and Gastroesophageal Varices in Liver Cirrhosis (PPIs: Proton pump inhibitors)	Liver cirrhosis; Hypertension, portal
Other	Human fetal liver cell transplantation	NCT01013194	1 or 2	Human Fetal Liver Cell Transplantation in Chronic Liver Failure	Liver cirrhosis
	G-colony stimulating factor and infusion of the mobilized monocyte cells	NCT01503749	1	Safety and Efficacy Study of Peripheral Blood Mononuc-leated Cells for Treatment of Liver Cirrhosis	Liver cirrhosis
	Leukapheresis; Infusion of stem cells *via* image-guided scan	NCT00147043	N/A	Adult Stem Cell Therapy in Liver Insufficiency	Liver cirrhosis

N/A: Not applicable; CVC: Cenicriviroc; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; NAFLD: non-alcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis.



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Figure 3 Treatment options for liver fibrosis. Currently, the preventive and therapeutic treatments for liver fibrosis include physical activity (*e.g.*, running), dietary change (*e.g.*, avoid of high-fat and high-sugar diet), dietary supplementation (*e.g.*, vitamin C), biological treatment (*e.g.*, simtuzumab), bariatric surgery (*e.g.*, Roux-en-Y-gastric procedure), drugs and herb medicines (*e.g.*, pegbelfermin), change of gut microbiota (*e.g.*, probiotics), nanoparticles (*e.g.*, BMS-986263), genetic regulation (*e.g.*, non-coding RNAs), and transplantation of stem cells (*e.g.*, hematopoietic stem cells). All cartoons in this figure were prepared using Biorender (https://biorender.com).

CONCLUSION

Liver fibrosis accompanies the progression of chronic liver diseases independent of their etiologies. The initiation and progression of liver fibrosis are mainly driven by liver inflammation and hepatocyte or cholangiocyte injury and damage, resulting in the activation of HSCs and their differentiation into ECM protein-producing myofibroblasts. Thus, current therapeutic options for liver fibrosis are to prevent the initial causing factors for liver inflammation, hepatocyte cell death and oxidative stress. Unfortunately,

Table 2 Recruiting and active clinical trials (Clinicaltrials.gov, accession date: March 1, 2023)

Method	Interventions	NCT number	Phases	Title	Conditions
Drug	ZED1227; Placebo	NCT05305599	2	Different Doses of ZED1227 *vs* Placebo in NAFLD	NAFLD; Fibrosis
	Tropifexor; Licogliflozin; Placebo	NCT04065841	2	Efficacy, Safety, and Tolerability of the Combination of Tropifexor & Licogliflozin and Each Monotherapy, Compared with Placebo in Adult Patients with NASH and Liver Fibrosis	NASH; Liver fibrosis
	Tenofovir disoproxil Fumarate; PEG-Interferon alfa 2a	NCT03957629	N/A	Optimized Treatment of Peginterferon Alfa 2a in Treatment Experienced Patients with HBV-Related Liver Fibrosis	Hepatitis B; Fibrosis
	Sildenafil	NCT04908657	4	Sildenafil for Liver Fibrosis in Adolescents and Adults After Fontan Operation	Fibrosis
	Saroglitazar magnesium	NCT05011305	2	Saroglitazar Magnesium for the Treatment of NASH with Fibrosis	NASH; Fibrosis
	Saroglitazar magnesium	NCT05045482	1	Hepatic Impairment with Cirrhosis Due to Cholestatic Liver Disease	Hepatic impairment; Cirrhosis
	Rivaroxaban apixaban	NCT04874428	1	Direct Oral Anticoagulants (Rivaroxaban and Apixaban) in Patients with Liver Cirrhosis	Cirrhosis
	Resmetirom; Placebo	NCT05500222	3	A Phase 3 Study to Evaluate the Effect of Resmetirom on Clinical Outcomes in Patients with Well-compensated NASH Cirrhosis (MAESTRO-NASH-OUTCOMES)	NASH; Cirrhosis
	Rencofilstat; Placebo	NCT05402371	2	A Study to Evaluate the Efficacy and Safety of Rencofilstat in Subjects with NASH and Advanced Liver Fibrosis	NASH; Fibrosis, Liver NAFLD
	Placebo; Esomeprazole	NCT04448028	4	Stop of Proton-pump Inhibitor Treatment in Patients with Liver Cirrhosis - a Double-blind, Placebo-controlled Trial	Liver cirrhosis
	PHIN-214	NCT05490888	1	Single Dose Escalation of PHIN-214 in Child-Pugh A and B Liver Cirrhotics	Cirrhosis; Fibrosis; Hepatic ascites
	Placebo zibotentan + dapagliflozin	NCT05516498	2	Zibotentan and Dapagliflozin Combination, EvAluated in Liver Cirrhosis (ZEAL Study)	Cirrhosis
	L-ornithine; L-aspartate	NCT05737030	4	Effect of L-ornithine-L-aspertate (LOLA) on the Gut Microbiome	Cirrhosis
	Hydronidone capsules; The placebo capsules	NCT05115942	3	Hydronidone for the Treatment of Liver Fibrosis Associated with Chronic Viral Hepatitis B Phase 3 Trial	Liver fibrosis
	Growth hormone	NCT05253287	2/3	Growth Hormone in Decompensated Liver Cirrhosis	Cirrhosis; Fibrosis
	Empagliflozin 10 MG; Placebo pills	NCT05147090	4	Effects of Empagliflozin on Fibrosis and Cirrhosis in Chronic Hepatitis B	NAFLD; Cirrhosis; Fibrosis
	Cotadutide; Placebo	NCT05364931	2/3	A Study to Evaluate the Safety and Efficacy of Cotadutide Given by Subcutaneous Injection in Adult Participants with Non-cirrhotic Non-alcoholic Steato-hepatitis with Fibrosis	Non-cirrhotic NASH with Fibrosis
	Candesartan; Ramipril	NCT03770936	3	Effect of Some Drugs on Liver Fibrosis	Liver fibrosis
	Branched-chain amino acid; Placebo	NCT03633279	4	Treatment of Sarcopenia Improves the Muscle Mass and Muscle Strength of Patients with Liver Cirrhosis-Child C	Liver cirrhosis
	BMS-986263; Placebo	NCT04267393	2	Safety and Effectiveness of BMS-986263 in Adults with Compensated Cirrhosis (Liver Disease) From Nonalcoholic Steatohepatitis (NASH)	NASH
	AZD4831; Placebo	NCT05638737	2	A Study in Participants with Non-cirrhotic NASH With Fibrosis	Non-cirrhotic NASH with fibrosis
	Atorvastatin; Placebo	NCT05028829	2	Safety and Efficacy of Atorvastatin v. Placebo on HCC Risk	Liver fibrosis; Cirrhosis
Dietary supplement	Leucine enriched essential amino acid; Balanced amino acid	NCT03208868	N/A	Leucine-Enriched Essential Amino Acid Mixture to Reverse Muscle Loss in Cirrhosis	Cirrhosis

	supplement (BAA)				
	Hydroxy methyl butyrate; Balanced Amino Acids	NCT05166499	N/A	HMB Enriched Amino Acids to Reverse Muscle Loss in Cirrhosis	Cirrhosis
Biological	Umbilical cord-derived mesenchymal stem cell comprehensive treatment	NCT03945487	2	Mesenchymal Stem Cells Treatment for Decompensated Liver Cirrhosis	Decompensated liver cirrhosis
	Mesenchymal stem cell	NCT03254758	1/2	A Study of ADR-001 in Patients with Liver Cirrhosis	Decompensated liver cirrhosis
	Human umbilical cord-derived mesenchymal stem cells	NCT05227846	1	Human Umbilical Cord-derived Mesenchymal Stem Cells for Decompensated Cirrhosis (MSC-DLC-1)	Decompensated cirrhosis
	Human umbilical cord-derived mesenchymal stem cell infusion	NCT05331872	1	Umbilical Cord-derived Mesenchymal Stem Cell Infusion in the Management of Adult Liver Cirrhosis	Liver cirrhosis
	Fecal microbiota transplantation; Placebo	NCT04932577	2/3	Fecal Microbiota Transplantation for Liver Cirrhosis	Cirrhosis
	Cellgram-LC	NCT04689152	3	Clinical Trial to Evaluate the Efficacy and Safety of Cellgram-LC Administration in Patients with Alcoholic Cirrhosis	Alcoholic cirrhosis
	Autologous BM MSC	NCT03626090	1/2	Mesenchymal Stem Cell Therapy for Liver Cirrhosis	Cirrhosis
	Allogeneic umbilical cord mesenchymal stem cell	NCT04357600	1/2	Umbilical Cord Mesenchymal Stem Cell for Liver Cirrhosis Patient Caused by Hepatitis B	Cirrhosis
Other	Weight loss	NCT05104541	N/A	Impact of Weight Loss in Cirrhosis with Obesity and MAFLD	Liver cirrhosis
	Lifestyle therapy bariatric surgery	NCT03472157	N/A	A Randomized Controlled Study Evaluating Bariatric Surgery as a Treatment for Severe NASH With Advanced Liver Fibrosis in Non-severe Obese Patients	Surgery; Obesity; NASH; Cirrhosis
	Indo mediterranean diet calorie restricted diet	NCT05073588	N/A	Effect of Indo-Mediterranean Diet on Hepatic Steatosis and Fibrosis in NAFLD Children	NAFLD

N/A: Not applicable; HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

the reverse of liver fibrosis is slowly and frequently impossible for advanced fibrosis or cirrhosis. Liver transplantation is the only therapeutic option for the late stage of liver cirrhosis and cancer. To avoid the life-threatening stage of advanced liver fibrosis and cirrhosis, anti-fibrotic treatments including biological, medicines, dietary change, and behavior prevention are needed. Currently, promising treatments for liver fibrosis are still the preventive strategies, such as treatment of hepatitis viral infection (*e.g.*, Peginterferon Alfa 2a), inhibition of the progression of MAFLD and obesity (*e.g.*, bariatric surgery), dietary modification (*e.g.*, Mediterranean diet or Calorie-restricted diet). In addition, nano-delivery systems have been applied to improve the treatment efficacy and specifically deliver the treatments. Pre-clinical and clinical evaluations for new treatments of liver fibrosis are required while we still lack currently effective strategies for liver fibrosis treatment. The treatment efficacy can be evaluated by histological staining methods, imaging methods, and serum biomarkers, as well as fibrosis scoring systems, such as FIB-4, APRI, and NFS. Although many anti-fibrotic candidate agents have shown robust effects in experimental animal models, their anti-fibrotic effects in clinical trials are less clear. The development of patient-derived organoid models for liver fibrosis may advance the development of compounds with anti-fibrotic properties in the future. In addition, new delivery systems can improve the efficacy of potent treatments and reduce the side effects of therapy. Meanwhile, additional clinical studies are required to confirm the efficacy and safety of treatments.

FOOTNOTES

Author contributions: Zhang CY, Liu S, and Yang M designed and collected data, wrote, revised, and finalized the manuscript; all authors contributed equally and shared the first authorship.

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MINIREVIEWS

Tumor budding as a potential prognostic marker in determining the behavior of primary liver cancers

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Abstract

Hepatocellular (HCC) and intrahepatic cholangiocarcinoma (ICC), the most common primary tumors of the liver, are among the most important causes of cancer deaths worldwide. Because patients with primary liver tumors are frequently diagnosed at an advanced stage and have high mortality, many efforts have been made to identify new markers to determine their behavior and treatment, similar to those in other solid organ tumors. Recently, morphological assessment of tumor budding (TB) has been revealed as a promising prognostic finding to predict tumor behavior and survival across several different tumor types. Currently, the TB score in colorectal cancer has been revealed as an important parameter in pathology report protocols to determine the course of the disease. Regarding the liver, despite enormous data showing that many mechanisms involved in TB are associated with tumor behavior in both HCC and ICC, studies focusing on the role of TB in predicting the behavior and prognosis of these tumors have started to be investigated very recently. The purpose of this review is to present data about TB in primary tumors of the liver, pointing out the potential role of this parameter in determining the course of the disease, and emphasize the need to increase the number of further studies focusing on the evaluation of this parameter with an overview of the mechanisms involved in TB.

Key Words: Tumor budding; Hepatocellular carcinoma; Cholangiocarcinoma; Prognosis; Liver cancer; Epithelial-mesenchymal transition

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Core Tip: This review aims to present recent data on the potential of tumor budding (TB) in determining tumor behavior in hepatocellular carcinoma and cholangiocarcinoma. Although the evidence from the published literature indicates that TB may be a promising prognostic factor for primary liver tumors, more multidisciplinary studies are needed to draw a conclusion. Besides, different assessment techniques in previous investigations indicate that a standard method should be established to provide a solid basis for further studies that may clarify whether this parameter will be included in pathology report protocols as in colorectal carcinoma in the near future.

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INTRODUCTION

Primary liver cancer is the seventh most common cancer worldwide and the fourth leading cause of cancer death[1]. Two types of liver cancer constitute a significant majority of cases: Hepatocellular carcinoma (HCC), originating from hepatocytes and usually accompanied by another underlying disease (75%-85%), and intrahepatic cholangiocarcinoma (ICC), arising from the bile duct epithelium (12%-15%)[2]. Their incidence rates are increasing in many countries and are expected to continue to rise in the next decade[3,4]. Considering that many patients are diagnosed at an advanced stage, there is a lack of current systemic therapy, especially for HCC, and the mortality rates are high, similar to that of other solid organ tumors. Thus, many efforts have been made to identify new markers to determine the course of the disease and the choice of treatment.

Recently, TB has emerged as a promising prognostic parameter to predict tumor behavior and survival across several tumor types[5,6]. After the international TB consensus conference, the first guideline for reporting TB was published in 2017[7]. Subsequently, the TB score in colorectal carcinoma (CRC) has been included as an important parameter in pathology report protocols[8]. These guidelines have also been confirmed to be helpful in cancers of the lung, stomach cancers, and ductal adenocarcinoma of the pancreas[9]. However, regarding primary liver tumors, studies focusing on the relationship between TB and clinicopathological parameters and prognosis are relatively new. Nevertheless, numerous studies have shown that many mechanisms involved in TB are associated with tumor behavior in HCC and ICC[10,11].

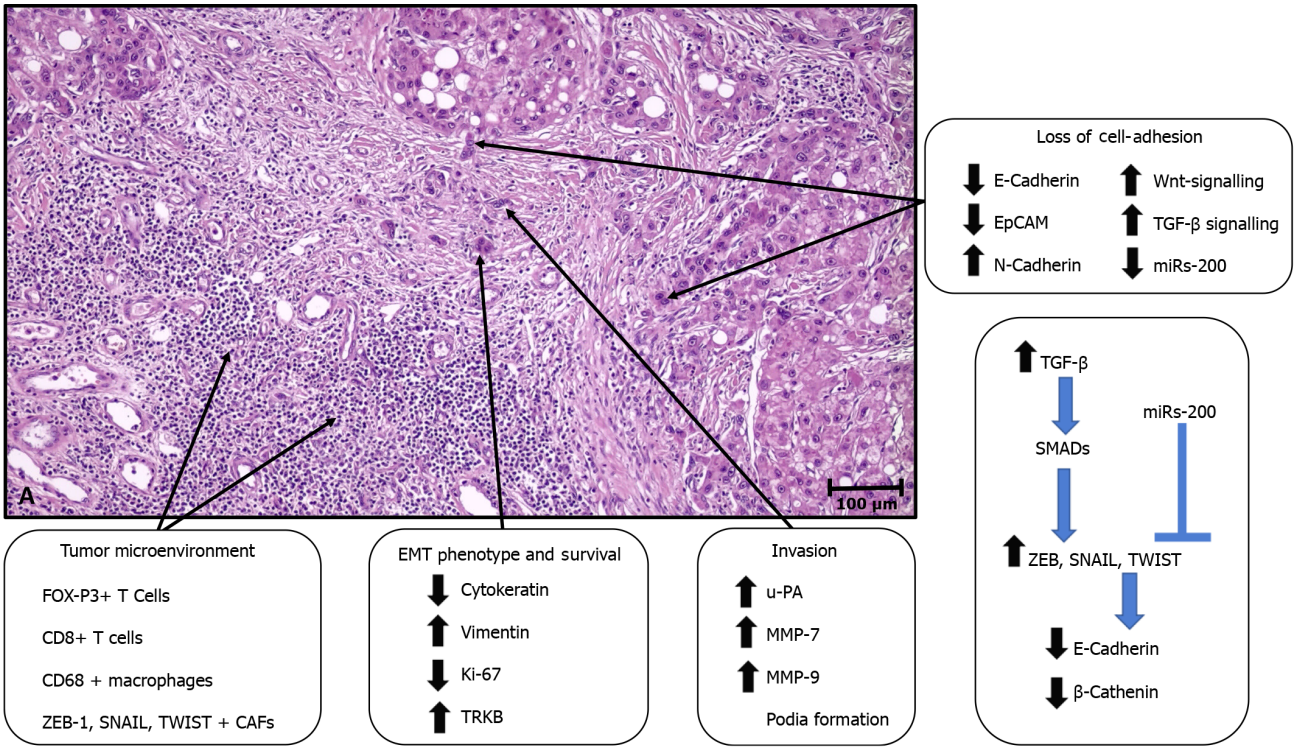
Therefore, this review aims to provide an overview of the events involved in TB, which is also observed in primary liver tumors. Additionally, this review presents the latest data in these tumors to draw attention to the potential role of this parameter in determining behavior and prognosis and underlines the need to increase the number of further studies focusing on the evaluation of this parameter.

GENERAL OVERVIEW OF THE MECHANISMS OF TB

During the invasion-metastasis process in cancers, tumor cells must undergo various changes to invade the surrounding tissue, transition to the vascular system, and finally engage in a parenchymal invasion of metastatic organs[12]. The mechanisms involved in TB are presented in Figure 1.

The epithelial-mesenchymal transition (EMT) program, which contributes to developmental events throughout embryogenesis, has been hypothesized to play a fundamental role in TB formation, particularly in the steps of cell dissociation and cell migration[10-14]. Indeed, accumulating evidence indicates that budding tumor cells might display the properties of cells undergoing EMT to acquire more invasive and migratory capacity.

E-cadherin, an essential cell-cell adhesion protein, plays a pivotal role in cellular dissociation. Therefore, the reports indicating a decrease or loss of expression of E-cadherin in the invasive margin and bud areas in many solid organ tumors, including esophageal, colon, pancreas, endometrial, and oral cancers, are not surprising[15-18]. In addition, the increase in the expression of EMT-related transcription factors in tumor buds that suppress the expression of this protein, including ZEB1, ZEB2, TWIST1, TWIST2, SNAI1 (SNAIL), and SNAI2 (SLUG), is also noted in many malignancies[17-19]. Recently, an increase in the expression of these transcription factors and a decrease in E-cadherin and b-catenin levels in tumor buds compared to tumor bulk have been observed in pancreatic and oral cancers [17,18]. In addition, it has been suggested that the decrease in β-catenin expression parallel to that of E-cadherin may be a finding of WNT-b-catenin signaling pathway activation in tumor buds[20-22].



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Figure 1 Tumor budding in primary liver cancers. A: Tumor budding consisting of small clusters of 4 or fewer tumor cells present at the invasive edge in a case of hepatocellular carcinoma; B: The main processes and mechanisms involved in tumor budding. EMT: Epithelial to mesenchymal transition; EpCAM: Epithelial cell adhesion molecule; CAFs: Cancer-associated fibroblasts; MMP: Matrix metalloproteinase; TRKB: Tyrosine kinase receptor B; u-PA: Urokinase plasminogen activator.

Moreover, data have also shown that TGFβ signaling activation in buds can induce transcriptional repression of E-cadherin by inducing E-cadherin repressors, such as ZEB, TWIST, and SNAIL, *via* deregulation of SMADs[18,23]. However, the observation that different subtypes of EMT transcription factors are increased in some tumors highlights that not all of them should be expected to be increased together in tumor buds[24]. It has been shown that both E-cadherin and molecules such as CD44 and EpCam are lost in TB areas[25-27]. Signature changes in some miRNAs have also been shown to contribute to TB. In particular, changes in the miR-200 family have been noted[28-31]. The levels of miR-200, which has a suppressive effect on the ZEB family that induces E-cadherin expression, were significantly decreased in tumor buds of colorectal and pancreatic ductal adenocarcinoma[32,33].

In TB, the effect of EMT is not limited to cell dislocation; moreover, it significantly affects cell migration through cytoskeletal reorganization, increased cell-associated proteolytic activity, and reprogramming of gene expression[34]. Recently, many studies have shown that these changes are found in budding tumor cells, and marked differences in the expression of genes involved in integrin-mediated cell adhesion, cell migration, cytoskeletal changes, and extracellular matrix degradation have been noted[35].

A monomeric form of laminin 5 gamma 2, which plays a role in the anchorage of epithelial cells to the underlying basement membrane, has been found to increase during tumor invasion and in tumor buds [35,36]. This finding was associated with aggressive tumor behavior, especially in pulmonary[37,38] and colorectal cancers[39-42]. Moreover, in the latter, the dendritic extensions of budding tumor cells are positive for laminin 5 gamma 2, which is associated with vascular invasion[43,44]. In addition, in line with the findings that β-catenin induces gene expression of this protein by binding to TCF and LEF family transcription factors, decreased membranous β-catenin levels, increased nuclear β-catenin levels, positivity for laminin 5 gamma 2, and decreased E-cadherin expression were associated with TB[40,45]. These data indicate that altered expression of β-catenin may participate in multiple events in TB. In addition, other cell migration markers, including motility class III β-tubulin and high-mobility Group A family proteins, are more abundant in invasive and TB sites[46,47]. Furthermore, the expression of proteins such as matrix metalloproteinase 7, matrix metalloproteinase 9 urokinase plasminogen activator and cathepsin B, which degrade the matrix of cells, was found to be significantly increased in tumor buds[41,48-50]. In this region, various metastasis suppressors (such as rapidly accelerated fibrosarcoma kinase inhibitor protein and maspin) are frequently disrupted and/or downregulated in tumor buds compared to the primary tumor mass[51-54].

The survival of malignant cells in the tumor bud largely depends on their adaptation to a hypoxic environment. Studies have shown that these cells overexpress TRKB, a marker of resistance to cell death, and hypoxia-inducible factor 1α[55,56]. In addition, cells in tumor buds have either shallow levels or the absence of proliferation markers (such as Ki-67)[57,58]. These findings support the view that cell proliferation and migration are mutually exclusive processes and that the transition from cell proliferation to invasion may be triggered by hypoxia. Moreover, the fact that budding tumor cells frequently overexpress stem cell markers, such as LGR5, ALDH1, and CD44, suggests the self-renewal capacity of these cells, including those at metastatic sites[26,59-62].

There are also data showing that T cells in the peritumoral stroma (CD8+ T cells and FOXP3+ T cells)[63-65], EMT marker-positive cancer-associated fibroblasts[66-68], the engulfment of budding tumor cells by CD68+ macrophages, and the loss of MHC class I expression may play roles in TB[69-71].

From a morphological point of view, TB is defined as small clusters of 4 or fewer tumor cells at the interface of invasive carcinoma. Although different methods are performed, TB is usually evaluated by determining the most invasive area of the tumor (hot spot) at 20x magnification on hematoxylin and eosin-stained slides. Regardless of tumor type, buds in these areas are counted, and according to the recommendation of TBCC, TB is classified into three grades: Low, intermediate, and high[7].

In the context of HCC and ICC, there is evidence from numerous studies focusing on the mechanisms involved in TB outlined above. Among these, it is noteworthy that the number of studies focusing on the EMT in primary liver tumors is over 200 per year[10,11].

This is not surprising, given the considerable roles of the EMT in tumor behavior and progression[72-74]. Accordingly, the number of studies aiming to detect tumor aggression using comprehensive immunohistochemical and molecular methods far exceeds the number of studies focusing on TB, which can be easily detected as a simple, cost-effective morphological finding from resection materials.

TB IN HCC

Unfortunately, according to the literature, there are very few studies on the relationship between TB and tumor behavior and prognosis in HCC (Table 1). Kairaluoma *et al*[75] studied the prognostic value of TB, including 259 patients with HCC, in a retrospective cohort study from a single institution. TB is evaluated according to the hot spot method, which is recommended when investigating TB in CRC. The overall 5-year survival in bud-negative patients was higher (72.1%) than that in bud-positive patients (29.2%) (P = 0.009). In addition, the difference between the disease-specific 5-year survival rates of these two groups was also significant, 86.5% (in bud-positive patients) *vs* 35.1% (in bud-negative patients) (P = 0.002). Multivariate analysis demonstrated that TB is an independent prognostic factor in surgically treated cases.

However, this parameter was not correlated with clinicopathological factors. This is the only study investigating TB in HCC in a Western population, although it had some limitations, as noted by the authors. There were relatively few patients, yielding wide confidence intervals in the surgical cohort. Additionally, instead of looking for the optimal threshold value, the analysis was performed by making a negative/positive distinction in TB. Again, the absence of significant results in biopsy samples warrants further studies.

Another study was performed in China by Wei *et al*[76] to classify HCC based on TB and immune scores in 423 patients. The authors developed a prognosis-relevant immune score based on five types of immune cells. A classification based on TB grade and immune type was established (IS-TB type). To explore the association between IS-TB type and molecular alterations of HCC, tumor samples and adjacent nontumor tissues from 100 patients were investigated by whole-exome sequencing. TB was classified into three grades. In addition, cases were also divided into high-grade TB (with ≥ 10 buds) and low-grade TB (with 0 to 9 buds) groups. TB was an independent prognostic indicator for overall survival (OS) and disease-free survival (DFS) in the training and validation cohorts. They also observed that high-grade TB was significantly associated with EMT markers and had higher incidences in patients with nonsteatotic, nonfibrolamellar HCC, stromal active (high α-SMA expression), and immature tumors. A link between TB and EMT markers (E-cadherin and vimentin) confirmed the hypothesis that TB might represent the EMT process.

Because the role of the immune milieu of HCC as a prognostic feature is only starting to emerge, they also divided cases by an immune score established based on Z scores that included five parameters (CD8 stromal, PD-L1 stromal, mast-cell stromal, CD68 stromal, and FOXP3 stromal) for each patient. According to the cutoff value (0.04), patients were divided into immune type A and B groups. DFS and OS were better in the type A group than in the type B group in both the training and validation cohorts. The combination of TB grade and immune type cases was also divided into four groups: ISA-TB_{high} (type I), ISB-TB_{high} (type II), ISA-TB_{low} (type III), and ISB- TB_{low} (type IV). While cases within IS-TB type II showed the worst long-term survival, cases within IS-TB type III had the best OS and DFS. These findings are in line with previous observations that indicated that a high lymphocyte-to-TB ratio was a good prognostic factor and that the integration of both TILs and TB was advantageous in the prediction of long-term prognosis in colorectal cancers. These findings provide a rationale for the pathological

Table 1 Relationship of tumor budding with clinicopathologic parameters and survival in hepatocellular and cholangiocarcinomas

Ref.	Tumor	No.	Correlations	Prognosis
Kairaluoma *et al*[75]	HCC	47-R; 212-NR	Not observed; Not observed	OS: TB negative *vs* TB positive; DSS: TB negative *vs* TB positive
Wei *et al*[76]	HCC	423	Tumor subtypes, EMT related marker expression, FOXP3, PD-L1 and CD68 expressions; Frequent mast cell infiltration, p53 mutation (IS-TB type I); CTNNB1 mutation (IS-TB type IV)	DFS: Type II *vs* Type I + Type IV; Type III *vs* Type I + Type IV; OS: Type II *vs* Type I + Type IV; Type III *vs* Type I + Type IV
Okubo *et al*[77]	CCC	299	Dif $^{G1/G2}$ *vs* Dif ^{G3}	OS: TB negative *vs* TB positive
Ogino *et al*[78]	EHCC-PH; EHCC-DC	195; 115	Grade, T, LI, VI, PN, LNM, RSM; Grade, Higher T, LI, VI, PN, LNM	OS: TB low *vs* TB ıntermediate *vs* TB high; OS: TB low *vs* TB high
Tanaka *et al*[80]	ICC	107	Stage, Hilar invasion, Grade, VI, LNM, SM	RFS: TB negative *vs* TB positive; OS: TB negative *vs* TB positive
	Type 1	49	NP	RFS: Not prognostic; OS: Not prognostic
	Type 2	58	NP	RFS: Not prognostic; OS: TB negative *vs* TB positive
	EHCC-PH	54	LI	RFS TB negative *vs* TB positive; OS TB negative *vs* TB positive
	EHCC-DC	40	VI	RFS: Not prognostic; OS: Not prognostic
Ito *et al*[81]	EHCC-PH	78	Grade, T, LNM, M	
		36 NT	Combined HA/PV Resection, Grade, T, LNM, M	DSS: TB low *vs* TB high; RFS: TB low *vs* TB high
		42 WT	Not observed	DSS: TB low *vs* TB high; RFS: Not prognostic
Agostini-Vulaj *et al*[83]	EHCC; ICC	58; 54	Gender, Location, Grade, LNI, PNI, RSM; Gender, Location, Grade, LNI, PNI	DSS: TB ıntermediate *vs* TB high; RFS: TB ıntermediate *vs* TB high
Budau *et al*[84]	ICC	89	NP	OS: TB Low *vs* TB Intermediate *vs* TB High; RFS TB Low *vs* TB Intermediate *vs* TB High ITTB, PTTB, TB
Kosaka *et al*[85]	ICC	235	Size, Tumor type, Grade, VI, MBI, LNM	DSS: TB Low/Intermediate *vs* TB High; RFS: TB Low/Intermediate *vs* TB High
Nakayama *et al*[82]	EHCC-DC	65	T, LNM, LI, VI, ZEB-1 expression, stage	OS: TB Low *vs* TB High

CCC: Cholangiocarcinoma; DFS: Disease free survival; DSS: Disease specific survival; Dif: Differentiation; DM: Distant metastasis; EHCC-DC: Extrahepatic cholangiocarcinoma-distal; EHCC-PH: Extrahepatic cholangiocarcinoma-perihilar; EMT: Epithelial to mesenchymal transition; G: Grade; HA/PV: Hepatic artery and portal vein; HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma; IS: Immunescore; ITTB: Intratumoral tumor budding; LI: Lymphatic invasion; LNM: Lymph node metastasis; M: Metastasis; MVI: Microvascular invasion; NCR: Noncurative resection; No: Number of cases; NR: Non-resectable; NT: Neoadjuvant therapy OS: Overall Survival; PN: Perineural invasion; PTTB: Peritumoral tumor budding; R: Resectable; RFS: Recurrence free survival; RSM: Residual tumor in surgical margin; T: Tumor invasion; TB: Tumor budding; VI: Type I: ISA-TB_{high}; Type II: ISB-TB_{high}; Type III: ISA-TB_{low}; Type IV: ISB-TB_{low}; VI: Vascular invasion; MBI: Major biliary invasion; WT: Without neoadjuvant therapy.

evaluation of the TME in addition to the current pathological classifications of HCC.

Another interesting finding of this study was the association between IS-TB type and molecular alterations. TP53 (mainly within IS-TB type I) and CTNNB1 (mainly within IS-TB type IV) mutations in two distinct HCC phenotypes exhibit different immune and pathological characteristics. While TP53 mutations were related to poor differentiation and a thick trabecular pattern, CTNNB1 mutations were associated with impaired antitumor immunity (immune type B), well-differentiated morphology, a pseudoglandular pattern, mature stroma, and low α-SMA (fibroblast activation protein) expression.

As noted above, despite the scarcity of studies examining TB in HCC, there is a wealth of data on the processes involved in this phenomenon.

TB IN CHOLANGIOCARCINOMA

Several studies focusing on TB in cholangiocarcinomas have recently been performed. The number of studies, including extrahepatic perihilar (EHCC-PH) and distal cholangiocarcinoma (EHCC-D) cases, exceeded the number of studies that included ICC cases in the study group. The characteristics and

results of these studies are summarized in Table 1.

In an earlier investigation of cholangiocarcinomas from all anatomical locations (CCC), TB was associated with the grade but not with the course of the disease[77]. However, in a more recent study, in addition to high grade, high TB was more frequently observed in males and patients with extrahepatic localization, perineural and lymphatic invasion, and presentation in settings with positive resection margins[78]. Moreover, TB is an independent prognostic factor for CCC. However, since TB scoring differed in these two studies, it is not possible to compare the results of one with the other (Tables 1 and 2), as noted by Regmi *et al*[79], who performed a meta-analysis of CCC samples from different locations, including tumors of the ampulla and gallbladder.

In EHCC-PH, TB is associated with tumor invasion, lymph node metastasis, perineural invasion, lymphovascular invasion, and positive resection margin status. It has also been shown to be an independent prognostic factor in determining the course of the disease in all of the studies[78,80,81]. In EHCC-D, higher TB was more frequent in tumors with deeper invasion, lymph node metastasis, and lymphovascular and perineural invasion[78,80,82]. The correlation between TB and stage and ZEB-1 expression was also noted[83]. Similar to EHCC-P, all but one study[81] showed that TB effectively determines the course of the disease, as shown by both univariate and multivariate analyses[77,78,81-84].

Regarding ICC, TB was shown to be correlated with stage, hilar invasion, grade, venous invasion, lymph node metastasis, and positive surgical margins, which are important parameters for determining the behavior of these tumors. Moreover, when ICCs were analyzed according to growth patterns, it was noted that 80% of mass-forming tumors had high TB. In contrast, this ratio was 16% and 2.3% in periductal infiltrating and intraductal growing subtypes, respectively[85]. In addition, the prognostic role of TB has been described[77,80,81,85]. Budau *et al*[84] analyzed TB using a three-tier grading system: high, intermediate, and low. While patients with low TB had the most favorable recurrence survival, high TB was associated with the most unfavorable outcomes.

Similarly, TB correlated significantly with the overall survival of patients in univariate and multivariate analyses ($P < 0.001$). In addition, their data demonstrated that in ICC, TB is significantly independent of the area of investigation (intratumoral or peritumoral). These findings indicate the possibility that TB assessment in preoperative tissue biopsies and in cases that would not be suitable for resection could be used to predict tumor behavior. Nevertheless, the evidence for intratumoral TB is still weak.

In another study, TB was observed to be a powerful prognostic factor for RFS and OS in ICC[80]. In patients stratified into negative and positive TB status, the median time to recurrence in cases with positive TB was 10.26 mo. This was significantly shorter than that of subjects with negative TB (35.57 mo), and the difference among median survival times was significant ($P < 0.001$). Furthermore, the results of the same study indicated that TB was a decisive and powerful prognostic factor for OS (HR: 4.547). Although these findings need to be supported by further large-scale studies, they suggest that TB may be an important prognostic parameter in these tumors.

Tanaka *et al*[80] presented an interesting finding about TB in ICC in an elegant study. When they evaluated TB by dividing ICC into two subgroups, Type 1 (hilar) and Type 2 (peripheral), according to the combined scores of mucin productivity and immunoreactivity of S100P, N-cadherin, and neural cell adhesion molecule, this parameter was determined to be a decisive prognostic factor in Type 2 but not in Type 1. They suggested that some differences exist in the biological behavior of these subtypes and pointed out that despite the prognostic importance of TB in ICC, its pathogenetic role in biliary tract carcinomas might differ by anatomic location. However, this finding needs to be supported in further studies. Nevertheless, the results of TB studies in ICC are similar and support the suggestion that TB is a relevant prognostic factor in the histopathological evaluation of these tumors.

Generally, different scoring methods have been used to investigate TB in cholangiocarcinomas. In a few studies, unlike the recommendation of TTBC, five cells were taken as the cutoff for the definition of TB[77,78,81]. The analyses were performed by categorizing the cases as negative vs. positive or low vs. high TB. In most other studies, including ICC cases, patients were assessed following the three-tiered system recommended by the TTBC for colorectal cancer[80,82-84]. However, different stratifications were used for further evaluations (Table 2). More recently, in an elegant study, Zlobec *et al*[86] observed that CRC without TB (TB0) is relatively frequent and provided additional information on tumor behavior, suggesting a new "zero budding" category for TB. There is currently no evidence about the prognostic value of TB0 in cholangiocarcinomas, and it would be interesting to conduct further studies in which this category is addressed separately.

Accumulated data indicate that the preferred staining method for scoring TB is HE. Recently, some studies on TB have reported that IHC is superior to HE regarding reproducibility and interobserver agreement in assessing this parameter in CRC. Regarding CCC, Ogino *et al*[78] obtained TB scores in HE-stained whole-tissue sections and PanCK immunostained tissue microarray (TMA) sections from 266 patients. They observed that the number of tumor buds in HE-stained slides was almost equal to that in PanCK-stained slides from TMA, with a strong correlation between them ($R = 0.763$, $P < 0.001$). This finding also supports that evaluating TB in HE-stained sections is a simple and reproducible method. Nevertheless, more studies are needed to standardize the assessment of TB in ICC because grading systems for this parameter vary between different types of cancer.

Table 2 Criteria applied for tumor budding in previous studies

Ref.	Tumor	Tumor budding criteria
Kairaluoma *et al* [75]	HCC	Evaluation was performed according to median values; Negative: No buds were found; Positive: At least one bud was present
Wei *et al*[76]	HCC	Association between TB and clinicopathological parameters; Grade 1 (0-4), Grade 2 (5-9), Grade 3 (≥ 10); For survival analysis; Low grade (0-9), High grade (≥ 10)
Okubo *et al*[77]	CCC	Negative: < 5 budding focus; Positive: ≥ 5 budding focus
Ogino *et al*[78]	EHCC-PH, EHCC-DC	Cut-off values of TB obtained by recursive partioning technique; For EHCC-PH; Low grade (0-4), Intermediate grade (5-11), High grade (≥ 12); For EHCC-DC; Low grade (0-4), High grade (≥ 5)
Tanaka *et al*[80]	ICC, EHCC-PH, EHCC-DC	Low grade (0-4), Intermediate grade (5-9), High grade (≥10)
Ito *et al*[81]	EHCC-PH	Low TB: < 5 budding focus; High TB: ≥ 5 budding focus
Agostini-Vulaj *et al*[83]	ICC, EHCC	Grade 1 (0-4), Grade 2 (5-9), Grade 3 (≥ 10)
Budau *et al*[84]	ICC	Grade 1 (0-4), Grade 2 (5-9), Grade 3 (≥ 10)
Kosaka *et al*[85]	ICC	Low grade (0-4), Intermediate grade (5-9), High grade (≥ 10)
Nakayama *et al* [82]	EHCC-DC	Low TB (0-4), High [TB Grade 2 (5-9) and 3 (≥ 10)]

CCC: Cholangiocarcinoma; EHCC-DC: Extrahepatic cholangiocarcinoma-distal; EHCC-PH: Extrahepatic cholangiocarcinoma-perihilar; HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma.

In CRC, TB, combined with other established biomarkers, may allow us to discriminate between patients who would benefit from oncological resection and patients who will receive adjuvant therapy and to classify different therapeutic options, especially in advanced-stage patients[87]. Thus, TB can predict prognosis and regulate treatment options in primary liver cancers. However, the role of TB in the treatment of these tumors remains to be investigated.

CONCLUSION

This review highlights that TB may be a promising prognostic factor for primary liver tumors. However, its clinical value in managing patients should be established in multidisciplinary studies. Evidence also suggests that TB in HCC can identify and reclassify tumors of molecular subtypes with different behavioral characteristics. The differences in the classification of TB in primary liver tumors indicate that a standard and validated method should be established to provide a solid basis for large-scale clinicopathological studies for further evaluation. In addition, the precise determination of the value of budding tumor assessment with multiple further studies may allow us to clarify whether this parameter will be included in pathology report protocols as in CRC in the near future.

FOOTNOTES

Author contributions: Celik MY, Gedik EO, Unal B performed the data acquisition; Bassorgun CI, Unal B and Elpek GO designed the outline and coordinated the writing of the paper; all authors equally contributed to the writing of the paper and preparation of the tables and illustrations.

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MINIREVIEWS

Role of vascular endothelial growth factor B in nonalcoholic fatty liver disease and its potential value

Yu-Qi Li, Lei Xin, Yu-Chi Zhao, Shang-Qi Li, Ya-Nuo Li

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Abstract

Nonalcoholic fatty liver disease (NAFLD) refers to fatty liver disease caused by liver injury factors other than alcohol. The disease is characterized by diffuse fat infiltration, including simple steatosis (no inflammatory fat deposition), nonalcoholic fatty hepatitis, liver fibrosis, and so on, which may cause liver cirrhosis, liver failure, and even liver cancer in the later stage of disease progression. At present, the pathogenesis of NAFLD is still being studied. The "two-hit" theory, represented by lipid metabolism disorder and inflammatory reactions, is gradually enriched by the "multiple-hit" theory, which includes multiple factors, such as insulin resistance and adipocyte dysfunction. In recent years, vascular endothelial growth factor B (VEGFB) has been reported to have the potential to regulate lipid metabolism and is expected to become a novel target for ameliorating metabolic diseases, such as obesity and type 2 diabetes. This review summarizes the regulatory role of VEGFB in the onset and development of NAFLD and illustrates its underlying molecular mechanism. In conclusion, the signaling pathway mediated by VEGFB in the liver may provide an innovative approach to the diagnosis and treatment of NAFLD.

Key Words: Nonalcoholic fatty liver disease; Vascular endothelial growth factor B; "Two-hit" theory; "Multiple-hit" theory; Obesity

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Core Tip: Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease with lipid accumulation caused by liver injury factors except alcohol. At present, vascular endothelial growth factor B (VEGFB) has been reported to play a special role in regulating lipid metabolism and improving the onset and development of NAFLD. Therefore, the use of VEGFB as a target for treatment has become the focus of current research. This review summarizes the role and potential mechanism of VEGFB in the pathogenesis of NAFLD to provide a theoretical basis for the clinical treatment of NAFLD.

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INTRODUCTION

With the growth of the economy and the increasing change in people's lifestyles, the prevalence and morbidity of nonalcoholic fatty liver disease (NAFLD) are rising rapidly worldwide. NAFLD occurs in one-fourth of the global population, and the highest incidence rate is in South America (31%) and the Middle East (32%), followed by Asia (27%), the United States (24%), and Europe (23%), while is not common in Africa (14%)[1]. In the United States, the number of NAFLD cases is expected to increase from 83.1 million in 2015 (approximately 24% of the population) to 100.9 million by 2030[2]. Because of its long course and high treatment cost, it has become a global medical and health problem.

NAFLD is an important cause of advanced liver disease, primary liver cancer, and liver transplantation and is also the world's fastest-growing cause of liver-related deaths[3]. In the United States, the burden of NAFLD-related cirrhosis is estimated to be twice that of hepatitis C virus (HCV) related cirrhosis, and it is expected to surpass HCV as the main indication for liver transplantation within 5 years[4]. In Asia, the incidence rate of hepatocellular carcinoma in patients with NAFLD is 1.8/1000 each year, and the total case fatality rate is 5.3/1000 each year[5]. In addition, insulin resistance, upregulation of insulin-like growth factor axis, downregulation of adiponectin expression, and elevated expression of tumor necrosis factor α (TNFα) caused by NAFLD may be potential factors to induce the development of tumors[6]. Meanwhile, NAFLD can also promote coronary atherosclerosis, significantly increase the risk of cardiomyopathy (mainly left ventricular hypertrophy), leading to valvular heart disease (mainly aortic valve and mitral valve), cardiac insufficiency, arrhythmia (mainly atrial fibrillation, prolonged QT interval) and some cardiac conduction system defects (such as an atrioventricular block)[7]. Therefore, more and more research is focusing on exploring the pathogenesis of NAFLD.

The physiological mechanism of NAFLD is very complex. The pathogenesis of early NAFLD is generally believed to be related to lipid metabolism and inflammatory reactions, which could not systematically and comprehensively explain the molecular mechanism and metabolic changes in NAFLD[8,9]. In recent years, studies have confirmed that insulin resistance is closely related to the pathogenesis of NAFLD[10]. In 2019, Lee *et al*[11] reported that NAFLD was related to liver and peripheral insulin resistance, leading to insufficient inhibition of liver insulin resistance, gluconeogenesis, reduced glycogen synthesis, and increased free fatty acid (FFA). Shi *et al*[12] confirmed that insulin resistance can promote the progression of liver fibrosis and NAFLD, and NAFLD can also accelerate insulin resistance in the liver.

With the deepening of research on NAFLD and the increasing understanding of its pathogenesis, it has been found that the onset of NAFLD is also related to "multiple-hit" such as liver insulin resistance, adipocyte dysfunction, gut microbiota imbalance, immune regulation imbalance, and dietary habits besides the "second-hit" caused by lipid metabolism disorder and inflammation reaction. Adolph *et al*[13] found that abnormal adiponectin secretion produced by adipocytes can aggravate high-fat diet (HFD)-induced obesity and related metabolic disorders, and the overexpression of adiponectin can hinder the progression of hepatic microsomal steatosis. Baker *et al*[14] found that the content of enzymes that can metabolize ethanol in the body of patients with NAFLD and intestinal flora imbalance increased significantly, which increased the permeability of the intestinal wall and was conducive to the entry of reactive oxygen species (ROS), bacterial endotoxins, ethanol and other toxic metabolites into the liver, resulting in increased liver damage andaccelerating the progression of NAFLD (Figure 1).

Early in 2008, Karpanen *et al*[15] unexpectedly found that vascular endothelial growth factor B (VEGFB) has a weak role in the vascular system but has a significant advantage in regulating lipid metabolism. In 2012, Hagberg *et al*[16] proved that targeting VEGFB as a novel treatment for insulin resistance and type 2 diabetes. In 2016, Robciuc *et al*[17] also found that transferring the VEGFB gene into HFD-induced obese mice can improve lipid metabolism and increase insulin supply and signal transduction. Hu *et al*[18] confirmed that VEGFB recombinant protein can reduce lipid accumulation and improve hyperlipidemia in NAFLD.

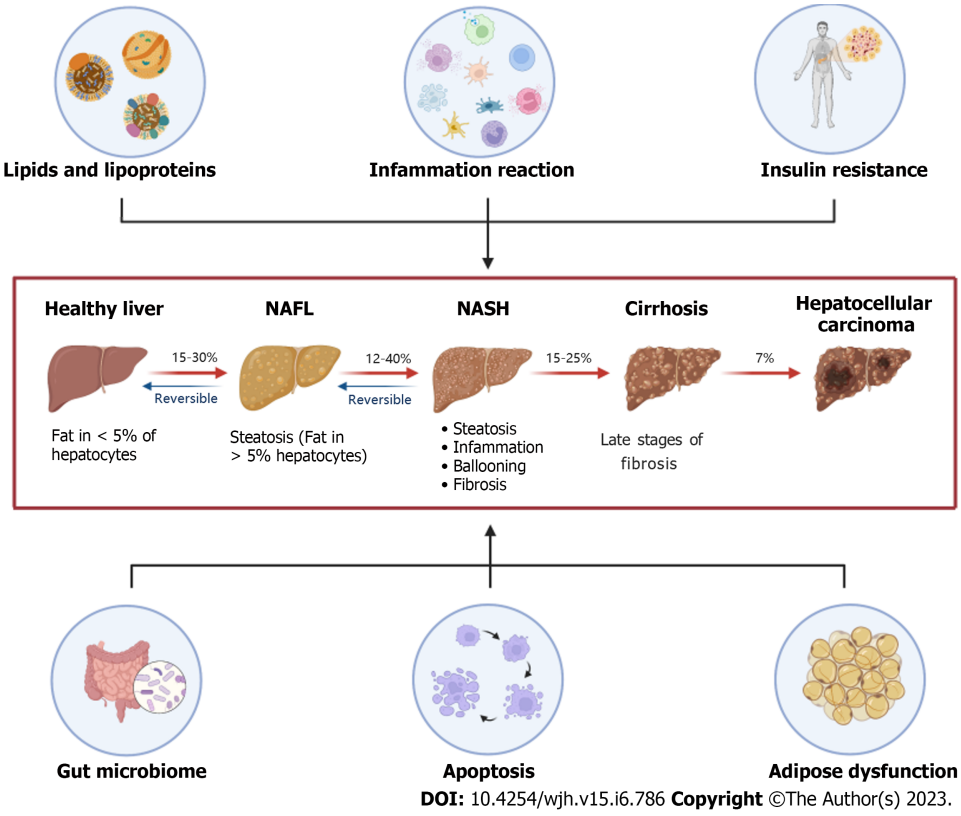


Figure 1 The "multiple-hit" theories are involved in the progress of nonalcoholic fatty liver disease. Lipids and lipoproteins represent the "first-hit", while the inflammation reaction illustrates the "second-hit" in the development of nonalcoholic fatty liver disease (NAFLD). Six aspects including lipids and lipoproteins, inflammation reaction, insulin resistance, gut microbiome, apoptosis, and adipose dysfunction have a common influence on the pathophysiological mechanism of NAFLD. NAFL: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis.

The regulatory role of VEGFB in the occurrence and development of metabolic diseases has attracted many scholars' attention. In this review, we mainly focus on the underlying mechanism of VEGFB in the onset of NAFLD and analyze how VEGFB participates in the "multiple-hit" of NAFLD by regulating lipid metabolism, inflammatory reactions, adipocyte dysfunction, and cell apoptosis. First, we introduce the positive regulatory effect of VEGFB on lipid metabolism and discuss how it affects fatty acid oxidation and lipid synthesis under the mediation of Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) signaling. Then, we summarize the role of VEGFB in anti-inflammation in NAFLD and further discuss the current mechanism of VEGFB in insulin resistance and the impact of targeted therapy. Finally, we also explain the controversial role of VEGFB in metabolic diseases and estimate whether VEGFB-mediated signal transduction could provide a theoretical and experimental basis for the pathogenesis of NAFLD and help identify potential treatment targets.

THE NOVEL ROLE OF VEGFB IN NAFLD

VEGFB is a special type of vascular endothelial growth factor. The total length of the VEGFB gene is 1197 bp, with 7 exons, and the total length of the CDS region is 566 bp, with two subtypes, VEGFB^{167} and VEGFB^{186}[19]. The VEGFB^{167} homotype has a similar effect to that of VEGFB^{186}[17]. VEGFB is a glycoprotein that forms a homodimer through the covalent binding of disulfide bonds. It needs to combine with a high-affinity tyrosine kinase receptor to exert biological effects[20]. The VEGF family includes seven members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor[21]. The VEGF receptor family includes VEGFR1, VEGFR2, VEGFR3, and neuropilin 1/2 (NRP1/2). VEGFA can combine with VEGFR1 or VEGFR2 to play a role in promoting angiogenesis[22]. Unlike other members of the VEGF family, the effect of VEGFB on vascular endothelial growth is not obvious. The biological function of VEGFB is exerted by forming a complex with VEGFR1. Moreover, the combination of VEGFB and NRP1 can also induce a series of reactions through a paracrine mechanism [23]. In recent years, studies have shown that the VEGFB/VEGFR1 pathway has therapeutic potential for obesity, type 2 diabetes, and other lipid metabolism disorder-related diseases[17].

VEGFB mainly exists in the heart, skeletal muscle, brown adipose tissue, and other tissues with high metabolic activity and plays a role in regulating blood vessel distribution and lowering blood lipids[24]. The level of VEGFB in the liver is also significantly higher than that in tissues with general metabolic

activity. Shang *et al*[25] found that cardiac-specific overexpression of VEGFB can reduce the activity of lipoprotein lipase and improve the metabolic level of myocardial cells. Wagenmakers *et al*[26] showed that VEGFB can control the expression of fatty acid transport protein (FATP) in the capillary endothelium and connect the uptake of endothelial FFA with the oxidation ability of the skeletal muscle to potentially prevent the accumulation of skeletal muscle lipotoxic FFA. Robciuc *et al*[17] confirmed that the complex of VEGFB and VEGFR1 can reshape the vascular distribution in adipose tissue and improve the insulin function of obese mice.

VEGFB also plays a biological role in the liver by forming a complex with VEGFR1. Cordeiro *et al*[27] showed that targeting VEGFB can effectively prevent lipid deposition in peripheral tissues in animal models. Hu *et al*[18] observed that the complex of VEGFB and VEGFR1 can increase the oxidation level of fatty acids in liver tissue and hepatocytes and reduce obesity-related hyperlipidemia and fatty liver disease in HFD-induced liver. Li *et al*[28] found that inhibiting VEGFB gene expression in liver tissue not only increased the weight and body fat rate of obese mice but also led to pathological changes, such as hepatocyte steatosis and liver fibrosis. These studies suggest that VEGFB is involved in the onset and development of simple steatosis and liver fibrosis in NAFLD (Figure 2).

VEGFB PARTICIPATES IN REGULATING THE "FIRST HIT" IN NAFLD

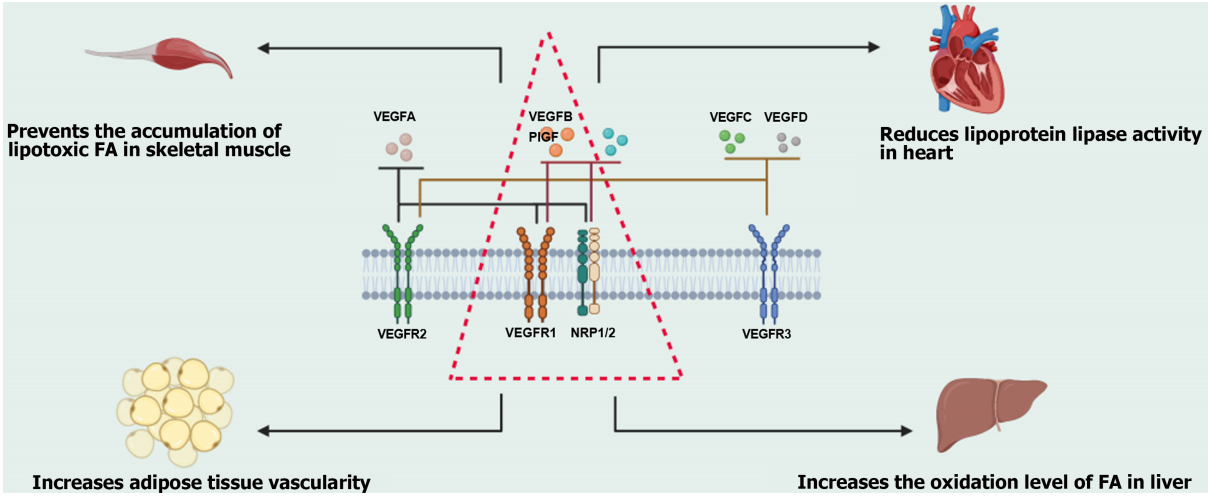
In 1998, Day *et al*[29] first proposed the "two-hit" theory of the pathogenesis of NAFLD. The "first hit" of NAFLD mainly involves lipid metabolism disorder caused by various factors. Hepatotoxicity is caused by FFA, which leads to an increase in the permeability of the cell membrane, destruction of mitochondrial function, and inhibition of related enzymes to produce genotoxicity. As the disease progresses, excess FFA undergoes β oxidation in mitochondria. When the capacity of the mitochondria to β oxidize FFA is overloaded, excess FFA accumulates in the liver and aggravates the steatosis of hepatocytes. Meanwhile, the triglyceride (TG) synthesized by excess FFA in the liver cannot be converted into very low-density lipoprotein for transport to the peripheral adipose tissue for storage. Therefore, TG can only be stored in the liver and eventually aggravate the onset of liver steatosis. Reducing lipid accumulation and restoring the balance of lipid metabolism are the key methods to improve the "first hit" of NAFLD.

The research findings on the role of VEGFB in improving the lipid disorder of the heart, skeletal muscle, and brown adipose tissue provide the theoretical and experimental basis for VEGFB to participate in the regulation of hepatic lipid metabolism in NAFLD. Shang *et al*[25] observed that rat heart lipoprotein lipase activity and lipid accumulation were decreased and insulin function was improved after cardiac-specific overexpression of VEGFB. Li *et al*[30] found that VEGFB can enhance the expression of FATP1 and FATP4 in C2C12 cells, promote the oxidation of FFA and the decomposition of TG in C2C12 myotubes, and inhibit the re-esterification of FFA to reduce lipid accumulation in myotubes. Chen *et al*[31] found that after inhibition of adipose-specific VEGFB, mice increased in size with more white adipose tissue, and the form and function of fat changed from those of brown adipose tissue to those of white adipose tissue, which indicated that VEGFB was the main regulator of the growth and function of fat.

Some scholars have proposed that the signaling pathway triggered by the combination of VEGFB with its receptor can promote lipid flow in the body, which may become a promising target to prevent the accumulation of ectopic lipids. In 2020, Tong *et al*[32] showed that VEGFB can reduce the levels of TG and FFA in the liver to prevent HFD-induced fatty liver disease by producing E. coli-expressed recombinant tPep-VEGFB. In 2021, Hu *et al*[18] found that recombinant VEGFB protein reduced the increase in high-density lipoprotein and low-density lipoprotein in the liver caused by HFD and reduced liver hyperlipidemia.

The mechanism of NAFLD involves multiple signaling pathways, of which the AMPK signaling pathway plays a key role in de novo synthesis and fatty acid oxidation[33]. Harjes *et al*[34] confirmed that the combination of VEGFB with its receptor VEGFR1 can activate AMPK, FATP3, and FATP4 to potentially promote the usage of FFA. AMPK activation is regulated by its upstream molecule Ca^{2+}/Calmodulin-dependent protein kinase β (CaMKKβ), which responds to increased intracellular calcium content[35]. Extracellular calcium ions enter the cell through a calcium channel carrier. The elevated intracellular calcium level causes the conformational change of CaMKKβ and the phosphorylation of AMPK[36]. Jia *et al*[37] showed that a high concentration of VEGFB recombinant protein can increase the level of calcium ions in MIN6 cells to increase insulin secretion. Li *et al*[28] suggested that inhibiting the expression of VEGFB in the liver can reduce the expression level of CaMKKβ and then affect the phosphorylation level of AMPK induced by CaMKKβ.

AMPK can control lipid metabolism by regulating its downstream molecules after its phosphorylation[38]. AMPK can directly phosphorylate and inhibit the activation of acetyl coenzyme A carboxylase (ACC), the rate-limiting enzyme that inhibits the synthesis of fatty acids, thereby activating carnitine palmitate transferase (CPT1) and transferring fatty acids into mitochondria for β oxidation[39, 40]. AMPK can also negatively regulate the expression of sterol-regulatory element-binding protein-1c (SREBP1c), downregulate the level of desaturase [stearoyl-CoA desaturase-1 (SCD1)], and inhibit the



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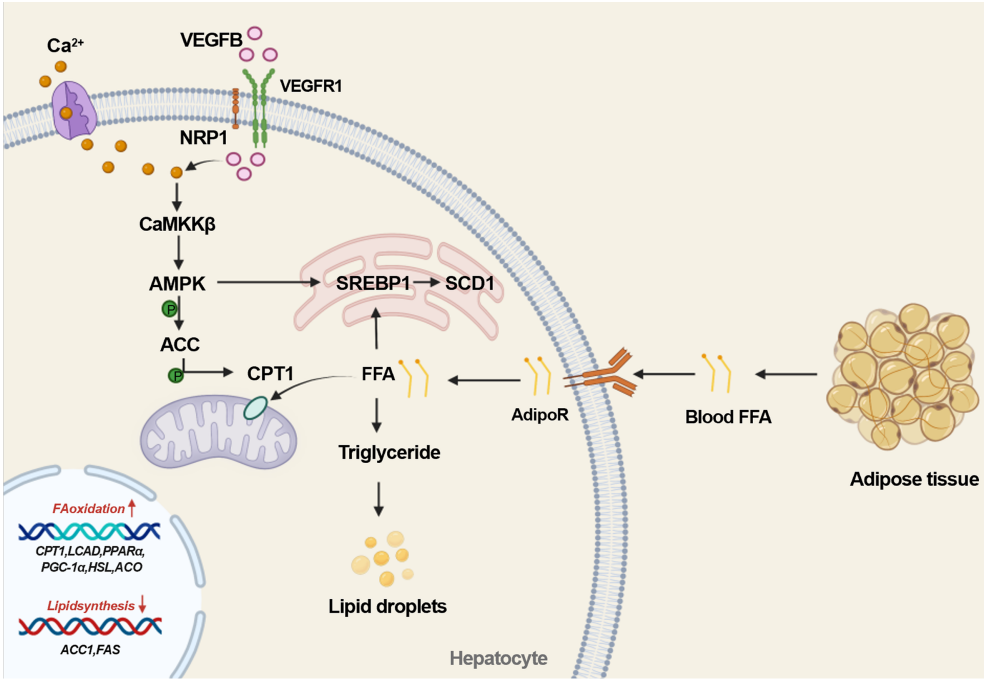
Figure 2 The vascular endothelial growth factor family and its receptors, and the biological function of vascular endothelial growth factor B. The vascular endothelial growth factor (VEGF) family includes VEGFA, vascular endothelial growth factor B (VEGFB), VEGFC, VEGFD, and so on. The VEGF receptor family includes VEGFR1, VEGFR2, VEGFR3, and neuropilin 1/2 (NRP1/2). VEGFA combines with VEGFR1, VEGFR2, or NRP1/2. VEGFB and placental growth factor combine with VEGFR1 or NRP1/2. VEGFC and VEGFD combine with VEGFR2 or VEGFR3 to exert their biological functions. VEGFB can prevent the accumulation of lipotoxic free fatty acid (FFA) in skeletal muscle, reduce lipoprotein activity in the heart, increase adipose tissue vascularity, and increase the oxidation level of FFA in the liver. PIGF: Placental growth factor; FA: Fatty acid; NRP1/2: Neuropilin 1/2; VEGF: Vascular endothelial growth factor; VEGFB: Vascular endothelial growth factor B.

synthesis of fatty acids and TG[41]. Hu *et al*[18] showed that VEGFB recombinant protein can upregulate the AMPK/ACC/CPT1 signaling pathway in the liver by binding to VEGFR1, promoting FFA oxidation and reducing lipid deposition. That study also found that VEGFB can simultaneously upregulate the expression levels of the lipid oxidation-related genes PPARα, PGC-1α, HSL, ACO, and CPT1 and that the downregulation of lipid synthesis can inhibit weight gain under HFD conditions and improve obesity-related hyperlipidemia and fatty liver disease[18]. Li *et al*[28] also found that VEGFB knockout can downregulate the CaMKKβ-mediated AMPK/ACC/CPT1 signaling pathway to inhibit fatty acid oxidation and activate the AMPK/SREBP1/SCD1 signaling pathway to promote lipid synthesis, thus affecting the level of lipid metabolism (Figure 3).

VEGFB PARTICIPATES IN REGULATING THE "SECOND HIT" IN NAFLD

Liver lipid accumulation induces overloaded lipid catabolism, causing lipid peroxidation. Excessive lipid peroxidation leads to oxidative stress, making it the "second hit" to the progression of NAFLD, which can accelerate inflammation and hepatocyte damage. Nuclear factor-kappa B (NF-kB) signaling plays an important role in the macrophage-mediated liver inflammatory response[42]. Research has confirmed that NF-kB can be activated by FFA in patients with NAFLD, and as the severity of NAFLD increases, the activity of NF-kB increases[43]. Moreover, liver mitochondrial dysfunction also accelerates the occurrence and development of NAFLD. The compensated acceleration of β oxidation in mitochondria can produce a large number of ROS[44]. When the antioxidant system mainly composed of reduced glutathione fails to eliminate ROS in time, oxidative stress develops[45], and a large number of peroxides are generated, which aggravate hepatocyte damage[46]. The apoptotic bodies produced by hepatocyte apoptosis are engulfed by Kupffer cells to decrease the activity of endothelial nitric oxide synthase (eNOS), which affects Mitochondrial function.

VEGFB can induce cell proliferation and differentiation, tumor immunity, and other biological effects through the signaling pathway mediated by the tyrosine-protein kinase receptor[47]. Kusuhara *et al*[48] observed that the VEGFR1 signal in monocytes and macrophages was significantly affected by the upregulation of VEGFB under inflammatory conditions. Akiyoshi U confirmed that VEGFR1 can regulate AKT signaling and affect the activity of NF-KB and eNOS respectively to regulate macrophage migration and mitochondrial function[49]. Mehlem *et al*[50] found that VEGFB signaling is involved in regulating pathological lipid accumulation in diabetes, obesity, and cardiovascular disease and mainly affects mitochondrial genes related to the regulation of fatty acid intake. Cao *et al*[51] showed that VEGFB/IL-17 inhibits the expression of fatty acid transporters to reduce the accumulation of renal lipids and inhibit renal oxidative stress and mitochondrial dysfunction, thus improving the inflammatory response. Shen *et al*[52] also found that VEGFB/IL-22 can not only regulate glucose and lipid metabolism but also reduce inflammation and ROS accumulation. Robciuc *et al*[17] transduced the



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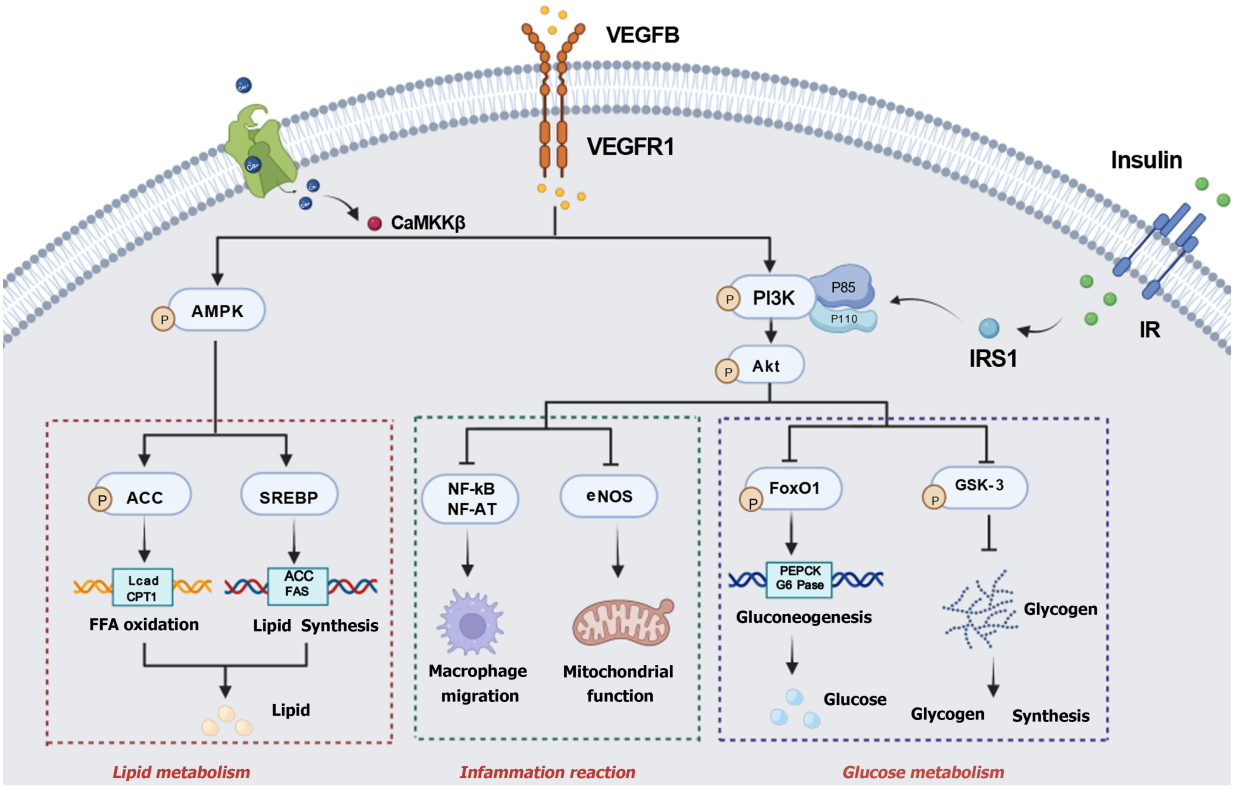
Figure 3 Vascular endothelial growth factor B regulates lipid metabolism in the "first hit" of nonalcoholic fatty liver disease *via* the activated protein kinase signaling pathway. Vascular endothelial growth factor B (VEGFB) performs its biological function by combining with VEGFR1. Once it enters the cell, it activates Calmodulin-dependent protein kinase β (CaMKKβ), which is induced by an increase in intracellular Ca^{2+} content. VEGFB activates the CaMKKβ-mediated activated protein kinase (AMPK)/A carboxylase (ACC)/carnitine palmitate transferase (CPT1) signaling pathway and related genes, such as CPT1 and long-chain acyl coenzyme A dehydrogenase, which regulate FFA oxidation in mitochondria. VEGFB activates AMPK/SREBP1/SCD1 and related genes, such as ACC1 and FAS, to inhibit lipid synthesis in the endoplasmic reticulum. CaMKKβ: Calmodulin-dependent protein kinase β; AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase; ACC: A carboxylase; CPT1: Carnitine palmitate transferase-1; FFA: Free fatty acid; NRP1/2: Neuropilin 1/2; SCD1: Stearoyl-CoA desaturase-1; SREBP1: Sterol-regulatory element-binding protein-1; VEGFB: Vascular endothelial growth factor B; VEGF: Vascular endothelial growth factor; VEGFB: Vascular endothelial growth factor B; FAS: fatty acid synthase; ACO: Acyl Coenzyme A Oxidase; HSL: hormone-sensitive lipase; LCAD: long-chain acyl-CoA dehydrogenase; PPARα: proliferator-activated receptor-α; PGC-1α: peroxidase proliferator activator receptor γ coactivator 1α.

VEGFB gene into mice to inhibit obesity-related inflammation and improve metabolic health.

The lipid deposition caused by the "first hit" can lead to an inflammatory cascade, causing the "second hit" to hepatocyte damage and accelerating pathological changes in NAFLD. VEGFB can affect inflammatory response by regulating lipid metabolism in NAFLD, thereby affecting the "first hit" and improving the "second hit" in NAFLD.

VEGFB PARTICIPATES IN REGULATING THE "MULTIPLE-HIT" IN NAFLD

"Multiple-hit" theory believes that the pathological mechanism of NAFLD involves insulin resistance, adipocyte dysfunction, gut microbiota disorder, aggregation of inflammatory factors, mitochondrial dysfunction, lipotoxicity, endoplasmic reticulum stress, and so on[53]. These factors collaborate and overlap with each other, accelerating hepatocyte damage and ultimately developing into cirrhosis, liver cancer, and end-stage liver failure. Insulin resistance is a common metabolic abnormality in patients with NAFLD and is considered the first step in the development of NAFLD[54]. Studies have shown that the activation of insulin receptor substrate 1 (IRS1) protein is downregulated and SREBP-1c is upregulated when insulin resistance occurs, which ultimately increases the expression of de novo synthesis of lipids, thus increasing the transport of FFA to the liver[55]. Meanwhile, hyperinsulinemia can inhibit the β-oxidative of FFA to further promote lipid accumulation in the liver. Excessive lipid accumulation in the liver can disrupt the homeostasis of glucose metabolism[56]. Hepatic insulin resistance participates in the inhibition of forkhead box protein 1 (FOXO1) and serine/threonine kinase (GSK-3) through phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), reduces Phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6 phosphatase (G6Pase) levels in the liver, which promotes gluconeogenesis and inhibits glycogen synthesis[57] (Figure 4). Recent studies have shown that VEGFB plays an active role in regulating metabolic diseases related to insulin resistance. Robciuc *et al*[17] found that VEGFB can increase the sensitivity of peripheral insulin and improve insulin resistance. Hu *et al*[18] found that VEGFB can reduce insulin resistance by reducing the content of FFA and total cholesterol, thus improving the disorder of lipid metabolism in NAFLD.



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Figure 4 Vascular endothelial growth factor B participates in the "multiple-hit" of nonalcoholic fatty liver disease. Vascular endothelial growth factor B (VEGFB) regulates lipid metabolism, inflammation reaction, and glucose metabolism, which co-exist in the nonalcoholic fatty liver disease progression. VEGFB activates the AMPK phosphorylation to regulate free fatty acid oxidation and lipid synthesis. Long-term lipid metabolism disorders will cause inflammatory reactions and glucose metabolism disorders. VEGFB promotes the phosphorylation of protein kinase B (AKT) *via* combining with the VEGFR1 to affect macrophage migration and mitochondrial inflammation reaction. Meanwhile, VEGFB/VEGFR1 also plays an important role in inhibiting gluconeogenesis and promoting glycogen synthesis by activating the phosphorylation of AKT to regulate glucose metabolism. CaMKKβ: Calmodulin-dependent protein kinase β; VEGFR1: Vascular endothelial growth factor receptor-1; VEGFB: Vascular endothelial growth factor B; IR: Insulin receptor; IRS1: Insulin receptor substrate-1; PI3K: Phosphoinositide 3-kinase; AKT: Protein kinase B; AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase; ACC: A carboxylase; CPT1: Carnitine palmitate transferase-1; Lcad: long-chain acyl-CoA dehydrogenase; SREBP: Sterol-regulatory element-binding protein; FAS: fatty acid synthase; NF-kB: Nuclear factor-kappa B; NF-AT: Nuclear factors of activated T; eNOS: endothelial nitric oxide synthase; FoxO1: Forkhead box protein-1; GSK-3: Serine/threonine kinase-3; PEPCK: Phosphoenolpyruvate carboxykinase; G6Pase: Glucose 6 phosphatase;

Insulin resistance has been proven to be an activator of cell apoptosis[58]. Cell apoptosis, an injury factor in the "multiple hits", is a common and important mechanism of NAFLD lesions and liver injury. Li *et al*[59] believe that the decrease in the number of hepatocytes may be due to apoptosis, and excessive apoptosis of hepatocytes is an important sign of NAFLD/NASH patients. As a member of the vascular growth factor family, VEGFB participates in many angiogenesis-dependent diseases, and its pathogenesis is related to cell apoptosis. Williams *et al*[60] showed that inhibition of VEGFB leads to increased apoptosis in cardiomyocytes of patients with diabetes. Dai *et al*[61] also demonstrated that VEGFB plays an antiapoptotic role in the context of tumors.

Adipocyte dysfunction has also been confirmed to be closely related to the pathogenesis of NAFLD [62]. The degree of adipocyte dysfunction is consistent with abnormal metabolism in NAFLD. Martina Rudnicki's study confirms that male mice fed with HFD exhibit adipocyte dysfunction[63]. Robciuc *et al* [17] has shown that the VEGFB/VEGFR1 pathway can be used to enhance vascular distribution in adipose tissue, which improves metabolic health and obesity. The VEGFB gene may affect the occurrence and development of NAFLD by affecting the expansion and loss of adipose tissue.

CONCLUSION

The role of VEGFB in regulating metabolic diseases, such as NAFLD, has attracted increasing attention from scholars. Research has shown that VEGFB can reduce lipid accumulation and restore insulin sensitivity in NAFLD. VEGFB activates the AKT signaling pathway by combining with VEGFR1, inhibits FOXO1 and GSK3 genes, blocks gluconeogenesis, accelerates glycogen synthesis, and improves insulin resistance. VEGFB not only improves liver insulin resistance, but also activates the AMPK signaling pathway, thereby activating the ACC signal to inhibit the expression of SREBP protein,

improving fatty acid oxidation, inhibiting lipid synthesis, and restoring lipid metabolism balance. The activated AKT protein inhibits nuclear factors and proteins such as NF-kB or eNOS after phosphorylation, regulates inflammatory factors such as macrophages and liver mitochondrial function, reduces the occurrence of inflammatory reactions in hepatocytes, and prevents the progression of NAFLD[43].

Although more and more studies support that VEGFB can be a new target for the treatment of NAFLD and type 2 diabetes, some studies have shown that VEGFB has not played a positive role in regulating lipid metabolism and insulin resistance. Ning *et al*[64] confirmed that the changes in VEGFB did not affect glucose metabolism or lipid uptake. Hagberg *et al*[65] suggested that VEGFB gene deletion can prevent ectopic lipid deposition and ameliorate dyslipidemia. Falkevall *et al*[66] showed that inhibition of VEGFB signaling can target liver steatosis by inhibiting lipolysis and preventing the development of NAFLD.

At present, the understanding of the role of VEGFB in regulating NAFLD and its mechanism remains controversial and is not completely clear. So more research focuses on the mechanism of VEGFB in the occurrence and development of NAFLD, which will provide a new idea for the study of pathophysiological mechanisms and therapeutic targets of NAFLD.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Acute pancreatitis in liver transplant hospitalizations: Identifying national trends, clinical outcomes and healthcare burden in the United States

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Abstract

BACKGROUND

Acute pancreatitis (AP) in liver transplant (LT) recipients may lead to poor clinical outcomes and development of severe complications.

AIM

We aimed to assess national trends, clinical outcomes, and the healthcare burden of LT hospitalizations with AP in the United States (US).

METHODS

The National Inpatient Sample was utilized to identify all adult (≥ 18 years old) LT hospitalizations with AP in the US from 2007–2019. Non-LT AP hospitalizations served as controls for comparative analysis. National trends of hospitalization characteristics, clinical outcomes, complications, and healthcare burden for LT hospitalizations with AP were highlighted. Hospitalization characteristics, clinical outcomes, complications, and healthcare burden were also compared between the LT and non-LT cohorts. Furthermore, predictors of inpatient mortality for LT hospitalizations with AP were identified. All *P* values ≤ 0.05 were considered statistically significant.

RESULTS

The total number of LT hospitalizations with AP increased from 305 in 2007 to 610 in 2019. There was a rising trend of Hispanic (16.5% in 2007 to 21.1% in 2018, P-trend = 0.0009) and Asian (4.3% in 2007 to 7.4% in 2019, p-trend = 0.0002) LT hospitalizations with AP, while a decline was noted for Blacks (11% in 2007 to 8.3% in 2019, P-trend = 0.0004). Furthermore, LT hospitalizations with AP had an increasing comorbidity burden as the Charlson Comorbidity Index (CCI) score ≥ 3 increased from 41.64% in 2007 to 62.30% in 2019 (P-trend < 0.0001). We did not find statistically significant trends in inpatient mortality, mean length of stay (LOS), and mean total healthcare charge (THC) for LT hospitalizations with AP despite rising trends of complications such as sepsis, acute kidney failure (AKF), acute respiratory failure (ARF), abdominal abscesses, portal vein thrombosis (PVT), and venous thromboembolism (VTE). Between 2007–2019, 6863 LT hospitalizations with AP were compared to 5649980 non-LT AP hospitalizations. LT hospitalizations with AP were slightly older (53.5 *vs* 52.6 years, *P* = 0.017) and had a higher proportion of patients with CCI ≥ 3 (51.5% *vs* 19.8%, *P* < 0.0001) compared to the non-LT cohort. Additionally, LT hospitalizations with AP had a higher proportion of Whites (67.9% *vs* 64.6%, *P* < 0.0001) and Asians (4% *vs* 2.3%, *P* < 0.0001), while the non-LT cohort had a higher proportion of Blacks and Hispanics. Interestingly, LT hospitalizations with AP had lower inpatient mortality (1.37% *vs* 2.16%, *P* = 0.0479) compared to the non-LT cohort despite having a higher mean age, CCI scores, and complications such as AKF, PVT, VTE, and the need for blood transfusion. However, LT hospitalizations with AP had a higher mean THC ($59596 *vs* $50466, *P* = 0.0429) than the non-LT cohort.

CONCLUSION

In the US, LT hospitalizations with AP were on the rise, particularly for Hispanics and Asians. However, LT hospitalizations with AP had lower inpatient mortality compared to non-LT AP hospitalizations.

Key Words: Liver transplantation; Pancreatitis; Mortality; Cost; Length of stay

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Core Tip: Liver transplant (LT) is a lifesaving intervention for patients with end-stage liver disease. Acute pancreatitis (AP) in LT recipients may lead to poor clinical outcomes and development of severe complications. In this study, we noted an increase in LT hospitalizations with AP at a national level from 305 in 2007 to 610 in 2019 with a rising trend for Hispanics and Asians. However, there was no trend for inpatient mortality, mean length of stay and mean total healthcare charge. After a comparative analysis, LT hospitalizations with AP had lower inpatient mortality compared to the non-LT cohort despite a higher mean age, comorbidity burden, and presence of complications.

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INTRODUCTION

Acute pancreatitis (AP), an inflammatory response to injury of the pancreas, is one of the leading causes of hospitalization amongst gastrointestinal disorders in the United States (US). In the general population, the incidence of AP is estimated to be 40-50 per 100000 persons and there are approximately 275000 AP hospitalizations annually in the US[1,2]. Risk factors implicated in the development of AP include cholelithiasis, heavy alcohol use (4-5 drinks daily for > 5 years), hypertriglyceridemia (> 1000 mg/dL), smoking, medications, autoimmune diseases, genetic predispositions, blunt/penetrating abdominal trauma, viral infections, and therapeutic endoscopic procedures such as endoscopic retrograde cholangiopancreatography (ERCP), among others[3-10]. The pathogenesis of AP is multifactorial, but ultimately involves the unregulated activation of proteolytic enzymes within the pancreas eventually leading to pancreatic ductal obstruction, subsequent inflammation, and in severe cases a systemic-inflammatory response syndrome[11]. The characteristic clinical features of AP include nausea, vomiting, loss of appetite, and epigastric abdominal pain radiating to the back[12]. A diagnosis of AP can be established by the presence of any two of the following three criteria: (1) Characteristic epigastric abdominal pain; (2) serum lipase and/or amylase greater than three times the upper limit of normal; and (3) evidence of AP on abdominal imaging[13]. Over the years, AP hospitalizations are on a rise in the US, with mortality rates ranging from 1%-2% and over 2.5 billion dollars being spent annually on healthcare costs[1,14].

Liver transplant (LT) has revolutionized management for chronic end-stage liver disease with excellent results. Since the first LT in 1967, the procedure has saved close to 500000 Life-years among patients with acute fulminant hepatic failure, hepatocellular carcinoma (HCC), and end-stage liver disease[15,16]. The recipients of the procedure have excellent survival rates. Per the Scientific Registry of Transplant Recipients data, the overall patient survival rate after deceased donor LT was 90% and 77% at 1 year and 5 years, respectively[17]. Moreover, the graft survival rate at 1 year and 5 years after LT was noted to be 89.6% and 72.8%, respectively[18].

AP is an important risk factor for poor surgical outcomes in patients with LT. Studies have reported an incidence rate ranging from 3%-8% for post-LT pancreatitis[19,20]. Common risk factors implicated in the development of post-LT pancreatitis include hepatitis B infection as an indication of transplant, re-transplantation, duration of venous bypass, hypotension with longer procedural time, utilization of ERCP, type of biliary reconstruction, intraoperative calcium chloride administration, and use of an aorto-hepatic graft[19,21,22]. Additionally, surgical manipulation, immunosuppression, infections, and biliary complications before LT may also increase the risk of developing post-LT pancreatitis[23]. In LT recipients, peri-transplant pancreatitis is associated with a two-fold increased risk of mortality[24]. Furthermore, early AP in LT recipients (within 1-2 mo of LT) may have mortality rates as high as 67% [25]. Given the acute-organ shortage worldwide, we must identify LT hospitalizations at high risk of developing AP to maximize patient survival.

Although studies investigating post-LT pancreatitis currently exist, they are primarily limited to small single-center experiences[19,20,22,25-27]. Hence, this study was designed to investigate trends in hospitalization characteristics and clinical outcomes for LT hospitalizations with AP. Furthermore, we performed a comparative analysis between LT and non-LT hospitalizations with AP to determine the influence of LT on clinical outcomes and healthcare burden. Predictors of inpatient mortality for LT hospitalizations with AP were also identified.

MATERIALS AND METHODS

Design and data source

This retrospective study derived the study population from the National Inpatient Sample (NIS) for 2007–2019 which was coded using the International Classification of Diseases, 9th and 10th Revision, Clinical Modification (ICD-9/10- CM) diagnosis codes, and procedure codes. The NIS, maintained by the Healthcare Cost and Utilization Project (HCUP), is one of the largest, publicly available, multi-ethnic databases in the US. HCUP is a family of healthcare databases, related software tools, and products developed through a Federal-State-Industry partnership and sponsored by the Agency for Healthcare Research and Quality. The NIS enables medical researchers to analyze data on more than seven million hospital stays each year in the US. It approximates a 20-percent stratified sample of all discharges from US community hospitals, excluding rehabilitation and long-term acute care hospitals. The NIS database is publicly available at: https://www.hcup-us.ahrq.gov/.

Study population and outcome measures

We utilized the NIS to identify all adult (≥ 18 years old) LT hospitalizations with AP in the US from 2007–2019. National trends of hospitalization characteristics, clinical outcomes, complications, and the healthcare burden were highlighted. Furthermore, non-LT AP hospitalizations served as controls for a comparative analysis of hospitalization characteristics, clinical outcomes, complications, and the healthcare burden with the LT cohort. Predictors of inpatient mortality for LT hospitalizations with AP were also identified.

Statistical analysis

Statistical analysis was conducted using SAS 9.4 (SAS Institute Inc, Cary, NC, United States) to account for weights in the stratified survey design of the NIS. During the statistical estimating process, weights were considered by incorporating the variables for strata, weight to discharges and cluster. Descriptive statistics including mean (± standard error) for continuous variables, and count (%) for categorical variables were provided after statistical analysis. The Cochran-Armitage trend tests were implemented to test the trends for proportions of binary variables. The trends for the averages of age, mean length of stay (LOS) and mean total healthcare charge (THC) were examined by using linear regression. The Rao-Scott design-adjusted chi-square test examined the association between binary variables in LT and non-LT hospitalizations with AP. F-statistics from the weighted regression model was used to test the differences in age, mean LOS, and mean THC in LT and non-LT hospitalizations with AP. Adjusted hazard ratios with 95% confidence interval were obtained through Cox proportional hazards regression to identify factors that influenced mortality. All analytical results were considered statistically significant when *P* values were less than or equal to 0.05.

Ethical considerations

The NIS database lacks patient and hospital-specific identifiers to protect patient privacy and maintain anonymity. Hence, our study was exempt from Institutional Review Board (IRB) approval as per guidelines put forth by our IRB for analysis of database studies.

RESULTS

Trends of hospitalization characteristics for LT hospitalizations with AP

There was an increase in the total number of LT hospitalizations with AP from 305 in 2007 to 610 in 2019. We did not find a statistically significant trend for gender or mean age; however, there was an increasing trend of LT hospitalizations with AP for patients aged ≥ 65 years (Table 1). Furthermore, LT hospitalizations with AP had an increasing comorbidity burden as the Charlson Comorbidity Index (CCI) score ≥ 3 increased from 41.64% in 2007 to 62.30% in 2019 (P-trend < 0.0001). Interestingly, we also noted a rising trend of LT hospitalizations with AP from 58.89% in 2007 to 82.79% in 2019 at urban teaching hospitals.

Racial differences in the trends of LT hospitalizations with AP were apparent. Whites made up a majority of the study cohort (Table 1) without a statistically significant trend. We noted an overall increasing trend of Hispanic (16.49% in 2007 to 21.09% in 2018, P-trend = 0.0009) and Asian (4.27% in 2007 to 7.44% in 2019, P-trend = 0.0009) LT hospitalizations with AP (Table 1 and Figure 1). However, Black LT hospitalizations with AP had a declining trend from 11% to 8.26%, P-trend = 0.0004) (Table 1).

Trends of clinical outcomes, healthcare burden and complications for LT hospitalizations with AP

We did not find a statistically significant trend for inpatient mortality, mean LOS, and mean THC for LT hospitalizations with AP (Table 2). However, we observed a rising trend of complications such as sepsis (1.25% in 2007 to 18.03% in 2019, P-trend < 0.0001), acute kidney failure (AKF) (17.13% to 34.43%, P-trend < 0.0001), acute respiratory failure (ARF) (1.44% to 6.56%, P-trend = 0.0002), abdominal abscesses

Table 1 Trends of hospitalization characteristics for liver transplant hospitalizations with acute pancreatitis in the United States from 2007–2019, *n* (%)

Epidemiologicalvariable	Years													Trend (*P* value)
	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	
Total number of hospitalizations	305	600	550	520	453	590	455	500	460	650	505	665	610	--
Mean age in yr (standard error)	53.08 (1.52)	51.00 (1.35)	52.01 (1.59)	51.32 (1.45)	51.79 (1.49)	52.02 (1.10)	52.86 (1.20)	55.16 (1.22)	50.27 (1.31)	51.84 (1.11)	53.70 (1.26)	53.32 (1.12)	54.73 (1.20)	No trend (0.1256)
Age groups (yr)														
18–34	30 (9.94)	82 (13.62)	76 (13.77)	62 (11.90)	50 (11.07)	80 (13.56)	45 (9.89)	55 (11.00)	90 (19.57)	105 (16.15)	50 (9.90)	70 (10.53)	55 (9.02)	No trend (0.1345)
35–49	61 (19.82)	180 (29.97)	117 (21.38)	111 (21.37)	84 (18.46)	145 (24.58)	100 (21.98)	90 (18.00)	115 (25.00)	120 (18.46)	100 (19.80)	185 (27.82)	165 (27.05)	No trend (0.2379)
50–64	164 (53.56)	236 (39.30)	270 (49.09)	281 (54.15)	276 (60.97)	280 (47.46)	255 (56.04)	225 (45.00)	170 (36.96)	295 (45.38)	260 (51.49)	265 (39.85)	200 (32.79)	Decrease (< 0.0001)
65–79	46 (14.90)	103 (17.12)	87 (15.76)	62 (11.86)	38 (8.40)	85 (14.41)	55 (12.09)	120 (24.00)	75 (16.30)	125 (19.23)	85 (16.83)	135 (20.30)	190 (31.15)	Increase (< 0.0001)
≥ 80	< 11 (1.78)	0 (0.00)	0 (0.00)	< 11 (0.71)	< 11 (1.09)	0 (0.00)	0 (0.00)	< 11 (2.00)	< 11 (2.17)	< 11 (0.77)	< 11 (1.98)	< 11 (1.50)	0 (0.00)	Increase (0.0157)
Gender														
Male	163 (53.30)	278 (46.32)	311 (56.61)	328 (63.08)	270 (59.48)	325 (55.08)	260 (57.14)	235 (47.00)	275 (59.78)	380 (58.46)	270 (53.47)	370 (55.64)	340 (55.74)	No trend (0.1383)
Female	143 (46.70)	322 (53.68)	238 (43.39)	192 (36.92)	184 (40.52)	265 (44.92)	195 (42.86)	265 (53.00)	185 (40.22)	270 (41.54)	235 (46.53)	295 (44.36)	270 (44.26)	No trend (0.1383)
Race														
White	139 (59.81)	387 (72.63)	310 (66.45)	322 (66.37)	285 (71.89)	395 (71.17)	290 (65.91)	375 (78.95)	265 (61.63)	390 (64.46)	360 (75.00)	385 (60.16)	405 (66.94)	No trend (0.0517)
Black	26 (11.00)	48 (9.04)	69 (14.68)	99 (20.39)	58 (14.49)	35 (6.31)	45 (10.23)	40 (8.42)	20 (4.65)	90 (14.88)	50 (10.42)	60 (9.38)	50 (8.26)	Decrease (0.0004)
Hispanic	38 (16.49)	63 (11.85)	54 (11.58)	43 (8.80)	39 (9.75)	65 (11.71)	70 (15.91)	35 (7.37)	95 (22.09)	80 (13.22)	35 (7.29)	135 (21.09)	80 (13.22)	Increase (0.0009)
Asian	< 11 (4.27)	20 (3.77)	0 (0.00)	11 (2.19)	< 11 (2.57)	35 (6.31)	20 (4.55)	15 (3.16)	20 (4.65)	30 (4.96)	< 11 (2.08)	25 (3.91)	45 (7.44)	Increase (0.0002)
Other	20 (8.42)	14 (2.71)	34 (7.28)	11 (2.26)	< 11 (1.31)	25 (4.50)	15 (3.41)	< 11 (2.11)	30 (6.98)	15 (2.48)	25 (5.21)	35 (5.47)	25 (4.13)	No trend (0.406)
CCI														
CCI = 1	130 (42.71)	232 (38.75)	174 (31.68)	147 (28.25)	138 (30.35)	185 (31.36)	130 (28.57)	155 (31.00)	150 (32.61)	160 (24.62)	120 (23.76)	160 (24.06)	145 (23.77)	Decrease (< 0.0001)
CCI = 2	48 (15.65)	101 (16.81)	106 (19.32)	155 (29.77)	61 (13.38)	115 (19.49)	115 (25.27)	130 (26.00)	90 (19.57)	110 (16.92)	75 (14.85)	115 (17.29)	85 (13.93)	Decrease (0.0036)
CCI ≥ 3	127 (41.64)	267 (44.44)	269 (49.01)	218 (41.98)	255 (56.27)	290 (49.15)	210 (46.15)	215 (43.00)	220 (47.83)	380 (58.46)	310 (61.39)	390 (58.65)	380 (62.30)	Increase (< 0.0001)
Hospital region														

Northeast	36 (11.70)	209 (34.76)	82 (14.99)	61 (11.73)	89 (19.75)	100 (16.95)	60 (13.19)	35 (7.00)	65 (14.13)	95 (14.62)	115 (22.77)	115 (17.29)	85 (13.93)	Decrease (< 0.0001)
Midwest	64 (20.97)	98 (16.26)	213 (38.69)	134 (25.71)	144 (31.79)	130 (22.03)	125 (27.47)	140 (28.00)	130 (28.26)	125 (19.23)	130 (25.74)	140 (21.05)	130 (21.31)	Decrease (0.0063)
South	105 (34.47)	121 (20.22)	119 (21.68)	244 (47.04)	169 (37.29)	185 (31.36)	180 (39.56)	205 (41.00)	170 (36.96)	270 (41.54)	145 (28.71)	265 (39.85)	230 (37.70)	Increase (< 0.0001)
West	100 (32.86)	173 (28.77)	135 (24.64)	81 (15.52)	51 (11.18)	175 (29.66)	90 (19.78)	120 (24.00)	95 (20.65)	160 (24.62)	115 (22.77)	145 (21.80)	165 (27.05)	No trend (0.2338)
Hospital bed-size														
Small	43 (13.91)	48 (7.97)	23 (4.59)	34 (6.64)	23 (5.15)	30 (5.08)	60 (13.19)	45 (9.00)	65 (14.13)	60 (9.23)	40 (7.92)	90 (13.53)	85 (13.93)	Increase (< 0.0001)
Medium	68 (22.16)	93 (15.44)	103 (20.26)	91 (17.70)	58 (13.10)	120 (20.34)	75 (16.48)	135 (27.00)	100 (21.74)	120 (18.46)	120 (23.76)	195 (29.32)	155 (25.41)	Increase (< 0.0001)
Large	195 (63.93)	459 (76.59)	381 (75.16)	389 (75.67)	363 (81.75)	440 (74.58)	320 (70.33)	320 (64.00)	295 (64.13)	470 (72.31)	345 (68.32)	380 (57.14)	370 (60.66)	Decrease (< 0.0001)
Hospital location and teaching status														
Rural	18 (5.97)	57 (9.51)	20 (3.98)	41 (7.98)	35 (7.95)	65 (11.02)	30 (6.59)	45 (9.00)	35 (7.61)	35 (5.38)	15 (2.97)	65 (9.77)	25 (4.10)	Decrease (0.0238)
Urban nonteaching	107 (35.14)	145 (24.21)	171 (33.68)	156 (30.35)	84 (18.89)	190 (32.20)	140 (30.77)	70 (14.00)	85 (18.48)	125 (19.23)	110 (21.78)	110 (16.54)	80 (13.11)	Decrease (< 0.0001)
Urban teaching	180 (58.89)	398 (66.28)	316 (62.34)	317 (61.67)	325 (73.16)	335 (56.78)	285 (62.64)	385 (77.00)	340 (73.91)	490 (75.38)	380 (75.25)	490 (73.68)	505 (82.79)	Increase (< 0.0001)
Disposition														
Discharge Home	276 (90.47)	473 (78.88)	397 (72.25)	359 (69.05)	333 (73.51)	440 (74.58)	345 (75.82)	395 (79.00)	375 (81.52)	525 (80.77)	370 (73.27)	490 (73.68)	455 (74.59)	No trend (0.1111)
Transfer to short-term hospital	< 11 (3.06)	46 (7.74)	37 (6.72)	68 (13.01)	35 (7.81)	50 (8.47)	25 (5.49)	35 (7.00)	40 (8.70)	30 (4.62)	60 (11.88)	30 (4.51)	35 (5.74)	No trend (0.0657)
Transfer to another facility (Includes SNF and ICF)	< 11 (3.25)	20 (3.28)	34 (6.11)	26 (5.09)	13 (2.97)	25 (4.24)	< 11 (2.20)	< 11 (1.00)	10 (2.17)	45 (6.92)	< 11 (0.99)	70 (10.53)	15 (2.46)	No trend (0.0532)
Home health care	< 11 (3.22)	30 (5.07)	51 (9.32)	62 (11.87)	42 (9.25)	60 (10.17)	60 (13.19)	50 (10.00)	30 (6.52)	45 (6.92)	55 (10.89)	55 (8.27)	60 (9.84)	Increase (0.0426)
Discharge against medical advice	0 (0.00)	25 (4.16)	11 (2.00)	< 11 (0.98%)	< 11 (1.11)	< 11 (1.69)	15 (3.30)	<11 (2.00)	< 11 (1.09)	0 (0.00)	< 11 (0.99)	20 (3.01)	25 (4.10)	No trend (0.1735)

CCI: Charlson comorbidity index; ICF: Intermediate care facility; SNF: Skilled nursing facility.

(0% in 2007 to 0.82% in 2019, P-trend = 0.0006), portal vein thrombosis (PVT) (0% to 4.10%, P-trend < 0.0001) and venous thromboembolism (VTE) (1.82% to 7.38%, P-trend < 0.0001) for LT hospitalizations with AP. Moreover, there was a decline in the need for blood transfusion from 6.09% in 2007 to 0% in 2019 (P-trend < 0.0001) for LT hospitalizations with AP.

Table 2 Trends of outcomes for liver transplant hospitalizations with acute pancreatitis in the United States from 2007–2019, *n* (%)

Outcomes	Years													Trend (*P* value)
	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	
Inpatient mortality	0 (0.00)	< 11 (0.87)	20 (3.60)	0 (0.00)	24 (5.35)	< 11 (0.85)	0 (0.00)	< 11 (1.00)	0 (0.00)	< 11 (0.77)	< 11 (1.98)	0 (0.00)	20 (3.28)	No trend (0.3879)
Length of stay (d)	5.62	5.44	7.09	6.02	7.85	5.40	8.53	4.53	4.86	7.33	6.62	6.23	4.55	No trend (0.6905)
Total healthcare charge ($)	36413	53418	50432	53115	68247	42107	95774	41613	42319	79746	80479	65054	56011	No trend (0.1946)
Complications														
Pancreatic psuedocyst	< 11 (3.41)	35 (5.88)	34 (6.13)	26 (5.06)	14 (3.01)	< 11 (1.69)	20 (4.40)	< 11 (2.00)	< 11 (1.09)	20 (3.08)	0 (0.00)	50 (7.52)	30 (4.92)	No trend (0.1273)
Abdominal abscess	0 (0.00)	0 (0.00)	0 (0.00)	11 (2.07)	< 11 (1.00)	0 (0.00)	< 11 (1.10)	< 11 (1.00)	0 (0.00)	< 11 (0.77)	0 (0.00)	20 (3.01)	< 11 (0.82)	Increase (0.0006)
Sepsis	< 11 (1.25)	41 (6.91)	46 (8.37)	30 (5.69)	32 (7.15)	20 (3.39)	35 (7.69)	30 (6.00)	40 (8.70)	60 (9.23)	55 (10.89)	70 (10.53)	110 (18.03)	Increase (< 0.0001)
Acute renal failure	52 (17.13)	121 (20.10)	149 (27.09)	123 (23.74)	143 (31.62)	160 (27.12)	130 (28.57)	145 (29.00)	155 (33.70)	205 (31.54)	180 (35.64)	245 (36.84)	210 (34.43)	Increase (< 0.0001)
Acute respiratory failure	< 11 (1.44)	37 (6.10)	21 (3.79)	21 (4.06)	28 (6.28)	0 (0.00)	15 (3.30)	< 11 (2.00)	25 (5.43)	55 (8.46)	20 (3.96)	40 (6.02)	40 (6.56)	Increase (0.0002)
Need for blood transfusion	19 (6.09)	72 (12.03)	33 (6.10)	40 (7.61)	91 (20.09)	95 (16.10)	45 (9.89)	35 (7.00)	30 (6.52)	20 (3.08)	20 (3.96)	25 (3.76)	0 (0.00)	Decrease (< 0.0001)
Portal vein thrombosis	0 (0.00)	< 11 (1.69)	< 11 (0.86)	0 (0.00)	0 (0.00)	0 (0.00)	< 11 (1.10)	< 11 (2.00)	< 11 (1.09)	15 (2.31)	15 (2.97)	15 (2.26)	25 (4.10)	Increase (< 0.0001)
Venous thromboembolism	< 11 (1.82)	< 11 (0.85)	35 (6.29)	16 (3.02)	< 11 (1.00)	< 11 (1.69)	< 11 (1.10)	< 11 (1.00)	< 11 (2.17)	25 (3.85)	25 (4.95%)	50 (7.52)	45 (7.38)	Increase (< 0.0001)

Comparative analysis of hospitalization characteristics for LT and non-LT hospitalizations with AP

Between 2007–2019, there were 6863 LT hospitalizations with AP which were compared to 5649980 non-LT AP hospitalizations. LT hospitalizations with AP had a slightly higher mean age (53.5 *vs* 52.55 years, $P = 0.017$) compared to the non-LT cohort. Furthermore, LT hospitalizations with AP also had a higher proportion of males (55.43% *vs* 51.13%, $P = 0.0046$) and patients with a CCI score ≥ 3 (51.46% *vs* 19.76%, $P < 0.0001$) compared to non-LT hospitalizations (Table 3). A majority of LT hospitalizations with AP were at large (69.47%), urban teaching (69.73%) hospitals.

Racial differences were observed between the LT and non-LT cohorts. We noted a higher proportion of Whites (67.91% *vs* 64.57%, $P < 0.0001$) and Asians (3.95% *vs* 2.3%, $P < 0.0001$) in the LT cohort, while there was a higher proportion of Blacks and Hispanics in the non-LT cohort (Table 3).

Comparative analysis of clinical outcomes, healthcare burden and complications for LT and non-LT hospitalizations with AP

Overall, the inpatient mortality for LT hospitalizations with AP was lower (1.37% *vs* 2.16%, $P = 0.0479$) than the non-LT cohort (Table 4). We did not find a statistical difference in the inpatient mortality rates after stratifying for age, gender, or race. Although the mean LOS was comparable between both groups, the mean THC was higher for LT hospitalizations with AP ($59596 *vs* $50466, P-trend = 0.0429) compared to the non-LT cohort. Furthermore, LT hospitalizations with AP also had a higher proportion

Table 3 Comparative analysis of hospitalization characteristics for liver and non-liver transplant hospitalizations with acute pancreatitis in the United States from 2007–2019, *n* (%)

Outcomes	Liver transplant hospitalizations with acute pancreatitis	Non-liver transplant hospitalizations with acute pancreatitis	*P* value
Total number of hospitalizations	6863	5649980	
Mean age ± standard error (yr)	53.50 (0.04)	52.55 (0.39)	0.017
Age group (yr)			< 0.0001
18–34	12.38	16.20	
35–49	22.91	26.36	
50–64	46.29	29.97	
65–79	17.55	18.43	
≥ 80	0.86	9.03	
Gender			0.0046
Male	55.43	51.13	
Female	44.57	48.87	
Race			≤ 0.0001
White	67.91	64.57	
Black	10.85	16.11	
Hispanic	13.11	13.12	
Asian	3.95	2.30	
Other	4.16	3.90	
Charlson comorbidity index			< 0.0001
CCI = 1	29.53	28.27	
CCI = 2	19.02	13.65	
CCI ≥ 3	51.46	19.76	
Hospital region			0.0753
Northeast	16.71	16.45	
Midwest	24.80	22.02	
South	35.10	40.16	
West	23.38	21.37	
Hospital bed-size			< 0.0001
Small	9.49	17.96	
Medium	21.04	28.13	
Large	69.47	53.91	
Hospital location and teaching status			< 0.0001
Rural	7.15	12.46	
Urban nonteaching	23.12	36.23	
Urban teaching	69.73	51.32	
Disposition			< 0.0001
Routine (Home)	76.26	77.39	
Transfer to short-term hospital	7.30	3.10	
Transfer to another type of facility (Includes SNF and ICF)	4.20	7.64	
Home health care	8.89	6.69	

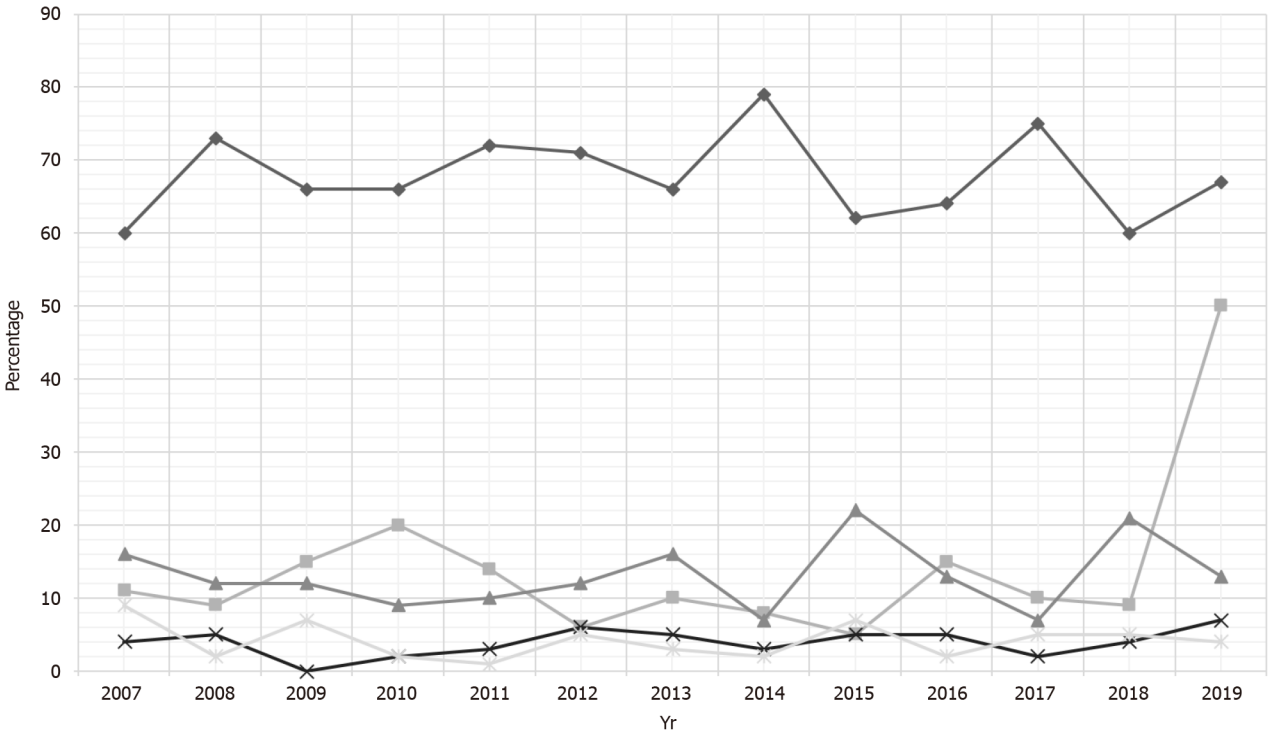
Discharge against medical advice	1.98	3.00

CCI: Charlson comorbidity index; ICF: Intermediate care facility; SNF: Skilled nursing facility.

Table 4 Comparative analysis of clinical outcomes for liver and non-liver transplant hospitalizations with acute pancreatitis in the United States from 2007–2019, *n* (%)

Outcomes	Liver transplant hospitalizations with acute pancreatitis	Non-liver transplant hospitalizations with acute pancreatitis	*P* value
Inpatient mortality	1.37	2.16	0.0479
Gender-specific inpatient mortality			
Male	1.43	2.34	0.1107
Female	1.31	1.97	0.2396
Race specific inpatient mortality			
White	1.48	2.23	0.1403
Black	1.45	2.00	0.6469
Hispanic	0.00	1.60	---
Asian	1.99	3.20	0.6289
Others	4.01	2.23	0.3868
Age group specific inpatient mortality			
18-34	0.60	0.64	0.9583
35-49	0.90	1.04	0.7855
50-64	1.12	2.08	0.0921
65-79	3.26	3.60	0.7784
≥ 80	0.00	5.46	---
Length of stay (d)	6.14	5.80	0.3189
Total healthcare charge ($)	59596	50466	0.0429
Complications (out of total hospitalizations)			
Pancreatic psuedocyst	3.85	5.46	0.0259
Abdominal abscess	0.81	0.53	0.1925
Sepsis	8.35	8.78	0.5834
Acute renal failure	29.41	14.91	< 0.0001
Acute respiratory failure	4.61	5.67	0.1018
Cholangiocarcinoma	0.21	0.11	0.2545
Need for blood transfusion	7.65	4.75	< 0.0001
Portal vein thrombosis	1.53	0.64	< 0.0001
Venous thromboembolism	3.50	2.19	0.0011

of patients with complications such as AKF (29.41% *vs* 14.91%, $P < 0.0001$), need for blood transfusion (7.65% *vs* 4.75%, $P < 0.0001$), PVT (1.53% *vs* 0.64%, $P < 0.0001$) and VTE (3.5% *vs* 2.19%, $P = 0.0011$) compared to non-LT hospitalizations; however, the non-LT cohort had a higher proportion of patients with pancreatic pseudocysts (5.46% *vs* 3.85%, $P = 0.0259$) (Table 4).



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Figure 1 Racial trends for liver transplant hospitalizations with acute pancreatitis in the United States from 2007–2019.

Predictors for inpatient mortality for LT hospitalizations with AP

After a regression analysis, Hispanics were noted to have lower odds of inpatient mortality compared to Whites (Table 5). Furthermore, after adjusting for all other variables, every one-point increase in the CCI score was associated with a 67.8% increase in inpatient mortality for LT hospitalizations with AP (Table 5). The presence of complications such as pancreatic pseudocysts (aHR: 14.158, 95%CI 1.642-122.094, $P = 0.016$), sepsis (aHR: 13.960, 95%CI 2.163-90.093, $P < 0.0001$), AKF (aHR: 2.684, 95%CI 1.109-6.494, $P = 0.029$), ARF (aHR: 24.758, 95%CI 1.063-576.522, $P = 0.046$), need for blood transfusion (aHR: 150.340, 95%CI 17.049-1325.754, $P < 0.0001$) and VTE (aHR: 75.422, 95%CI 1.637-3475.134, $P = 0.027$) were also associated with higher odds inpatient mortality for LT hospitalizations with AP after adjusting for all other variables.

DISCUSSION

AP is a well-known clinical entity. Although it has been thoroughly studied in the general population, there is a significant paucity of data on AP in solid-organ transplant recipients, particularly those undergoing LT. This is the only study in current literature that investigates trends, clinical outcomes, and the healthcare burden of LT hospitalizations with AP at a national level. In this study, we noted an increase in LT hospitalizations with AP with a rising trend for ethnic minorities *i.e.* Hispanics and Asians; however, we did not find a statistically significant trend of inpatient mortality, mean LOS and mean THC. Although the LT cohort was slightly older and had a higher comorbidity burden, the overall inpatient mortality was lower (1.37% *vs* 2.16%, $P = 0.0479$) compared to the non-LT cohort. Furthermore, LT hospitalizations with AP had a higher proportion of patients with AKF, PVT, VTE, and the need for blood transfusion compared to the non-LT cohort. Increasing CCI and the presence of pancreatic pseudocysts, sepsis, ARF, AKF, VTE, and the need for blood transfusion were associated with increased odds of inpatient mortality for LT hospitalizations with AP. With the increasing rates of liver transplants being performed and relative organ shortage in the US, it is vital to understand patient characteristics, outcomes, and complications of LT hospitalizations with AP to potentially reduce adverse clinical outcomes in these high-risk individuals[18].

As per data available from United Network for Organ Sharing (UNOS), the total number of LT increased from 6494 in 2007 to 8896 in 2019[18]. However, in our study, the total number of LT hospitalizations with AP increased disproportionally, essentially doubling in the same time frame. In the US, the rates of LT for patients ≥ 65 years of age have also been on the rise as there is a general consensus that LT in the elderly is feasible with acceptable short-term and long-term results[28,29]. Similarly, in this study, we noted an increase in the rates of LT hospitalizations with AP for patients > 65 years of age

Table 5 Predictors of inpatient mortality for liver transplant hospitalizations with acute pancreatitis in the United States from 2007–2019

Variable	Adjusted hazard ratio	95%CI	*P* value
Gender			
Male	Reference		
Female	0.596	(0.150, 2.365)	0.461
Race			
White	Reference		
Black	0.306	(0.017, 5.368)	0.418
Hispanic	< 0.001	(< 0.001, < 0.001)	< 0.0001
Asian	0.042	(< 0.001, 14.916)	0.289
Other	0.064	(< 0.001, 10.989)	0.295
Charlson comorbidity index	1.678	(1.055, 2.668)	0.029
Hospital region			
Northeast	Reference		
Midwest	1.574	(0.148, 16.692)	0.706
South	1.435	(0.230, 8.955)	0.699
West	1.723	(0.423, 7.014)	0.447
Hospital bed size			
Small	Reference		
Medium	1.427	(0.093, 21.893)	0.798
Large	1.974	(0.093, 42.139)	0.663
Hospital location and teaching status			
Rural	Reference		
Urban nonteaching	< 0.001	(< 0.001, 0.048)	0.003
Urban teaching	0.551	(0.053, 5.689)	0.617
Complications (reference = Without the complication)			
Pancreatic psuedocyst	14.158	(1.642, 122.094)	0.016
Abdominal abscess	< 0.001	(< 0.001, < 0.001)	< 0.0001
Sepsis	13.960	(2.163, 90.093)	0.006
Acute renal failure	2.684	(1.109, 6.494)	0.029
Acute respiratory failure	24.758	(1.063, 576.522)	0.046
Need for blood transfusion	150.340	(17.049, 1325.754)	< 0.0001
Portal vein thrombosis	< 0.001	(< 0.001, < 0.001)	< 0.0001
Venous thromboembolism	75.422	(1.637, 3475.134)	0.027

(Table 1). However, it should be noted that AP carries a higher morbidity and mortality burden in the elderly population at baseline, and this is compounded in organ transplant recipients[30].

In the US, there was an increase in LT for Hispanics and Asians from 912 in 2007 to 1498 in 2019 and 325 in 2007 to 363 in 2019, respectively as per the UNOS registry. Current literature lacks data on the racial distribution of AP in LT recipients, particularly for ethnic minorities *i.e.* Blacks, Hispanics, and Asians. However, studies have demonstrated that ethnic minorities, at baseline, are at a higher risk of developing AP and have greater severity of disease compared to the general population[2,31-35]. In our study, there was an increasing trend of Hispanic and Asian LT hospitalizations with AP (Figure 1) which was disproportionate to the increase in LT for this population. Interestingly, Black LT hospitalizations with AP were noted to have a declining trend between 2007–2019. After a comparative analysis, we observed a higher proportion of Asians in the LT cohort, while there was a higher proportion of Blacks and Hispanics in the non-LT cohort. The exact reason for this variable racial distribution is currently unknown but needs further investigation through large, multi-center prospective studies.

Furthermore, we emphasize the need for early recognition and prompt treatment of AP in Hispanic and Asian LT hospitalizations to prevent adverse clinical outcomes.

Statistics have demonstrated continuous improvements in survival rates for liver transplant recipients [36-38]. Over the last few decades, AP-related mortality has also declined due to prompt recognition and improvement in management strategies[1,39]. However, prior literature offers conflicting evidence on ethnic variations in AP-related mortality with some studies reporting increased mortality rates in Whites, while others noted higher mortality rates in Blacks among the general population[14,40]. There continues to be a significant paucity of data on mortality for AP in LT recipients in current literature. In our study, we did not find a statistically significant trend for inpatient mortality in LT hospitalizations with AP (Table 2). Interestingly, after a comparative analysis, LT hospitalizations with AP had lower inpatient mortality rates compared to the non-LT cohort despite a higher mean age, greater comorbidity burden, and higher proportion of patients with complications. Furthermore, we did not find a statistical difference in the inpatient mortality rates after stratifying for age, gender, or race. The exact reason for lower inpatient mortality rates in LT hospitalizations with AP is unknown. However, it may, in part, be due to increased vigilance for complications in these high-risk hospitalizations, overall improvements in management strategies, and a multi-disciplinary team approach for management of these highly complex patients. Additional multi-center prospective studies are needed to further investigate these findings. Nonetheless, lower mortality suggests improved survival rates for LT hospitalizations which is in line with current literature.

Healthcare utilization by LT recipients is on the rise. A study by Habka *et al*[41] in 2015 predicted that the cost of LT will increase by 33% in 10 years and 81% in the next 20 years. The inpatient cost of management of AP has also almost doubled from 1996 ($3.9 billion) to 2016 ($7.7 billion)[42]. On the contrary, the utilization of the inpatient service (bed days per prevent case) for AP has declined over the years[42]. No data currently exists on healthcare utilization for AP in LT recipients. In our study, we did not find a statistically significant trend in mean LOS and mean THC for LT hospitalizations with AP indicating that the healthcare burden has remained relatively stable over the years despite a higher proportion of patients with complications such as sepsis, AKF, ARF, PVT, VTE, and abdominal abscesses. After a comparative analysis, the mean LOS was comparable between the LT and non-LT cohorts; however, the mean THC for the LT cohort was $9130 higher than that of the non-LT cohort. This may, in part, be attributed to a higher proportion of patients with complications in the LT cohort compared to the non-LT cohort requiring a higher level of care and multi-disciplinary team management (Table 4). Furthermore, after adjusting for all other variables, increasing CCI, and the presence of complications such as pancreatic pseudocysts, sepsis, ARF, AKF, VTE, and need for blood transfusions were associated with higher odds of inpatient mortality for LT hospitalizations with AP. These findings somewhat mirror predictors of inpatient mortality for AP that have been reported in previous population-based studies[43].

Our study has several strengths and a few limitations. Our study population, which was drawn from one of the largest, publicly available, multi-ethnic databases in the US, is a key strength of this study. This is the only study in the current literature that offers a national perspective on hospitalization characteristics, clinical outcomes, complications, and the healthcare burden of LT hospitalizations with AP over 13 years, compared to other single-center experiences which offer limited information. Through a comprehensive and unique analysis technique, we were also able to compare LT and non-LT hospitalizations to understand the influence of AP on LT hospitalizations thereby giving gastroenterologists real world data. Furthermore, as the NIS covers approximately 97% of the US population, the results of our study are applicable to all LT hospitalizations with AP in the US.

However, we do acknowledge the limitations associated with our study. The retrospective study design makes our study susceptible to the biases that are associated with retrospective studies. Additionally, the NIS database does not contain information on the indication of liver transplant, time from LT to development of AP, disease severity, hospital course, treatment aspects of the disease, time from any procedure to development of complications, procedural complications (pre, intra, and post), intraprocedural operator preferences, or performance of any procedure. Lastly, the NIS is an administrative database that uses ICD codes to store data; hence, the possibility of human coding errors always exists. Despite these limitations, our large sample size, unique analysis technique, and multi-faceted outcomes add valuable data to limited literature.

CONCLUSION

LT is a lifesaving procedure for chronic end-stage liver disease patients. However, the development of post-LT pancreatitis may lead to poor surgical outcomes and development of complications. In our study, we noted an increase in LT hospitalizations with AP, particularly for ethnic minorities *i.e.* Hispanics and Asians; however, there was no trend for inpatient mortality. We also did not find a statistically significant trend mean LOS and mean THC indicating that healthcare utilization has remained relatively stable for LT hospitalizations with AP between 2007–2019. On comparison, LT hospitalizations with AP had lower inpatient mortality compared to non-LT AP hospitalizations despite

a higher proportion of patients that were older, had CCI ≥ 3, and had complications such as AKF, PVT, VTE, and need for blood transfusion. Increasing CCI, presence of pancreatic pseudocysts, sepsis, ARF, ARF, VTE, and need for blood transfusion were identified to be independent predictors of inpatient mortality for LT hospitalizations with AP.

ARTICLE HIGHLIGHTS

Research background

The development of Acute Pancreatitis (AP) in Liver Transplant (LT) recipients may be associated with poor clinical outcomes and severe complications.

Research motivation

Although studies investigating post-LT pancreatitis currently exist, they are primarily limited to small single-center experiences. Currently, a national perspective in the United States (US) does not exist. Therefore, this study was designed to investigate trends and outcomes of LT hospitalization with AP.

Research objectives

We aimed to assess national trends of hospitalization characteristics, clinical outcomes, and the healthcare burden of LT hospitalizations with AP in the US. Non-LT hospitalizations with AP were also identified as controls to compare hospitalization characteristics, clinical outcomes, and the healthcare burden with the LT cohort. Furthermore, predictors of inpatient mortality for LT hospitalizations with AP were identified.

Research methods

The National Inpatient Sample was utilized to identify LT and non-LT hospitalizations with AP. The Cochran-Armitage trend was used to test the trends for proportions of binary variables. Linear regression examined the trends for the averages of age, mean length of stay (LOS), and mean total healthcare charge (THC). Rao-Scott design-adjusted chi-square test examined the association between binary variables in LT and non-LT Hospitalizations with AP. F-statistics were used to test the differences in age, mean LOS, and mean THC in LT and non-LT Hospitalizations with AP. Cox proportional hazards regression was used to identify factors that influenced mortality.

Research results

The total number of LT hospitalizations with AP increased from 305 in 2007 to 610 in 2019. We did not find statistically significant trends in inpatient mortality, mean LOS, and mean THC for LT hospitalizations with AP. LT hospitalizations with AP had lower inpatient mortality compared to the non-LT cohort despite having a higher mean age, comorbidity burden, and complications. Increasing CCI, presence of pancreatic pseudocysts, sepsis, acute respiratory failure, acute renal failure, venous thromboembolism, and need for blood transfusion were independent predictors of inpatient mortality for LT hospitalizations with AP.

Research conclusions

LT is a lifesaving procedure for chronic end-stage liver disease patients. In the US, LT hospitalizations with AP increased between 2007 to 2019, particularly for Hispanics and Asians. However, LT hospitalizations with AP had lower inpatient mortality compared to non-LT AP hospitalizations.

Research perspectives

This is the only study in the current literature that offers a national perspective on hospitalization characteristics, clinical outcomes, complications, and the healthcare burden of LT hospitalizations with AP in the US.

FOOTNOTES

Author contributions: Dahiya DS, Jahagirdar V, Chandan S, Inamdar S, Sharma N, and Al-Haddad M contributed to conception and design; Dahiya DS, Cheng CI, and Al-Haddad M contributed to administrative support; Dahiya DS and Cheng CI contributed to provision, collection, and assembly of data; all Authors contributed to review of literature, drafting the manuscript, revision of key components of the manuscript, final approval of manuscript, agreement to be accountable for all aspects of the work.

Institutional review board statement: The NIS database lacks patient and hospital-specific identifiers to protect patient privacy and maintain anonymity. Hence, our study was exempt from Institutional Review Board (IRB) approval as

per guidelines put forth by our IRB for analysis of database studies.

Informed consent statement: The data for this study was collected from the National Inpatient Sample (NIS) database. As the NIS database lacks patient-specific and hospital-specific identifiers, this study did not require informed consent. The NIS database is available at: https://www.hcup-us.ahrq.gov.

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

Data sharing statement: The NIS database is publicly available at: https://www.hcup-us.ahrq.gov/.

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ORIGINAL ARTICLE

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Lower alanine aminotransferase levels are associated with increased all-cause and cardiovascular mortality in nonalcoholic fatty liver patients

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Abstract

BACKGROUND

Serum alanine aminotransferase (ALT) levels are often considered a marker to evaluate liver disease and its severity.

AIM

To investigate the association between ALT levels and all-cause and cause-specific mortality in patients with nonalcoholic fatty liver disease (NAFLD).

METHODS

The Third National Health and Nutrition Examination Survey (NHANES-III) from 1988 to 1994 and NHANES-III-related mortality data from 2019 onward were used to obtain the necessary data for the study. NAFLD was defined as hepatic steatosis, as diagnosed by ultrasound, with no other liver diseases. ALT levels were categorized into four groups according to the different recommended upper limits of normal (ULN) in men and women: < 0.5 ULN, 0.5-1 ULN, 1-2 ULN, and ≥ 2 ULN. The hazard ratios for all-cause mortality and cause-specific mortality were analyzed using the Cox proportional hazard model.

RESULTS

Multivariate logistic regression analysis demonstrated that the odds ratio of NAFLD correlated positively with increased serum ALT levels. In patients with NAFLD, all-cause mortality and cardiovascular mortality were the highest when ALT was < 0.5 ULN, yet cancer-related mortality was the highest when ALT was ≥ 2 ULN. The same results could be found in both men and women. Univariate analysis showed that severe NAFLD with normal ALT levels had the highest all-cause and cause-specific mortality, but the difference was not statistically significant after adjustment for age and multivariate factors.

CONCLUSION
The risk of NAFLD was positively correlated with ALT level, but all-cause and cardiovascular mortality were the highest when ALT was < 0.5 ULN. Regardless of the severity of NAFLD, normal or lower ALT levels were associated with higher mortality than elevated ALT levels. Clinicians should be aware that high ALT levels indicate liver injury, but low ALT levels are associated with a higher risk of death.

Key Words: Non-alcoholic fatty liver disease; Alanine aminotransferase; Mortality; NHANES-III

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Core Tip: The risk of nonalcoholic fatty liver disease (NAFLD) was positively correlated with alanine aminotransferase (ALT) level, but all-cause and cardiovascular mortality were the highest when ALT < 0.5 upper limits of normal. Regardless of the severity of NAFLD, normal or lower ALT levels are associated with higher mortality than elevated ALT levels. Clinicians should be aware of not only high ALT, indicating liver injury, but also low ALT associated with higher risk of death.

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INTRODUCTION

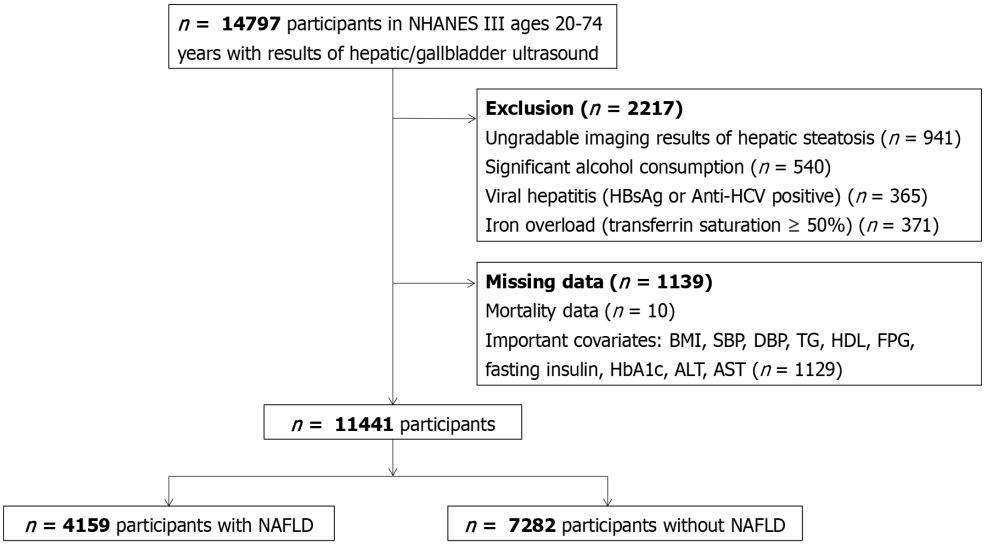
The number of patients suffering from nonalcoholic fatty liver disease (NAFLD) has reached one billion worldwide, replacing viral hepatitis as the most common chronic liver disease[1-3], as well as increasing the risk of hepatocellular carcinoma[4]. Research has demonstrated that NAFLD is no longer restricted to the liver itself but is also the main manifestation of metabolic syndrome in the liver, which is closely related to obesity, dyslipidemia, hypertension, type 2 diabetes, insulin resistance, and cardiovascular disease[5,6]. The all-cause mortality of patients with NAFLD is significantly increased, and cardiovascular disease, malignant tumors, and end-stage liver disease are the main causes of death in patients with NAFLD[7-9]. Therefore, it is very important to find an effective treatment for NAFLD.

The elevation of alanine aminotransferase (ALT) is a measure of liver disease activity and liver injury severity. Several studies have shown that an increase in ALT levels has a close correlation with an increased risk of NAFLD. It has also been proven to be an independent predictor of NAFLD and is related to nonalcoholic steatohepatitis and advanced liver fibrosis[10-12]. Research has shown that elevated ALT levels are also positively correlated with metabolic syndrome-related diseases, such as cardiovascular disease and type 2 diabetes[13,14]. Based on these previous findings, it could be inferred that all-cause and cardiovascular mortality are correlated with elevated ALT levels. However, this conclusion appears to be inconsistent and controversial[15-17]. Elevated ALT levels are related to mortality from various causes in some studies[18,19], but not in others[20,21]. In contrast, some studies have indicated that normal or lower ALT levels appear to be negatively correlated with a higher risk of all-cause mortality[22,23]. Moreover, the relationship between ALT levels and all-cause and cause-specific mortality in patients with NAFLD has not been fully reported. Therefore, this study focuses on the relationship between serum ALT levels and all-cause mortality and cause-specific mortality in patients with NAFLD in the Third National Health and Nutrition Examination Survey (NHANES-III) database, so as to further explore the clinical significance of using ALT as a potential treatment target and to improve the survival and prognosis of patients with NAFLD.

MATERIALS AND METHODS

Dataset and study population

The NHANES-III produced a national dataset that evaluates the health status of people in the United States, and it was conducted in two phases (1988-1991 and 1991-1994). The NHANES-III conducted interviews, physical examinations, and laboratory tests, and, at the same time, it used ultrasonic examination to evaluate hepatic steatosis, which is the main reason the authors chose this dataset. Data from NHANES-III was also linked to death certificates from the National Death Index (NDI) as of



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Figure 1 Flow-chart of the Study. NHANE III: The National Health and Nutrition Examination Survey III (1988-1994); HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TC: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein cholesterol; FPG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NAFLD: Nonalcoholic fatty liver disease.

December 31, 2019, allowing for mortality analysis. The NHANES protocol was approved by the Ethics Review Committee of the National Center for Health Statistics, which obtained informed consent from all subjects.

Among the adult participants (aged 20-74 years old) in the NHANES-III survey with liver/gallbladder ultrasound and laboratory test results (n = 14797), individuals without a liver ultrasound steatosis grade and those with significant alcohol consumption (men > 21 drinks/week, women > 14 drinks/week), viral hepatitis (serum hepatitis B surface antigen and/or serum hepatitis C antibody positive), or iron overload (transferring saturation ≥ 50%) (n = 2217) were excluded from this study. Individuals with incomplete mortality information and certain important indicators were also excluded (n = 1139). Finally, this study included a total of 11,441 individuals aged 20-74 years (Figure 1).

Clinical variables

An elevated ALT level was defined as ALT > 30 U/L in males and > 19 U/L in females, and the ALT levels were classified into four groups [< 0.5 upper limits of normal (ULN), 0.5-1 ULN, 1-2 ULN, ≥ 2 ULN]. Race/ethnicity was categorized as non-Hispanic white, non-Hispanic black, Mexican–American, or other. Diabetes was defined as a high fasting blood sugar (> 126 ng/dL), a high glycosylated hemoglobin (HbA1c) (> 6.5%), or a history of diabetes and/or use of diabetic medication. A homeostasis model assessment of insulin resistance > 2.5 was considered as insulin resistant[24]. Hypertension was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg and/or treatment with antihypertensive medication. A self-reported questionnaire on the frequency and amount of alcohol consumption was used to identify drinking status[25]. If people answered "no" to having done any of the following activities in the previous month, they were classified as being "sedentary" in terms of sports activities: jogging/running, cycling, swimming, aerobic exercise, other dancing, aerobics, yard work/gardening, weightlifting, or other sports[26].

Definition of NAFLD

The gallbladder ultrasound image files were reviewed by three board-certified clinicians to assess hepatic steatosis. In this study, NAFLD was defined as any degree (mild to severe) of steatosis, according to five criteria, without a competing etiology for secondary liver steatosis.

Mortality

Participants who were aged over 20 years in the NHANES-III were followed up for passive mortality as of December 31, 2019. Probability matching was performed using NDI records to assess the death status (including the date of death) and the cause of death; the potential cause of death 113 code was used to code deaths before 1998, and deaths between 1999 and 2015 were coded according to the Ninth Revision of the International Classification of Diseases. The Centers for Disease Control and Prevention restricted the liver-related mortality data in the NHANES III for public use.

Statistical analysis

All data were analyzed using SPSS 27.0. Weighted analyses were performed using NHANES survey weights[27]. The Student's *t*-test was used to compare continuous variables, and the Rao-Scott chi-square was used to test categorical variables. Multivariate logistic regression was used to confirm an independent relationship between ALT status and NAFLD after adjustment for potential clinical and demographic variables. Cox proportional hazards regression analysis was used to analyze all-cause and cause-specific mortality. A P value < 0.05 was considered to have a significant statistical difference.

RESULTS

Baseline characteristics of patients

Of the 11441 NHANES-III survey participants (mean age, 44.4 years; male, 44.6%) enrolled in this study, the prevalence of NAFLD was 36.4%, among which moderate to severe steatosis accounted for 22.8%. In this cohort, 84.8% of the participants had normal ALT levels, and 15.2% had elevated ALT levels. Table 1 summarizes the baseline characteristics of the overall population and patients with NAFLD. In the whole population, compared with the individuals with normal ALT levels, those with elevated ALT levels were more likely to be young people, women, or Mexican Americans, those who did little physical activity, and those who were diabetic and insulin resistant. In addition, body mass index (BMI), waist circumference, TC, TG, HbA1c, FPG, and AST were higher in these people, and HDL was lower. The prevalence of NAFLD was higher in individuals with elevated ALT levels compared with those with normal ALT levels (59% *vs* 32.3%, P < 0.001). In patients with NAFLD, compared with individuals with normal ALT levels, those with elevated ALT levels were also younger, more likely to be women, Mexican American, diabetic, insulin resistant, and they had higher BMI, waist circumference, TC, TG, and AST levels. The proportion of patients with moderate and severe steatosis was higher (47.7% and 30.1%, respectively). When ALT was classified into different levels (< 0.5 ULN, 0.5-1 ULN, 1-2 ULN, and ≥ 2 ULN), in the univariate model, the incidence of NAFLD increased with the increase in ALT level [odds ratio (OR): 1, 1.65, 3.56, and 6.72 respectively, P < 0.001]. When adjusted for age, this relationship still existed. Multivariate analysis showed that with the increase in ALT level, the risk of NAFLD increased by 37%, 128%, and 217%, respectively (Supplementary Table 1).

ALT levels and mortality in the overall population

The average follow-up time of the 11,441 individuals was 23.8 years. A total of 3,976 people died during the follow-up period, and cardiovascular disease (n = 1104) and cancer (n = 953) were the two leading causes of death. The results of the Cox proportional hazards regression analysis of the overall population are given in Table 2. The univariate analysis showed that all-cause mortality gradually decreased with the increase in ALT level [hazard ratio (HR): 0.76, 0.66, and 0.60, respectively, P < 0.001]. When considering known demographic variables and traditional risk factors, all-cause mortality was still the highest when ALT was at the lowest level (< 0.5 ULN). With regard to NAFLD, in univariate analysis, patients with NAFLD had a 41% higher risk of all-cause death than the patients who did not have NAFLD [HR: 1.41, 95% confidence interval (CI): 1.33-1.51, P < 0.001]. However, NAFLD was no longer correlated with all-cause mortality when other demographics and covariates were controlled.

When mortality was limited to cardiovascular disease (Table 3), the multivariable analysis indicated that elevated ALT levels were correlated with decreased cardiovascular mortality in individuals overall and in patients with NAFLD, but there was no significant statistical difference when ALT was ≥ 2 ULN. Death caused by other diseases, such as respiratory and cerebrovascular diseases, with different ALT levels also showed a similar result. The analysis of cancer-related mortality had a different result; when ALT was ≥ 2 ULN, the risk of cancer-related death in the whole population increased by 11% and, in patients with NAFLD, it increased by 39%, but there was no significant statistical difference.

ALT levels and mortality in patients with NAFLD

Further research was done concerning the patients with NAFLD (Supplementary Table 2 in the ESM). Because the ULN of the ALT level differs in men and women, an analysis was performed according to gender. The baseline characteristics of the enrolled patients with NAFLD are summarized in Supplementary Table 3 in the ESM. A total of 1932 men and 2227 women are included, with an average age of 47.9 and 45.8 years, respectively. The median ALT level was 25 U/L in men and 18 U/L in women. In most patients with NAFLD, ALT levels were within the normal range, of which 0.5-1 ULN was the most common (found in 49.9% of males and 50.9% of females, respectively). All-cause mortality and cause-specific mortality were investigated based on ALT levels in men and women. In male patients with NAFLD, the univariate model showed that all-cause and cancer-related mortality decreased with the increase in ALT level, but the age-adjusted and multivariate models showed that mortality increased when ALT was ≥ 2 ULN. However, the difference was not statistically significant. Regarding death caused by cardiovascular disease and other causes, the three models showed that the risk of death decreased with the increase of ALT level. Among female patients, cancer-related mortality was the

Table 1 Baseline characteristics of the overall population and nonalcoholic fatty liver disease patients with normal or elevated alanine aminotransferase level

	Overall population (*n* = 11441)			NAFLD population (*n* = 4159)		
	Normal ALT level (*n* = 9698)	Elevated ALT level (*n* = 1743)	*P* value	Normal ALT level (*n* = 3131)	Elevated ALT level (*n* = 1028)	*P* value
Age (yr)	44.4 ± 0.17	41.5 ± 0.35	< 0.001	48.0 ± 0.29	43.2 ± 0.45	< 0.001
Sex, male (%)	4397 (45.3)	710 (40.7)	< 0.001	1496 (47.8)	436 (42.4)	0.003
Race/ethnicity, *n* (%)			< 0.001			< 0.001
Non-Hispanic White	3725 (38.4)	527 (30.2)		1186 (37.9)	300 (29.2)	
Non-Hispanic Black	2950 (30.4)	325 (18.6)		861 (27.5)	143 (13.9)	
Mexican American	2620 (27.0)	815 (46.8)		967 (30.9)	540 (52.5)	
Others	403 (4.2)	76 (4.4)		117 (3.7)	45 (4.4)	
Waist circumference (cm)	92.3 ± 0.15	98.3 ± 0.35	< 0.001	97.9 ± 0.30	102.6 ± 0.45	< 0.001
BMI (kg/m^2)	27.0 ± 0.06	29.7 ± 0.15	< 0.001	28.9 ± 0.12	31.4 ± 0.20	< 0.001
Hypertension, *n* (%)	3892 (40.1)	698 (40.0)	0.946	1434 (45.8)	458 (44.6)	0.486
Diabetes, *n* (%)	836 (8.6)	253 (14.5)	< 0.001	476 (15.2)	214 (20.8)	< 0.001
HOMA-IR, *n* (%)	3652 (37.7)	1117 (64.1)	< 0.001	1712 (54.7)	785 (76.4)	< 0.001
Fasting plasma glucose (mmol/L)	5.6 ± 0.02	5.9 ± 0.06	< 0.001	6.0 ± 0.05	6.3 ± 0.09	0.002
HbA1c (%)	5.5 ± 0.01	5.7 ± 0.03	< 0.001	5.8 ± 0.02	5.9 ± 0.05	0.027
TG (mg/dL)	135.2 ± 1.0	182.4 ± 3.2	< 0.001	165.9 ± 2.09	207.0 ± 4.49	< 0.001
Total cholesterol (mg/dL)	204.2 ± 0.44	209.7 ± 1.07	< 0.001	208.6 ± 0.80	212.6 ± 1.37	< 0.001
HDL (mg/dL)	51.3 ± 0.15	47.0 ± 0.36	< 0.001	48.3 ± 0.27	45.0 ± 0.47	< 0.001
ALT (IU/L)	13.7 ± 0.06	39.5 ± 0.63	< 0.001	14.9 ± 0.10	41.7 ± 0.90	< 0.001
AST (IU/L)	19.0 ± 0.06	34.2 ± 0.64	< 0.001	19.5 ± 0.11	35.6 ± 0.92	< 0.001
Albumin (g/L)	41.4 ± 0.04	41.7 ± 0.09	0.007	41.1 ± 0.07	41.8 ± 0.11	< 0.001
Smoked at least 100 cigarettes, *n* (%)	4872 (50.2)	752 (43.3)	< 0.001	1637 (52.3)	448 (43.6)	< 0.001
Sedentary lifestyle, *n* (%)	2811 (29.0)	583 (33.4)	< 0.001	1048 (33.5)	373 (36.3)	0.099
NAFLD, *n* (%)	3131 (32.3)	1028 (59.0)	< 0.001			
Mild	1326 (13.7)	229 (13.1)		1326 (42.4)	229 (22.3)	
Moderate	1268 (13.1)	490 (28.1)		1268 (40.5)	490 (47.7)	
Severe	537 (5.5)	309 (1.7)	< 0.001	537 (17.2)	309 (30.1)	< 0.001

Categorical values are shown as *n* (%). Continuous variables are shown as mean ± SEs. Elevated alanine aminotransferase (ALT) level was defined as ALT > 30 U/L in men or > 19 U/L in women. BMI: Body mass index; HOMA-IR: Homeostasis model assessment of insulin resistance; HbA1c: Glycosylated hemoglobin; TG: Triglyceride; HDL: High-density lipoprotein cholesterol; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NAFLD: Nonalcoholic fatty liver disease.

highest in the univariate, age-adjusted, and multivariate models when ALT was ≥ 2 ULN, which was similar to the results in the whole population with NAFLD (Tables 4 and 5).

The different models and mortality in patients with NAFLD

The different NAFLD statuses (mild to moderate or severe) were then combined with the different ALT levels (normal or elevated) for further analysis of all-cause and cause-specific mortality. In patients with NAFLD, the univariate and age-adjusted models showed that severe NAFLD with normal ALT levels had the highest all-cause mortality (HR: 1.45, $P < 0.001$ and 1.11, $P = 0.139$), but this relationship no longer existed after multivariable adjustment (HR: 0.98, $P = 0.727$) (Table 6). In terms of cause-specific mortality, Model 1 (mild to moderate NAFLD with normal ALT level) and Model 3 (severe NAFLD with normal ALT level) had a higher risk of death than Model 2 (mild to moderate NAFLD with

Table 2 Association between alanine aminotransferase level or nonalcoholic fatty liver disease status and all-cause mortality in overall population

ALT level	Unadjusted		Age-adjusted		Multivariate-adjusted	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
< 0.5 ULN	1		1		1	
0.5-1 ULN	0.76 (0.71-0.81)	< 0.001	0.74 (0.70-0.80)	< 0.001	0.76 (0.71-0.81)	< 0.001
1-2 ULN	0.66 (0.60-0.74)	< 0.001	0.73 (0.66-0.81)	< 0.001	0.70 (0.63-0.79)	< 0.001
≥ 2 ULN	0.60 (0.49-0.74)	< 0.001	0.92 (0.75-1.14)	0.442	0.74 (0.57-0.97)	0.017
NAFLD						
No NAFLD	1		1		1	
NAFLD	1.41 (1.33-1.50)	< 0.001	1.13 (1.06-1.20)	< 0.001	0.99 (0.92-1.06)	0.758

The multivariate model was adjusted for age, sex, race/ethnicity, body mass index, waist circumference, aspartate aminotransferase, albumin, triglyceride, total cholesterol, high-density lipoprotein cholesterol, smoking status, diabetes, hypertension, and sedentary lifestyle. CI: Confidence interval; HR: Hazard ratio; ULN: Upper limits of normal; ALT: Alanine aminotransferase; NAFLD: Nonalcoholic fatty liver disease.

Table 3 Association of alanine aminotransferase level, cardiovascular disease, cancer-related and others-related mortality stratified by the presence/absence of nonalcoholic fatty liver disease

	Total population		No NAFLD		NAFLD	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
ALT level						
Cardiovascular						
< 0.5 ULN	1		1		1	
0.5-1 ULN	0.74 (0.65-0.85)	< 0.001	0.82 (0.68-0.97)	0.022	0.66 (0.52-0.82)	< 0.001
1-2 ULN	0.63 (0.50-0.79)	< 0.001	0.78 (0.56-1.09)	0.149	0.55 (0.39-0.78)	< 0.001
≥ 2 ULN	0.63 (0.35-1.12)	0.113	0.70 (0.26-1.86)	0.469	0.68 (0.31-1.50)	0.342
Cancer						
< 0.5 ULN	1		1		1	
0.5-1 ULN	0.74 (0.64-0.86)	< 0.001	0.70 (0.58-0.84)	< 0.001	0.85 (0.66-1.11)	0.230
1-2 ULN	0.67 (0.52-0.86)	0.002	0.61 (0.42-0.88)	0.009	0.77 (0.54-1.12)	0.171
≥ 2 ULN	1.11 (0.67-1.86)	0.682	0.86 (0.35-2.11)	0.738	1.39 (0.70-2.75)	0.346
Others						
< 0.5 ULN	1		1		1	
0.5-1 ULN	0.89 (0.80-0.99)	0.029	0.92 (0.81-1.05)	0.227	0.80 (0.67-0.95)	0.011
1-2 ULN	0.98 (0.83-1.16)	0.809	1.25 (0.98-1.60)	0.074	0.73 (0.57-0.93)	0.012
≥ 2 ULN	0.75 (0.51-1.09)	0.131	0.69 (0.33-1.45)	0.331	0.53 (0.32-0.89)	0.015

The multivariate model was adjusted for age, sex, race/ethnicity, body mass index, waist circumference, aspartate aminotransferase, albumin, triglyceride, total cholesterol, high-density lipoprotein cholesterol, smoking status, diabetes, hypertension, and sedentary lifestyle. Others: Chronic lower respiratory diseases, accidents (unintentional injuries), cerebrovascular diseases, Alzheimer's diseases, diabetes mellitus, influenza and pneumonia, nephritis, nephrotic syndrome and nephrosis and all other causes (residual). CI: Confidence interval; HR: Hazard ratio; ULN: Upper limits of normal; ALT: Alanine aminotransferase; NAFLD: Nonalcoholic fatty liver disease.

elevated ALT level) and Model 4 (severe NAFLD with elevated ALT level). Further analysis was made according to gender (Supplementary Tables 4 and 5). Among male patients with NAFLD, those with severe NAFLD and elevated ALT levels had higher cancer-related mortality (HR: 1.18, $P = 0.602$). The risk factors of ALT at the lowest and highest levels were explored (Supplementary Table 6 in the ESM). In male patients, the univariate analysis indicated that the age of those with ALT ≥ 2 ULN was

Table 4 Association of alanine aminotransferase level, all-cause, cardiovascular disease, cancer-related and others-related mortality among nonalcoholic fatty liver disease patients in men

Mortality outcome, ALT level	No. of death, *n* (%)	Unadjusted		Age-adjusted		Multivariate-adjusted	
		HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
All-cause							
< 0.5 ULN	306 (61.1)	1		1		1	
0.5-1 ULN	436 (45.2)	0.60 (0.52-0.70)	< 0.001	0.69 (0.59-0.80)	< 0.001	0.68 (0.58-0.80)	< 0001
1-2 ULN	115 (30.8))	0.37 (0.30-0.46)	< 0.001	0.62 (0.50-0.77)	< 0.001	0.60 (0.47-0.76)	< 0001
≥ 2 ULN	21 (22.6)	0.26 (0.16-0.40)	< 0.001	0.69 (0.44-1.09)	0.11	0.72 (0.42-1.21)	0.214
Cardiovascular disease							
< 0.5 ULN	102 (20.4)	1		1		1	
0.5-1 ULN	119 (22.3)	0.50 (0.39-0.65)	< 0.001	0.58 (0.44-0.76)	< 0.001	0.57 (0.43-0.77)	< 0.001
1-2 ULN	34 (9.1)	0.34 (0.23-0.50)	< 0.001	0.58 (0.39-0.87)	0.007	0.55 (0.33-0.92)	0.022
≥ 2 ULN	3 (3.2)	0.11 (0.04-0.36)	< 0.001	0.33 (0.10-1.05)	0.06	0.51 (0.13-1.97)	0.331
Cancer							
< 0.5 ULN	67 (13.4)	1		1		1	
0.5-1 ULN	103 (10.7)	0.65 (0.48-0.89)	0.006	0.73 (0.54-1.00)	0.051	0.79 (0.57-1.10)	0.169
1-2 ULN	25 (6.7)	0.37 (0.23-0.58)	< 0.001	0.62 (0.39-0.99)	0.047	0.76 (0.45-1.29)	0.308
≥ 2 ULN	5 (5.4)	0.28 (0.11-0.69)	0.006	0.81 (0.32-2.05)	0.654	1.05 (0.35-3.12)	0.935
Others							
< 0.5 ULN	137 (44.8)	1		1		1	
0.5-1 ULN	214 (49.1)	0.81 (0.65-1.00)	0.048	0.81 (0.66-1.01)	0.058	0.73 (0.57-0.92)	0.921
1-2 ULN	56 (48.7)	0.68 (0.50-0.92)	0.014	0.75 (0.55-1.03)	0.073	0.57 (0.38-0.85)	0.851
≥ 2 ULN	13 (61.9)	0.70 (0.39-1.23)	0.212	0.76 (0.42-1.37)	0.361	0.40 (0.19-0.84)	0.844

The multivariate model was adjusted for age, sex, race/ethnicity, body mass index, waist circumference, aspartate aminotransferase, albumin, triglyceride, total cholesterol, high-density lipoprotein cholesterol, smoking status, diabetes, hypertension, and sedentary lifestyle. Others: Chronic lower respiratory diseases, accidents (unintentional injuries), cerebrovascular diseases, Alzheimer's diseases, diabetes mellitus, influenza and pneumonia, nephritis, nephrotic syndrome and nephrosis and all other causes (residual). CI: Confidence interval; HR: Hazard ratio; ULN: Upper limits of normal; ALT: Alanine aminotransferase.

significantly lower than that of those with ALT < 0.5 ULN, with more patients aged 20-39 years and fewer patients aged over 60 years. The multivariable-adjusted analysis also indicated that age was a protective factor in male patients. However, in female patients, no significant statistical difference was seen between the two age groups.

DISCUSSION

The main findings of the present large cohort study were that elevated serum ALT levels were closely related to the increased risk of NAFLD but did not increase the risk of all-cause and cardiovascular mortality in the whole population and in patients with and without NAFLD. On the contrary, in the patients with NAFLD, all-cause and cardiovascular mortality was the highest when ALT was < 0.5 ULN, and this was the same for both sexes and all ages. Cancer-related mortality was the highest when ALT was ≥ 2 ULN, but there was no significant statistical difference. With different degrees of NAFLD (mild to moderate or severe), whether the ALT level was elevated or not seems to have had no significant effect on all-cause and cause-specific mortality.

Previous research has shown that NAFLD is related to a higher risk of all-cause mortality[28]. In addition, the correlation between NAFLD and increased risk of cardiovascular events was proved in a meta-analysis[29]. Cardiovascular disease, malignant tumors, and end-stage liver disease are the main causes of death in patients with NAFLD[7,8], but cardiovascular disease is the primary cause of death [7]. Therefore, it is of great importance to recognize the significant influence of metabolic complications

Table 5 Association of alanine aminotransferase level, all-cause, cardiovascular disease, cancer-related and others-related mortality among nonalcoholic fatty liver disease patients in women

Mortality outcome, ALT level	No. of death, *n* (%)	Unadjusted		Age-adjusted		Multivariate-adjusted	
		HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
All-cause							
< 0.5 ULN	144 (34.0)	1		1		1	
0.5-1 ULN	440 (38.8)	1.16 (0.96-1.40)	0.127	0.88 (0.73-1.06)	0.176	0.91 (0.74-1.10)	0.348
1-2 ULN	187 (36.0)	1.05 (0.85-1.31)	0.632	0.83 (0.67-1.03)	0.095	0.76 (0.60-0.97)	0.027
≥ 2 ULN	46 (30.7)	0.89 (0.64-1.24)	0.484	1.10 (0.79-1.53)	0.585	0.82 (0.52-1.31)	0.416
Cardiovascular disease							
< 0.5 ULN	41 (9.7)	1		1		1	
0.5-1 ULN	119 (10.5)	1.10 (0.77-1.58)	0.594	0.82 (0.58-1.17)	0.282	0.86 (0.59-1.26)	0.44
1-2 ULN	442 (8.1)	0.83 (0.54-1.28)	0.41	0.65 (0.42-1.00)	0.051	0.62 (0.38-1.02)	0.061
≥ 2 ULN	99 (6.0)	0.61 (0.30-1.26)	0.182	0.78 (0.38-1.61)	0.504	0.85 (0.29-2.43)	0.756
Cancer							
< 0.5 ULN	31 (7.3)	1		1		1	
0.5-1 ULN	97 (8.6)	1.18 (0.79-1.77)	0.425	0.93 (0.62-1.40)	0.728	1.02 (0.66-1.57)	934
1-2 ULN	37 (7.1)	0.96 (0.60-1.55)	0.877	0.76 (0.47-1.23)	0.261	0.83 (0.49-1.44)	0.482
≥ 2 ULN	15 (10.0)	1.34 (0.72-2.48)	0.352	1.38 (0.74-2.57)	0.309	1.49 (0.61-3.68)	0.395
Others							
< 0.5 ULN	71 (49.7)	1		1		1	
0.5-1 ULN	224 (50.9)	0.91 (0.70-1.20)	0.526	0.92 (0.71-1.20)	0.549	0.89 (0.67-1.18)	0.411
1-2 ULN	108 (57.8)	1.01 (0.75-1.37)	0.935	1.08 (0.80-1.46)	0.627	0.85 (0.61-1.20)	0.366
≥ 2 ULN	22 (47.8)	0.99 (0.62-1.61)	0.978	1.16 (0.72-1.88)	0.548	0.62 (0.30-1.29)	0.199

The multivariate model was adjusted for age, sex, race/ethnicity, body mass index, waist circumference, aspartate aminotransferase, albumin, triglyceride, total cholesterol, high-density lipoprotein cholesterol, smoking status, diabetes, hypertension, and sedentary lifestyle. Others: Chronic lower respiratory diseases, accidents (unintentional injuries), cerebrovascular diseases, Alzheimer's diseases, diabetes mellitus, influenza and pneumonia, nephritis, nephrotic syndrome and nephrosis and all other causes (residual). CI: Confidence interval; HR: Hazard ratio; ULN: Upper limits of normal; ALT: Alanine aminotransferase.

on NAFLD, especially as seen in patients with NAFLD who died of cardiovascular disease. Since ALT is often considered a marker to evaluate the activity of liver disease and the severity of liver injury in clinical practice, many believe that monitoring ALT levels can be an effective means of following the progression of NAFLD[30,31]. Because of the invasive nature of liver biopsy, clinicians often take ALT levels as the basis of liver protection treatment. The association between elevated ALT levels and increased mortality has been reported in several epidemiological studies[18,32]. It seems to be a reasonable inference that elevated ALT levels may increase the risk of death. However, in different countries and cohorts, the opposite relationship between ALT levels and all-cause mortality has been observed. One study of NHANES-III data found that the relationship between ALT levels and all-cause mortality was similar to a U-shaped curve[22]. Another large cohort study also indicated that ALT at the lowest levels (≤ 10 U/L) and elevated ALT levels (> 40 U/L) were associated with increased all-cause mortality, especially among people aged over 60 years[33]. Two recent studies also showed that lower ALT levels within the normal range were related to a higher risk of all-cause and cardiovascular mortality in the elderly population[34,35]. These findings consistently indicate that there is a certain non-linear correlation between ALT levels and all-cause mortality although some unmeasured or residual confounding cannot be excluded.

The results of the current study showed that in patients with NAFLD, the ALT levels of the vast majority, both male and female, are within the normal range, and the number of patients with an ALT level in the range of 0.5-1 ULN is the largest, while the number in the range ≥ 2 ULN is the smallest. In addition, nearly half of the male patients with NAFLD with an ALT level < 0.5 ULN were elderly, while nearly 3/4 of those with an ALT level ≥ 2 ULN were young. Another study also showed that the ALT level first increased and then decreased with age[36], which may explain why in patients with NAFLD

Table 6 Association of different models, all-cause, cardiovascular disease, cancer-related and others-related mortality among nonalcoholic fatty liver disease patients

Mortality outcome	No. of death, *n* (%)	Unadjusted		Age-adjusted		Multivariate-adjusted	
		HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
All-cause							
Model 1	1055 (41.9)	1		1		1	
Model 2	248 (31.2)	0.68 (0.59-0.78)	< 0.001	0.89 (0.77-1.02)	0.1	0.87 (0.74-1.01)	0.067
Model 3	271 (53.7)	1.45 (1.26-1.65)	< 0.001	1.11 (0.97-1.27)	0.139	0.98 (0.85-1.12)	0.727
Model 4	121 (35.5)	0.82 (0.68-0.99)	0.037	0.91 (0.75-1.10)	0.312	0.81 (0.66-0.99)	0.042
Cardiovascular disease							
Model 1	309 (12.3)	1		1		1	
Model 2	68 (8.6)	0.64 (0.49-0.83)	< 0.001	0.86 (0.66-1.12)	0.254	0.89 (0.65-1.20)	0.437
Model 3	72 (14.3)	1.30 (1.01-1.68)	0.045	0.99 (0.76-1.27)	0.907	0.82 (0.63-1.07)	0.146
Model 4	20 (5.9)	0.46 (0.30-0.73)	< 0.001	0.52 (0.33-0.82)	0.005	0.53 (0.33-0.87)	0.011
Cancer							
Model 1	241 (9.6)	1		1		1	
Model 2	50 (6.3)	0.60 (0.44-0.81)	0.001	0.74 (0.55-1.01)	0.055	0.86 (0.62-1.21)	0.387
Model 3	57 (11.3)	1.31 (0.98-1.75)	0.067	1.02 (0.76-1.36)	0.904	1.01 (0.75-1.37)	0.928
Model 4	32 (9.4)	0.94 (0.65-1.36)	0.743	1.02 (0.70-1.47)	0.938	1.07 (0.71-1.61)	0.755
Others							
Model 1	504 (47.8)	1		1		1	
Model 2	130 (53.4)	0.94 (0.77-1.13)	0.492	1.03 (0.84-1.25)	0.799	0.93 (0.74-1.16)	0.506
Model 3	142 (53.4)	1.28 (1.06-1.55)	0.009	1.25 (1.04-1.51)	0.019	1.15 (0.95-1.40)	0.159
Model 4	69 (57.0)	1.09 (0.85-1.41)	0.483	1.14 (0.89-1.47)	0.312	0.89 (0.67-1.20)	0.453

Model 1: Mild to moderate nonalcoholic fatty liver disease (NAFLD) with normal alanine aminotransferase (ALT) level; Model 2: Mild to moderate NAFLD with elevated ALT Level; Model 3: Severe NAFLD with normal ALT level; Model 4: Severe NAFLD with elevated ALT Level. The multivariate model was adjusted for age, sex, race/ethnicity, body mass index, waist circumference, aspartate aminotransferase, albumin, triglyceride, total cholesterol, high-density lipoprotein cholesterol, smoking status, diabetes, hypertension, and sedentary lifestyle. Others: Chronic lower respiratory diseases, accidents (unintentional injuries), cerebrovascular diseases, Alzheimer's diseases, diabetes mellitus, influenza and pneumonia, nephritis, nephrotic syndrome and nephrosis and all other causes (residual). CI: Confidence interval; HR: Hazard ratio.

the mortality rate is the highest when ALT is < 0.5 ULN. That is to say, during the follow-up period of over 31 years, individuals with an ALT level < 0.5 ULN at baseline were mostly elderly, so the rate of all-cause and cause-specific death outcomes was higher. However, most of the patients with ALT level ≥ 2 ULN at baseline were young people, so the rate of endpoint events was low. However, in the age-adjusted model and multivariable model, the results were still approximately the same, which shows that other factors besides age may play a more important role, and this possibility needs further study.

Three mechanisms might explain the underlying association between ALT levels and death. First, as an enzyme, ALT plays a vital role in converting L-alanine and α- ketoglutarate to pyruvate and glutamic acid in the heart, liver, kidney, skeletal muscle, and brain, and a low ALT level increases the risk of death by reducing the catalytic capacity for the vital metabolic steps of amino acid metabolism and gluconeogenesis[37-40]. Second, a low ALT level indicates a vitamin B6 deficiency, and epidemiological evidence indicates that a deficiency of vitamin B6 can increase the risk of cardiovascular disease, immune dysfunction, depression, and neurocognitive impairment[41,42]. Finally, other unmeasured confounding factors, rather than vitamin B6, may lead to death.

It was also found that the BMI of male and female patients with NAFLD with ALT levels < 0.5 ULN were 26.8 kg/m^2 and 27.8 kg/m^2, respectively. Recent epidemiological studies have shown that mild obesity (BMI: 23-29 kg/m^2) has a protective effect on overall mortality[43,44]. However, the underlying mechanism is unclear and needs further research.

Some limitations exist to the current study. First, it is a cross-sectional observation of the relationship between ALT levels and NAFLD, so the time correlation cannot be determined. Second, the diagnosis of NAFLD was carried out by hepatic ultrasound, which does not distinguish fat accumulation of less than

30%[45]. However, the advantages of ultrasound include safety, repeatability, low cost, high sensitivity, and specificity[46]. Therefore, ultrasound is considered a first-line imaging technology in epidemiological research and clinical practice[47]. Third, despite the adjustments of multiple covariates affecting all-cause and cause-specific mortality, some unmeasured or residual confounding factors may still exist. It was also not possible to extract specific information about liver-related mortality (due to NHANES), and obtaining such data could provide more clarity. Finally, since the participants in NHANES-III represent the population from 1988 to 1994, there may have been a much lower prevalence of diabetes and NAFLD than would be expected today.

However, this study also has its strengths. This is the first study focusing on ALT levels and all-cause and cause-specific mortality in patients with NAFLD. In a large cohort of the United States population, there are rich demographic and metabolic variables, with a median follow-up period of 23.8 years. Unlike other NHANES cycles that lack liver ultrasound results, NHANES-III includes liver steatosis diagnosed by ultrasound, which makes the diagnosis of NAFLD more accurate than that using non-invasive laboratory markers.

CONCLUSION

In conclusion, the present study's findings confirmed that all-cause and cardiovascular mortality in patients with NAFLD was the highest when ALT was < 0.5 ULN, and, regardless of the severity of the NAFLD, normal or lower ALT levels are associated with higher mortality than elevated ALT levels. This knowledge may be of importance to clinicians who, because of the invasive nature of liver perforation, often use ALT levels as the basis for liver protection treatment. According to the results of this study, in terms of treatment, it may not be beneficial to reduce ALT levels as low as possible. The findings suggest that, on the contrary, clinicians should be more vigilant of patients with NAFLD who have ALT levels < 0.5 ULN.

ARTICLE HIGHLIGHTS

Research background

Serum alanine aminotransferase (ALT) levels are greatly important in the liver disease but the role ALT levels play in the nonalcoholic fatty liver disease (NAFLD) is not clear.

Research motivation

This study aimed to investigate the association between ALT levels and all-cause and cause-specific mortality in patients with NAFLD.

Research objectives

To give the clinicians a hint about the patients with NAFLD who have lower ALT levels.

Research methods

The Third National Health and Nutrition Examination Survey (NHANES-III) from 1988 to 1994 and NHANES-III-related mortality data from 2019 onward were used to obtain the necessary data for the study. NAFLD was defined as hepatic steatosis, as diagnosed by ultrasound, with no other liver diseases. ALT levels were categorized into four groups according to the different recommended upper limits of normal (ULN) in men and women: < 0.5 ULN, 0.5-1 ULN, 1-2 ULN, and ≥ 2 ULN. The hazard ratios for all-cause mortality and cause-specific mortality were analyzed using the Cox proportional hazard model.

Research results

In patients with NAFLD, all-cause mortality and cardiovascular mortality were the highest when ALT was < 0.5 ULN, yet cancer-related mortality was the highest when ALT was ≥ 2 ULN. The same results could be found in both men and women. Univariate analysis showed that severe NAFLD with normal ALT levels had the highest all-cause and cause-specific mortality, but the difference was not statistically significant after adjustment for age and multivariate factors, both the underlying mechanism is unclear and needs further research.

Research conclusions

The risk of NAFLD was positively correlated with ALT level, but all-cause and cardiovascular mortality were the highest when ALT was < 0.5 ULN. Regardless of the severity of NAFLD, normal or lower ALT levels were associated with higher mortality than elevated ALT levels. Clinicians should be aware that high ALT levels indicate liver injury, but low ALT levels are associated with a higher risk of death.

Research perspectives

The underlying mechanism about the lower ALT levels and high mortality death is unclear and needs further research.

FOOTNOTES

Author contributions: Feng B designed the study; Zheng JR and Wang ZL contributed to the acquisition, analysis and interpretation of data and drafted the manuscript; Jiang SZ contributed to the critical revision of the manuscript for important intellectual content and Chen HS contributed to the study supervision; all authors have made a significant contribution to this study and have approved the final manuscript.

Institutional review board statement: The NHANES protocol was approved by the Ethics Review Committee of the National Center for Health Statistics, which obtained informed consent from all subjects.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: The authors have declared no conflict of interest.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Randomized intervention and outpatient follow-up lowers 30-d readmissions for patients with hepatic encephalopathy, decompensated cirrhosis

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Abstract

BACKGROUND

We previously reported national 30-d readmission rates of 27% in patients with decompensated cirrhosis (DC).

AIM

To study prospective interventions to reduce early readmissions in DC at our tertiary center.

METHODS

Adults with DC admitted July 2019 to December 2020 were enrolled and randomized into the intervention (INT) or standard of care (SOC) arms. Weekly phone calls for a month were completed. In the INT arm, case managers ensured outpatient follow-up, paracentesis, and medication compliance. Thirty-day

readmission rates and reasons were compared.

RESULTS

Calculated sample size was not achieved due to coronavirus disease 2019; 240 patients were randomized into INT and SOC arms. 30-d readmission rate was 33.75%, 35.83% in the INT *vs* 31.67% in the SOC arm (P = 0.59). The top reason for 30-d readmission was hepatic encephalopathy (HE, 32.10%). There was a lower rate of 30-d readmissions for HE in the INT (21%) *vs* SOC arm (45%, P = 0.03). There were fewer 30-d readmissions in patients who attended early outpatient follow-up (n = 17, 23.61% *vs* n = 55, 76.39%, P = 0.04).

CONCLUSION

Our 30-d readmission rate was higher than the national rate but reduced by interventions in patients with DC with HE and early outpatient follow-up. Development of interventions to reduce early readmission in patients with DC is needed.

Key Words: Decompensated cirrhosis; Hospital readmissions; Interventions

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Core Tip: Our 30-d readmission rate was higher than the national rate but reduced by interventions in patients with decompensated cirrhosis (DC) with hepatic encephalopathy and early outpatient follow-up. Development of interventions to reduce early readmission in patients with DC is needed.

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INTRODUCTION

Cirrhosis affects approximately 5 million annually[1] and has been reported to be the 8th leading cause of death with more than 40000 deaths annually in the United States[2]. A study on the burden of gastrointestinal (GI), liver, and pancreatic diseases in the United States revealed that liver diseases had the highest mortality at 3.1%[3]. In addition to high mortality, cirrhosis is also associated with high morbidity. The sequelae of decompensated cirrhosis (DC) are often managed during hospital admissions and include volume overload in the form of ascites, edema or hepatic hydrothorax, portal hypertension leading to bleeding esophageal or gastric varices, as well as hepatic encephalopathy (HE), hyponatremia, acute kidney injury (AKI), and spontaneous bacterial peritonitis (SBP)[4].

Several studies have demonstrated hospital readmissions in DC place a large financial burden on the United State healthcare system. The 30-d readmission rate has been reported to be 20%-37%[5-14]. We have recently published on early readmission rates up to 27% in patients with DC and developed the Mumtaz readmission risk score based on United States data[15]. We also reported that nearly one-third of patients with HE were readmitted within 30 d, and early readmission adversely impacted healthcare utilization and calendar-year mortality[16].

Interventions to reduce readmissions have been shown to be safe and effective. For instance, Morales *et al*[17] developed a program including a hepatologist follow-up exam within 7 d after discharge. This program resulted in a reduction in 30-d readmissions, 60-d mortality, emergency department visits and associated costs[17]. Similarly, another group demonstrated that follow-up with a "care management check-up" as opposed to "standard outpatient care" reduced 30-d readmission, 12-mo mortality and saved 1500 euros per patient month of life[18].

There is a paucity of prospective studies on interventions to reduce early readmission rates in patients with DC. Therefore, we prospectively studied 30-d readmission rates in patients with DC and compared various interventions (INT) with standard of care (SOC) to reduce early readmission rates. We hypothesized that DC patients in the INT arm would have decreased 30-d readmission *vs* the SOC arm.

MATERIALS AND METHODS

This study was conducted at the Ohio State University Wexner Medical Center (OSUWMC), Columbus, Ohio from July 2019 to December 2020. Our study was approved by OSUWMC Institutional Review Board. All aspects of the studying involving human participants including informed consent for enrollment were in accordance with the ethical standards of our Institutional Review Board and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Screening

All patients admitted with DC to the hepatology (inpatient or consult) service were screened for enrollment. Patients meeting inclusion criteria were approached for study consent. Of note, due to the global coronavirus disease 2019 (COVID-19) pandemic, beginning March 2020, only COVID negative patients were approached for informed consent. Elective readmissions for inpatient procedures including endoscopy, trans-arterial chemoembolization, transjugular intrahepatic portosystemic shunt (TIPS), paracentesis or readmissions unrelated to DC such as motor vehicle accidents were excluded.

Randomization and data collection

Study data were collected and managed using research electronic data capture (REDCap) hosted at The Ohio State University Wexner Medical Center[19,20]. Informed consent was obtained from all individual participants included in the study. Consented patients were randomly assigned to either the INT arm or the SOC arm in a 1:1 ratio using the REDCap randomization tool. The following data were collected on all patients *via* REDCap software including demographics (age, sex, insurance type, income based on the zip code), hospitalization data [date of index admission defined as initial admission during which patient consented for study, reason for admission, length of stay (LOS) defined as difference in days between index admission date and index admission discharge date, discharge disposition, associated cost of care of admission as obtained through medical record billing tab], etiology of cirrhosis (alcoholic and non-alcoholic including viral, non-alcoholic fatty liver disease, autoimmune, primary biliary cirrhosis, primary sclerosing cholangitis or cryptogenic), complications of cirrhosis (HE, AKI, ascites, variceal bleeding, SBP, hepatorenal syndrome, coagulopathy, portal hypertension, hepato-pulmonary syndrome, hepatocellular carcinoma), and procedures performed during admission [esophago-gastro-duodenoscopy, colonoscopy or flexible sigmoidoscopy, paracentesis, TIPS and hemodialysis (HD) on admission and discharge]. We also collected data including Elixhauser comorbidity index, discharge medications, and laboratory data (complete blood counts, serum creatinine, liver function tests including total bilirubin, INR, and sodium). Child Turcotte Pugh (CTP) and Sodium-model for end stage liver disease (MELD-Na) score were calculated from the data. The nurse case manager (CM) also recorded labs & medications at readmission and discharge and associated cost of readmission. Status of early readmission, liver transplantation, and mortality at one year were also collected.

Follow-up

The CM phoned each patient enrolled in either arm weekly for 30 d after index discharge to find out if the patient has been readmitted to OSUWMC or another hospital. In the INT arm, during the call CM also ensured i) early (defined as within 30 d from index admission discharge) outpatient hepatology follow-up ii) compliance of medication, iii) arrangement of outpatient paracentesis if needed, and reviewed outpatient hepatology clinic follow-up records. SOC arm as per our center's protocol had to be taken care of by the primary inpatient team. This included arranging early outpatient clinic follow-up, providing list of medications, and advice for outpatient paracentesis if needed at the time of discharge. Due to the nature of intervention, the study could not be blinded.

Definition of outcomes

Early readmission was defined as admission within 30 d of index admission discharge. Reasons for readmission were gathered by CM by reviewing the electronic medical record (EMR) of all enrolled patients readmitted at OSUWMC or outside hospital within 30 d of index admission. Predictors of early readmission were also compared in the two arms.

Sample size

Based on the sample size calculation, target of recruitment for the study was 848 patients, admitted to the hospital with DC under the hepatology (inpatient and consult) services. Patients were randomly assigned in a 1:1 ratio into INT or SOC arms. Based on our previous study using the National Readmissions Administrative Database, we expected a 30-d readmission rate of 27% among patients meeting inclusion criteria, which yield 114/424 patients with 30-d readmission events, thus meeting the target sample size. Based on this calculation, a total sample size of 848 (424 per group) provided 80% power to detect a 30% decrease in 30-d readmission rate (from 27% to 19%) with a type I error rate of 0.05. However, planned sample size could not be achieved due to the COVID-19 pandemic related restriction started in our center in March 2020. Therefore, we end up with available sample size of a total

of 240 patients. The modified consort flow diagram for enrollment in our study trial is illustrated in Figure 1.

Statistical analysis

Means of continuous response variables between two groups were compared using robust t-test (Welch test). Proportions were compared using χ-test or Fisher's exact test as applicable. Logarithmic transformation was used for comparing the LOS and admission cost across groups. Level of significance was kept at 0.05 for each comparison. JMP Version 15 (SAS Institute, NC) was used for all the analyses.

RESULTS

Initial screening data

From July 1, 2019, to December 1, 2020, 1392 patients were screened. Due to the COVID-19 pandemic, recruitment was held from March 2020 to July 2020 and subsequently resumed until December 2020. Out of the patients screened, only 499 (35.85%) were eligible for inclusion; however, 240 patients consented and randomized: 120 each into the INT and SOC arm (Figure 1).

Patient demographics and clinical characteristics

The mean age of patients was 56.34 ± 11.19 years, majority were males (135, 56.25%), belonged to White race (n = 202, 84.17%) and non-Hispanic or Latino ethnicity (n = 227, 94.58%). Almost two-thirds of the patients had public insurance (n = 76, 31.67% on Medicare and n = 70, 29.17% on Medicaid); 73 (30.42%) had private insurance. At admission, the mean MELD-Na score and mean CTP Score were 21.89 ± 8.03 and 9.36 ± 1.96, respectively. Major etiology of cirrhosis was alcohol (n = 121, 50.42%) followed by non-alcoholic fatty liver disease (n = 79, 32.92%) and viral hepatitis (n = 43, 17.92%). Furthermore, 116 (48.33%) patients were actively under evaluation for liver transplantation.

Characteristics of index admissions

The index admission mean LOS was 8.13 ± 5.83 d (median 6, range 1-43 d). The mean cost of index admission was $60595 ± $47174 ($n$ = 225, median $42932, range $1630-251991). The top five reasons for index admission included volume overload (n = 111, 46.25%), AKI (n = 65, 27.08%), hepatic encephalopathy (n = 45, 18.75%), variceal bleed (n = 42, 17.50%), lower GI bleed (n = 19, 7.92%) and hyponatremia (n = 16, 6.67%). The top five interventions performed were esophago-gastro-duodenoscopy (n = 136, 56.67%), paracentesis (n = 115, 47.92%), colonoscopy/flexible sigmoidoscopy (n = 24, 10 %), HD (n = 15, 6.25%) and TIPS (n = 10, 4.17%). Most patients were discharged from index admission to home (n = 159, 66.25%) followed by home with health care (n = 42, 17.50%) and skilled nursing facility (n = 32, 13.33 %, Table 1).

Characteristics and reasons for early readmissions

Overall, 81 (33.75%) patients were readmitted within 30 d of discharge. The major reasons for first readmission included hepatic encephalopathy (n = 26, 32.10%) followed by volume overload (n = 22, 27.16%), AKI (n = 16, 19.75%), variceal bleed (n = 12, 14.82%) and hyponatremia (n = 10, 12.35%). 14 patients were readmitted twice, 3 admitted thrice and one admitted 5 times within 30 d. The mean time to first readmission was 12.65 ± 7.55 d (median 12 d, range 1-30 d). The mean LOS of first readmission was 8.11 ± 8.98 days. The mean cost of stay of first readmission was $55548.29 ± $65164.91 (Table 2). Those readmitted had a higher MELD-Na score on index admission (23.54 ± 7.80 *vs* 21.05 ± 8.03, P = 0.02) and index discharge (21.67 ± 7.95 *vs* 19.39 ± 6.89, P = 0.03) than those not readmitted. Similarly, those readmitted had a higher index admission creatinine (1.80 ± 1.53 *vs* 1.39 ± 1.16, P = 0.03), index discharge creatinine (1.61 ± 1.34 *vs* 1.20 ± 0.97, P = 0.02), and higher index admission INR (1.80 ± 0.64 *vs* 1.63 ± 0.50, P = 0.05) than those not readmitted.

Comparison of demographics and clinical characteristics in two intervention arms

Demographics including age, race, ethnicity, income, and insurance were comparable in two groups, as well as etiology of cirrhosis, MELD-Na score, CTP score, status of evaluation for liver transplant. There were majority females in the INT arm (60/120, 50% *vs* 45/120, 32.50%) and males in SOC arm (75/120, 62.50% *vs* 60/120, 50%, P = 0.03, Table 3). Index admission characteristics, disposition and index admission were also comparative in two arms (Tables 4 and 5).

Comparison of reasons of 1st readmission and outcomes in the INT vs SOC arm

There was no difference in the readmission rates for patients in the INT (n = 4, 35.83%) *vs* SOC arm (n = 38, 31.67%, P = 0.59, Table 6). Other outcomes including number of readmissions within 30 d (P = 0.65), index admission cost (P = 0.49), index admission LOS (P = 0.63), 1st readmission LOS (P = 0.58), all readmissions' LOS (P = 0.82) and waiting time for 1st readmission (P = 0.06) were comparable in two arms.

Table 1 Characteristic features of index admission by readmission status, *n* (%)

	Total	Not readmitted (*n* = 159)	Readmitted (*n* = 81)	*P* value
Index admission characteristics				
Reasons for admission[1]				
Acute kidney injury	65, 27.08	41, 25.79	24, 29.63	0.54
Hyponatremia	16, 6.67	11, 6.92	5, 6.17	1.00
Hepatic encephalopathy	45, 18.75	26, 16.35	19, 23.46	0.22
Volume overload	111, 46.25	81, 50.94	30, 37.04	0.06
Variceal bleed	42, 17.50	31, 19.50	11, 13.58	0.29
Lower GI bleed	19, 7.92	11, 6.92	8, 9.88	0.45
SBP	21, 8.75	14, 8.81	7, 8.64	1.00
Complications of cirrhosis during admission[1]				
Presence of AKI	80, 33.33	50, 31.45	30, 37.04	0.39
HE	49, 20.42	31, 19.50	18, 22.22	0.62
Ascites	139, 57.92	95, 59.75	44, 54.32	0.49
Variceal bleeding	37, 15.42	26, 16.35	11, 13.58	0.71
SBP	16, 6.67	12, 7.55	4, 4.94	0.59
HRS	14, 5.83	8, 5.03	6, 7.41	0.56
Coagulopathy	56, 23.33	36, 22.64	20, 24.69	0.75
Portal hypertension	46, 19.17	34, 21.38	12, 14.81	0.30
HPS	15, 6.25	8, 5.03	7, 8.64	0.27
HCC	11, 4.58	6, 3.77	5, 6.17	0.51
Procedures performed during admission[1]				
EGD	136, 56.67	92, 57.86	44, 54.32	0.68
Paracentesis	115, 47.92	73, 45.91	42, 51.85	0.41
Emergent TIPS	10, 4.17	9, 5.66	1, 1.23	0.17
HD	15, 6.25	7, 4.40	8, 9.88	0.16
Colonoscopy/flex sig	24, 10.00	18, 11.32	6, 7.41	0.37
Disposition[1]				
Home	159, 66.25	107, 67.30	52, 64.20	0.66
Home with Home Health Newly Arranged	39, 16.25	24, 15.09	15, 18.52	
Home with Home Health Previously Arranged	3, 1.25	2, 1.26	1, 1.23	
SNF newly Arranged	21, 8.75	16, 10.06	5, 6.17	
SNF Previously Arranged	11, 4.58	5, 3.14	6, 7.41	
Left Against Medical Advice	2, 0.83	1, 0.63	1, 1.23	
Transfer (long term acute care hospital)	3, 1.25	2, 1.26	1, 1.23	
Homeless	2, 0.83	2, 1.26	0, 0.00	

[1]Patient can have more than one of variable listed.

SBP: Spontaneous bacterial peritonitis; AKI: Acute kidney injury; HE: Hepatic encephalopathy; HRS: Hepatorenal syndrome; HPS: Hepato-pulmonary syndrome; HCC: Hepatocellular carcinoma; EGD: Esophago-gastro-duodenoscopy; GI: Gastrointestinal; TIPS: Transjugular intrahepatic portosystemic shunt; HD: Hemodialysis; SNF: Skilled Nursing Facility.

Table 2 Characteristics and reasons for readmission

Readmission status	*n*	%
No	159	66.25
Yes	81	33.75
Number of readmissions within 30 d		
0	159	66.25
1	63	26.25
2	14	5.83
3	3	1.25
5	1	0.42
Location of 1st readmission		
OSUWMC	59	72.84
Outside hospital	22	27.16
Reason for 1st readmission[1]		
Hepatic encephalopathy	26	32.10
Volume overload	22	27.16
Acute kidney injury	16	19.75
Variceal bleed	12	14.82
Hyponatremia	10	12.35
Lower GI bleed	4	4.94
Spontaneous Bacterial Peritonitis	3	3.70
LOS of first readmission (*n* = 81, mean ± SD), median = 5, range = 1 to 69	8.11 ± 8.98	
LOS of all readmissions (*n* = 105, mean ± SD), median = 4, range = 0 to 124	9.03 ± 14.42	
Cost of first readmission (*n* = 45, mean ± SD), median= $31848.95, range $765-325656.38	$55548.29 ± 65164.91	
Waiting time for first readmission (*n* = 81, mean ± SD), median = 12, range = 1-30	12.65 ± 7.55	

[1]Patient can have more than one of variable listed.
OSUWMC: The Ohio State University Wexner Medical Center; GI: Gastrointestinal; LOS: Length of stay.

Statistically significant differences were noticed in INT arm in location of 1st readmission (*n* = 36, 83.72% at OSU as compared to *n* = 23, 60.5% outside hospital, *P* = 0.03), and lesser 1st readmission with HE in the INT arm (*n* = 9, 20.9%) *vs* SOC (*n* = 17, 44.7%, *P* = 0.03). Finally, contingency analysis of readmission data showed fewer readmissions in patients who attended outpatient follow-up within 30 days of discharge from index admission (*n* = 17, 23.61% *vs n* = 55, 76.39%, *P* = 0.04).

At the end of our study, 47 (19.58%) patients received a liver transplant and 62 (25.83%) died; among those who died, 5 patients were post-transplant and 22 died in hospice. Due to the COVID-19 pandemic we were unable to achieve the anticipated sample size. Therefore, multivariate analysis was not performed.

DISCUSSION

This prospective randomized study investigated early readmission rates and healthcare utilization in patients with DC. Our readmission rate of 33.75% is higher than the United States national average (27%). While our nurse CM interventions did not reduce told readmissions, we found that HE was the top reason for readmission and such interventions were helpful in reducing early readmissions in patients with HE. This is an important lesson learned given increased burden of HE on hospitalizations, falls, mortality, impaired quality of life and caregiver burden[21]. In the validation of readmission using the liver-renal-risk score or "LIRER score", Freitas *et al*[22] showed that HE was not only a predictor of

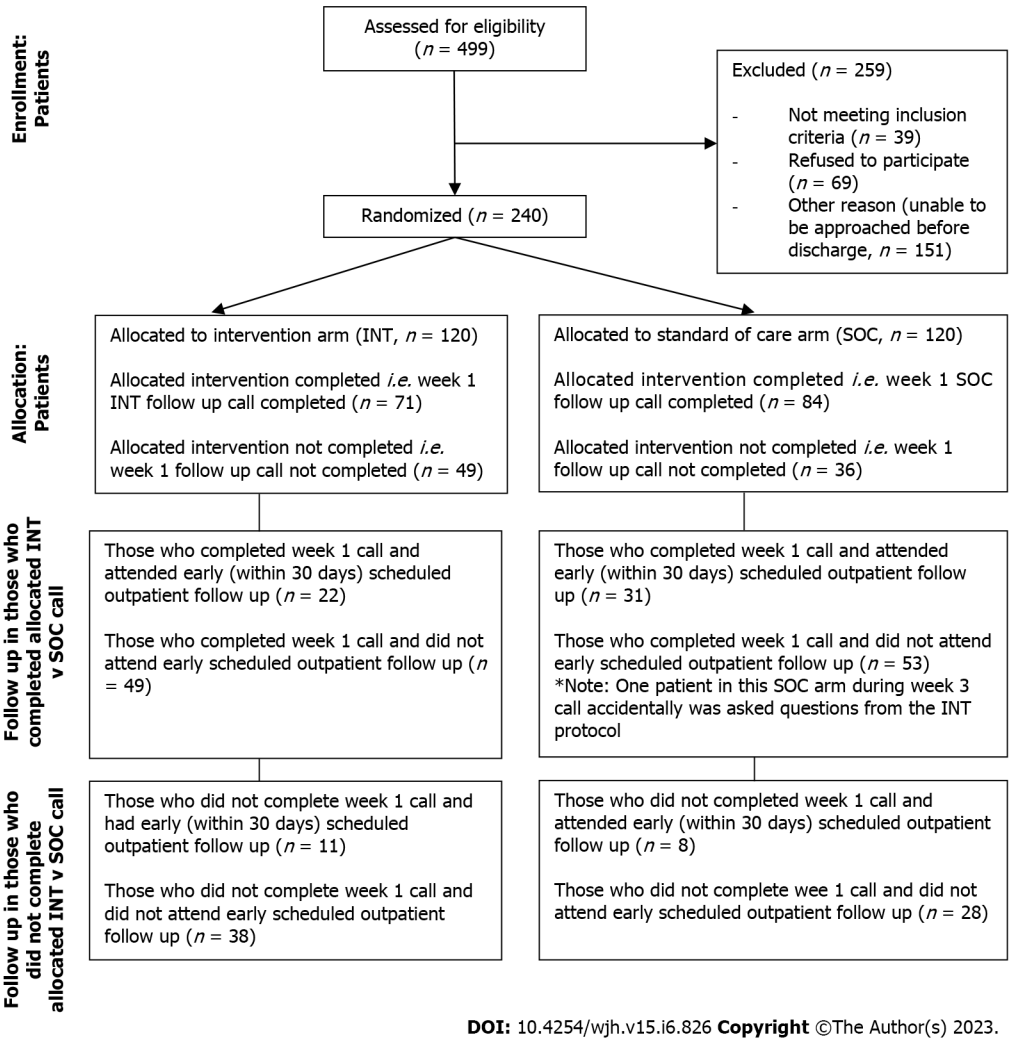
Table 3 Comparison of patient demographics and clinical characteristics by randomization arm, *n* (%)

	Intervention (*n* = 120)	Standard of care (*n* = 120)	*P* value
Patient demographics			
Age (mean ± SD)	56.54 ± 11.21	56.14 ± 11.21	0.78
Age group			
65+	32, 26.67	28, 23.33	0.79
40-64	75, 62.50	80, 66.67	
18-39	13, 10.83	12, 10.00	
Gender			
Male	60, 50.00	75, 62.50	0.03
Female	60, 50.00	45, 32.50	
Race			
White	105, 87.50	97, 80.83	0.22
Other	15, 12.50	23, 19.17	
Ethnicity			
Not Hispanic or latino	113, 94.17	114, 95.00	0.81
Hispanic or latino	3, 2.50	1, 0.83	
Unknown / Not reported	4, 3.33	5, 4.17	
Zip code income (mean ± SD)	$68045 ± $21370	$68455 ± $21651	0.88
Employment status			
Unemployed	33, 27.50	30, 25.00	0.78
Disabled	24, 20.00	24, 20.00	
Retired	26, 21.67	30, 20.00	
Employed, part time	5, 4.17	3, 2.50	
Employed, full time	23, 19.17	28, 23.33	
Other / Unknown	9, 7.50	14, 11.67	
Insurance type			
Self-pay	4, 3.33	3, 2.50	0.54
No Charge / Other / Unknown	7, 5.83	7, 5.83	
Private insurance	38, 31.67	35, 29.17	
Medicare	32, 26.67	44, 36.67	
Medicaid	39, 32.50	31, 25.83	
Number of admissions at OSU for DC in last 1 year (mean ± SD)	1.99 ± 1.61	1.84 ± 1.48	0.45
MELD-Na score admit (mean ± SD)	21.32 ± 8.19	22.47 ± 7.85	0.27
MELD-Na score discharge (mean ± SD, *n* = 117+118)	20.07 ± 7.74	20.25 ± 6.93	0.84
CTP score admit (mean ± SD)	9.31 ± 2.02	9.41 ± 1.89	0.69
CTP score discharge (mean ± SD)	8.44 ± 1.86	8.73 ± 1.89	0.24
Etiology of cirrhosis (Index admission[1])			
Alcoholic	61, 50.83	60, 50.00	1.00
Non-alcoholic fatty liver	42, 35.00	37, 30.83	0.58
Viral	21, 17.50	22, 18.33	1.00
Hep B	1, 4.76	3, 13.64	0.80
Hep C	19, 90.48	18, 81.82	

Hep B and C	1, 4.76	1, 4.55	
Cryptogenic	6, 5.00	7, 5.83	1.00
Autoimmune	1, 0.83	1, 0.83	1.00
Primary sclerosing cholangitis	2, 1.67	2, 1.67	1.00
Hemochromatosis	0, 0.0	3, 2.5	0.25
Alpha 1 anti-trypsin deficiency	3, 2.5	0, 0.0	0.25
Under evaluation for liver transplant			
No	45, 37.50	61, 50.83	0.08
Yes	63, 52.50	53, 44.17	
Unknown	12, 10.00	6, 5.00	

[1]Patient can have more than one of variable listed.

OSUWMC: The Ohio State University Wexner Medical Center; DC: Decompensated cirrhosis; MELD-Na: Model of End Stage Liver Disease Score; CTP: Child Pugh Score.



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Figure 1 Modified consort flow diagram of patients eligible for enrollment in study trial. INT: Intervention; SOC: Standard of care.

30 d readmission independent of MELD score, index, first-year, two-years and overall mortality, but also HE at admission had significantly higher mean LIRER scores. Furthermore HE patients on Medicare and geographically from the South or Midwest have higher in-hospital mortality[23]. Considerable research has been done to address HE readmissions. Bajaj *et al*[24] found that efforts to reduce medication-precipitated HE, prevent aspiration pneumonia and optimize HE medications on hospital

Table 4 Characteristic features during index admission in two randomization arms, *n* (%)

Index admission characteristics	Intervention (*n* = 120)	Standard of care (*n* = 120)	*P* value
Reasons for admission[1]			
Acute kidney injury	30, 25.00	35, 29.17	0.56
Hyponatremia	10, 8.33	6, 5.00	0.44
Hepatic encephalopathy	22, 18.33	23, 19.17	1
Volume overload	59, 49.17	52, 43.33	0.44
Variceal bleed	21, 17.50	21, 17.50	1
Lower GI bleed	8, 6.67	11, 9.17	0.63
SBP	9, 7.50	12, 10.00	0.65
Complications of cirrhosis during admission[1]			
Presence of AKI	39, 32.50	41, 34.17	0.89
HE	25, 20.83	24, 20.00	1
Ascites	70, 58.33	69, 57.50	1
Variceal bleeding	21, 17.50	16, 13.33	0.48
SBP	10, 8.33	6, 5.00	0.44
HRS	7, 5.83	7, 5.83	1
Coagulopathy	32, 26.67	24, 20.00	0.29
Portal hypertension	19, 15.83	27, 22.50	0.25
HPS	10, 8.33	5, 4.17	0.29
HCC	6, 5.00	5, 4.17	1
Procedures performed during admission[1]			
EGD	68, 56.67	68, 56.67	1
Paracentesis	60, 50.00	55, 45.83	0.61
TIPS	7, 5.83	3, 2.50	0.33
HD	5, 4.17	10, 8.33	0.29
Colonoscopy/flex sig	13, 10.83	11, 9.17	0.83
Disposition			
Home	83, 69.17	76, 63.33	0.44
Home with home health newly arranged	17, 14.17	22, 18.33	
Home with home health previously arranged	2, 1.67	1, 0.83	
SNF newly arranged	7, 5.83	14, 11.67	
SNF previously arranged	6, 5.00	5, 4.17	
Left against medical advice	1, 0.83	1, 0.83	
Transfer (Long term acute care hospital)	3, 2.50	0, 0.00	
Homeless	1, 0.83	1, 0.83	

[1]Patient can have more than one of variable listed.

SBP: Spontaneous Bacterial Peritonitis; AKI: Acute kidney injury; HE: Hepatic encephalopathy; HRS: Hepatorenal syndrome; HPS: Hepato-pulmonary syndrome; HCC: Hepatocellular carcinoma; EGD: Esophago-gastro-duodenoscopy; TIPS: Transjugular intrahepatic portosystemic shunt; HD: Hemodialysis; SNF: Skilled Nursing Facility; GI: Gastrointestinal.

discharge should be areas of focus to decrease HE readmissions. Tapper *et al*[25] demonstrated that development of a checklist for HE protocols integrated into the EMR and order entry system reduced odds of 30-d readmission for patients with HE (from 39.2% to 27.6%). Thus, our results are congruent with existing evidence that interventions should be invested in post-discharge education and

Table 5 Clinical and laboratory features during index admission and discharge in two randomization arms, *n* (%)

	Intervention (*n* = 120)	Standard of care (*n* = 120)	*P* value
Index admission labs (mean ± SD)			
Sodium	132.59 ± 5.58	132.28 ± 6.28	0.68
Serum creatinine (mg/dL)	1.42 ± 1.11	1.64 ± 1.47	0.19
Total bilirubin (mg/dL)	5.90 ± 9.10	6.19 ± 7.80	0.79
Albumin (g/dL)	2.83 ± 0.59	2.85 ± 0.55	0.72
INR	1.68 ± 0.52	1.70 ± 0.59	0.80
Hemoglobin (g/dL)	10.22 ± 2.34	10.02 ± 2.04	0.48
Ascites			
Absent	35, 29.17	35, 29.17	0.44
Slight	26, 21.67	34, 28.33	
Moderate	59, 49.17	51, 42.50	
Encephalopathy			
None	91, 75.83	96, 80.00	0.78
Grade 1-2	22, 18.33	18, 15.00	
Grade 3-4	7, 5.83	6, 5.00	
Dialysis at least twice in last week			
No	117, 97.50	115, 95.83	0.72
Yes	3, 2.50	5, 4.17	
Index admission discharge labs (mean ± SD)			
Sodium (mmol/L)	134.72 ± 4.14	134.95 ± 3.57	0.64
Serum creatinine (mg/dL)	1.31 ± 1.06	1.37 ± 1.18	0.69
Total bilirubin (mg/dL, *n* = 237)	5.50 ± 8.80	5.39 ± 6.96	0.92
Albumin (g/dL, *n* = 237)	2.98 ± 0.64	2.94 ± 0.61	0.65
INR (*n* = 238)	1.71 ± 0.49	1.69 ± 0.45	0.65
Hemoglobin (g/dL)	9.30 ± 1.69	9.21 ± 1.68	0.68
Ascites			
Absent	42, 35.00	39, 32.50	0.35
Slight	56, 46.67	66, 55.00	
Moderate	22, 18.33	15, 12.50	
Encephalopathy			
None	117, 97.50	112. 93.33	0.10
Grade 1-2	2, 1.67	8, 6.67	
Grade 3-4	1, 0.83	0, 0.00	
Dialysis at least twice in last week			
No	114, 95.00	110, 91.67	0.44
Yes	6, 5.00	10, 8.33	

communication for all patients with cirrhosis, especially with HE.

One of the components of intervention in our study was to arrange appointment of patients in the clinic within a week with their hepatologist. Patients with DC who attended their follow up appointment within 30 d of discharge from index admission had fewer readmissions. This suggests that overall, in our cohort, outpatient linkage with a hepatologist should be a priority to reduce readmission rates [26]. Morales *et al*[17] in their retrospective program looked at the impact of follow-up of cirrhotics within 7 d after discharge with a hepatologist. They reported reduced 30-d readmission, 60-d mortality

Table 6 Outcomes and reasons of readmission characteristics by randomization arms, *n* (%)

	Intervention (*n* = 120)	Standard of care (*n* = 120)	*P* value
Readmission			
No	77, 64.17	82, 68.33	0.59
Yes	43, 35.83	38, 31.67	
Number of readmissions within 30 d			
0	77, 64.17	82, 68.33	0.65
1	31, 25.83	32, 26.67	
2	9, 7.50	5, 4.17	
3	2, 1.67	1, 0.83	
5	1, 0.83	0, 0.00	
Location of 1st readmission			
Our institution	36, 83.72	23, 60.53	0.03
Outside hospital	7, 16.28	15, 39.47	
Reason for 1st readmission[1]			
Acute kidney injury	10, 23.26	6, 15.79	0.58
Hyponatremia	4, 9.30	6, 15.79	0.50
Hepatic encephalopathy	9, 20.93	17, 44.74	0.03
Volume overload	13, 30.23	9, 23.68	0.62
Variceal bleed	6, 13.95	6, 15.79	1.00
Lower GI bleed	1, 2.33	3, 7.89	0.34
Spontaneous bacterial peritonitis	2, 4.65	1, 2.63	1.00
Other	20, 46.51	22, 57.89	0.37
Index admission cost (mean ± SD, *n* = 116 + 109)	61581 ± 47825	59547 ± 46669	0.46
Index admission LOS (mean ± SD)	8.17 ± 5.56	8.08 ± 6.11	0.63
First readmission LOS (*n* = 43 + 38, mean ± SD)	7.58 ± 7.57	8.71 ± 10.41	0.58
All readmissions LOS (*n* = 60 + 45, mean ± SD)	9.28 ± 16.88	8.69 ± 10.44	0.82
Waiting time for first readmission (*n* = 43 + 38, mean ± SD)	11.16 ± 7.10	14.34 ± 7.77	0.06

[1]Patient can have more than one of variable listed.
GI: Gastrointestinal; LOS: Length of stay.

and rate of emergency department visits and associated costs in those who followed up within 7 d. Morando *et al*[18] demonstrated that follow up with a "care management check-up" group as opposed to "standard outpatient care" reduced 30-d readmission, reduced 12-mo mortality, and saved almost 1500 euros per patient month of life. While Kanwal *et al*[9] found early outpatient follow-up after discharge was associated with a small increase in readmissions, they found an lower overall mortality in their patients with cirrhosis admitted to Veterans Affairs hospitals. Thus our results are also consistent with the current evidence that patients with DC likely benefit from early post-hospitalization follow up with specialty providers[27,28].

One of the major limitations of our study was inability to enroll patients according to the proposed sample size due to the COVID-19 pandemic. Our study was underpowered to perform multiple regression analysis to detect differences in readmission rates in INT *vs* SOC arm. From March 2020 to July 2020 our recruitment process was put on hold due to hospital regulations to reduce patient and staff exposure. Despite this major limitation, we were able to enroll 80.17% (279 consented out of 348 approached) of patients in our study.

This study was also performed in the setting of a large academic medical center and a high-volume liver transplant center. While our methods and results may be applicable to the clinical practice of other such centers, the same impact may not be appreciated by smaller, community hospitals that are not liver transplant centers.

Future work in patients with DC should continue to focus on prospective intervention strategies to reduce early readmissions and educate patients and providers. To achieve desired sample size, we would suggest collaborations with various centers to identify and recruit patients with DC into a multicenter prospective cohort. Given our finding that there were fewer readmissions in patients with follow-up within 30 d, studies should evaluate the use of telehealth visits for follows up, especially in the COVID19 era, as outlined by Stotts *et al*[29].

CONCLUSION

In conclusion, this prospective randomized study investigated the impact of various pragmatic interventions to reduce early readmission and healthcare utilization in patients with DC. Our study was underpowered to detect statistically significant differences in readmission rates in INT *vs* SOC arm. We reported that readmission rate of our medical center was 33.75% and HE was the top reason for readmission. We found a reduction in early readmission in patients with HE in the INT arm and those who attended their follow up appointment within 30 d of discharge from index admission. We demonstrated that simple interventions in patients with DC are pragmatic and there is need for more prospective multicenter trials in this area of research.

ARTICLE HIGHLIGHTS

Research background

Decompensated cirrhosis (DC) is a leading cause of morbidity and mortality in the United States often requiring multiple hospitalizations to manage. Studies show 20%-37% of patients with DC are readmitted within 30 d of index admission, which has significant burden on patients, their families and the healthcare system.

Research motivation

We were motivated to study and reduce readmissions as we see the physical, mental and emotional toll repeated hospitalizations for DC take on our patients and their families.

Research objectives

We sought to enroll patients in a randomized trial seeing if a nurse case manager (CM) ensuring early outpatient follow up, medication compliance and outpatient paracentesis if needed reduced readmissions in patients with DC.

Research methods

We sought to enroll patients in a randomized trial seeing if a nurse CM ensuring early outpatient follow up, medication compliance and outpatient paracentesis if needed reduced readmissions in patients with DC.

Research results

While our calculated sample size was not achieved due to the coronavirus disease 2019 pandemic, we found a 33.75% 30 d readmission rate in our patients admitted with DC. There was no difference in readmission between intervention and standard of care arms. Most patients were re-admitted with hepatic encephalopathy. There was a lower 30 d readmission rate in patients with hepatic encephalopathy in the intervention arm and those who attended early outpatient follow up.

Research conclusions

Our 30 d readmission was higher than the national rate. Further efforts should explore the positive impact of a nurse CM and early outpatient follow up in reducing readmissions for patients with cirrhosis, especially with hepatic encephalopathy.

Research perspectives

Further development of strategies to predict and reduce readmissions for patients with DC should be done.

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FOOTNOTES

Author contributions: Pusateri A and Mumtaz K study design, team administration, training team members for recruiting, recruiting patients for study, interpreting data, drafting manuscript; both approved the final submitted version of this manuscript; Litzenberg K, Griffiths C, Hayes C, Gnyawali B and Manious M recruiting patients for study, drafting manuscript, approved the final submitted version of manuscript; Jalil S, Kelly S and Conteh L reviewed and edited the final draft of the manuscript; Nagaraja K analyzed data, edited manuscript, and approved the final submitted version of this manuscript.

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Institutional review board statement: This study was conducted at the Ohio State University Wexner Medical Center (OSUWMC), Columbus, Ohio from July 2019 to December 2020. Our study was approved by OSUWMC Institutional Review Board. All aspects of the studying involving human participants including informed consent for enrollment were in accordance with the ethical standards of our Institutional Review Board and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Clinical trial registration statement: Since our randomized trial was of a nursing intervention only, it was not an official randomized control clinical trial that needed registering on a national level.

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

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SYSTEMATIC REVIEWS

Liver injury from direct oral anticoagulants

Deven Juneja, Prashant Nasa, Ravi Jain

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Abstract

BACKGROUND

Drug-induced liver injury (DILI) can be caused by any prescribed drug and is a significant reason for the withdrawal of newly launched drugs. Direct-acting oral anticoagulants (DOACs) are non-vitamin K-based antagonists recently introduced and increasingly used for various clinical conditions. A meta-analysis of 29 randomised controlled trials and 152116 patients reported no increased risk of DILI with DOACs. However, it is challenging to predict the risk factors for DILI in individual patients with exclusion of patients with pre-existing liver disease from these studies.

AIM

To determine the risk factors and outcomes of patients who developed DILI secondary to DOACs by systematic review and meta-summary of recent case reports and series.

METHODS

A systematic search was conducted on multiple databases including PubMed, Science Direct, *Reference Citation Analysis*, and Google Scholar. The search terms included "Acute Liver Failure" OR "Acute-On-Chronic Liver Failure" OR "Acute Chemical and Drug Induced Liver Injury" OR "Chronic Chemical and Drug Induced Liver Injury" AND "Factor Xa Inhibitors" OR "Dabigatran" OR "Rivaroxaban" OR "apixaban" OR "betrixaban" OR "edoxaban" OR "Otamixaban". The results were filtered for literature published in English and on adult patients. Only case reports and case studies reporting cases of DILI secondary to

DOACs were included. Data on demographics, comorbidities, medication history, laboratory investigations, imaging, histology, management, and outcomes were extracted.

RESULTS

A total of 15 studies (13 case reports and 2 case series) were included in the analysis, comprising 27 patients who developed DILI secondary to DOACs. Rivaroxaban was the most commonly implicated DOAC (n = 20, 74.1%). The mean time to onset of DILI was 40.6 d. The most common symptoms were jaundice (n = 15, 55.6%), malaise (n = 9, 33.3%), and vomiting (n = 9, 33.3%). Laboratory investigations showed elevated liver enzymes and bilirubin levels. Imaging studies and liver biopsies revealed features of acute hepatitis and cholestatic injury. Most patients had a favourable outcome, and only 1 patient (3.7%) died due to liver failure.

CONCLUSION

DOACs are increasingly used for various clinical conditions, and DILI secondary to DOACs is a rare but potentially serious complication. Prompt identification and cessation of the offending drug are crucial for the management of DILI. Most patients with DILI secondary to DOACs have a favourable outcome, but a small proportion may progress to liver failure and death. Further research, including post-marketing population-based studies, is needed to better understand the incidence and risk factors for DILI secondary to DOACs.

Key Words: Anticoagulants; Direct-acting oral anticoagulants; Drug induced liver injury; Drug reactions; Hepatotoxicity; Novel oral anticoagulants

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Core Tip: Drug-induced liver injury (DILI) can be ascribed to practically any prescribed drug. The side effect profile of relatively newer direct-acting oral anticoagulants (DOACs) is yet to be completely determined. Even though the data from earlier clinical trials suggested no significant liver toxicity, several case reports and series describing DOAC-induced DILI have been recently published. Most of these cases have been reported in elderly patients, not on concomitant hepatotoxic drugs. However, these patients may have good clinical outcomes, with complete recovery of liver function, if an early diagnosis is made and the offending agent is stopped.

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INTRODUCTION

In general, drug-induced liver injury (DILI) can be ascribed to any sort of prescribed drug. DALI remains a major reason behind the premature termination of the development of a promising therapeutic agent or even the withdrawal of a newly launched drug in the market. On the contrary, the true incidence of DILI remains unknown because of under-reporting, missed diagnosis and the usage of different criteria to define DILI. The incidence of DILI varies from 2.3 and 2.7 per 100000 exposed individuals in the United Kingdom and the United States, respectively[1,2]. The incidence of the drug-induced acute liver failure (ALF) has been reported to be 1.61/1000000 persons-years[3]. However, the incidence varies with geographic, socio-economic, and cultural status of the population. For example, a recent retrospective study from China reported an incidence of 23.8 per 100000 persons. Moreover, DILI is the most common cause of ALF in the United States and Europe, accounting for almost 10% of all the causes of hepatitis. In these countries, the over-the-counter medicines and herbal or dietary supplements are the leading causes of DILI[3,4]. In comparison, the traditional Chinese and herbal medicines, dietary supplements and anti-tubercular drugs remain the major reasons for DILI in China[5]. In case of India, both Ayurvedic medicines as well as anti-tubercular drugs are responsible for most of the DILI cases[6].

Traditionally, the mechanism of DILI can be divided into direct (dose-dependent, predictable) and indirect (idiosyncratic, non-predictable) causes. Further, the idiosyncratic reactions may be immune-mediated (*e.g.*, phenytoin) or metabolic (*e.g.*, isoniazid). Acetaminophen is the best example for dose-dependent DILI. However, the recent studies demonstrated a dose-dependent mechanism in case of idiosyncratic type of DILI. While the exact pathogenesis of DILI remains unknown, genetic risk factors

(including human leucocyte antigen and its associations) have been identified in the literature[7,8]. Female gender, race (higher need for liver transplantation among Asians and chronicity among African Americans), age (elderly or young based on the type of drug), pre-existing liver disease, alcohol abuse, malnutrition, and mutations in the P450 gene are the main risk factors identified for DILI. However, it is challenging to predict the risk factors for DILI in individual patients. High index of suspicion is recommended for DILI, especially among those patients with large number of risk factors and when no other etiology is identified. A detailed review of the drug prescriptions should be performed for at least 3-6 mo. Prompt cessation of the suspected drug(s) is the first step in the management of DILI.

Direct-acting oral anticoagulants (DOACs) are non-vitamin K-based antagonists that have been recently introduced and are increasingly used for varied clinical conditions. Ximelagatran, a direct thrombin inhibitor, was withdrawn from the market because of its hepatoxicity[9]. There was no signal of hepatoxicity recorded during the randomised controlled trials (RCTs) of other novel DOACs. However, the patients with significant pre-existing liver diseases were excluded from the pre-approved RCTs for DOACs. Further, these RCTs remain inadequately powered to detect any difference in rare events such as DILI. A meta-analysis conducted by Caldeira *et al*[10], included 29 RCTs and 152116 patients and the study did not find any increased risk of DILI with DOACs. On the contrary, it reported a "protective effect" against hepatoxicity, when compared with the low molecular weight heparin. Though the meta-analysis overcame the limitations of inadequate power of individual studies, the absence of the individual patient data, variable follow-up among the individual studies, usage of DOACs for different indications and hepatotoxicity being measured as a secondary outcome measure cannot reliably prove the safety of the DOACs. Hence, the post-marketing population-based, case-control, real-world data, and pharmacovigilance reports are required to assess the risk of hepatoxicity of the DOACs.

More recently, multiple case reports and series have reported DILI secondary to the use of these drugs. However, the incidence, probability, and the risk factors for the development of DILI are not entirely elucidated and may also vary with different DOACs. Hence, the aim of the current study is to collate the data from recent case reports and series and analyse them to determine the risk factors and the outcomes of patients, who developed DILI secondary to DOACs.

MATERIALS AND METHODS

The authors conducted a systematic search for this meta-summary from multiple databases such as PubMed, Science Direct, *Reference Citation Analysis* and Google Scholar. The search terms included were "Acute Liver Failure" OR "Acute-On-Chronic Liver Failure" OR "Acute Chemical and Drug Induced Liver Injury" OR "Chronic Chemical and Drug Induced Liver Injury" AND "Factor Xa Inhibitors" OR "Dabigatran" OR "Rivaroxaban" OR "Apixaban" OR "Betrixaban" OR "Edoxaban" OR "Otamixaban".

The results were filtered for the literature published in the English language and on adult (> 18 years) humans. All the search results were manually screened by the authors while only those relevant literature for DOAC-induced-DILI was analysed. Duplicate articles from different search databases were excluded (Figure 1). All the case reports and case series were evaluated and the data in terms of patient demographics, clinical symptomatology, type, dose and duration of the DOACs, clinical interventions, intensive care unit course, need for organ support and outcomes was extracted. The concomitant usage of the hepatotoxic drugs was also noted down. A datasheet was prepared for further evaluation.

Statistical analysis

The prepared datasheet was evaluated using MS Excel and Microsoft office, 2019. The categorical variables were presented as frequency and percentage. Median (interquartile range) or mean ± SD was used for continuous variables. Tabulation and final documentation were done using the MS Office software (MS office 2019, Microsoft Corp, WA, United States).

RESULTS

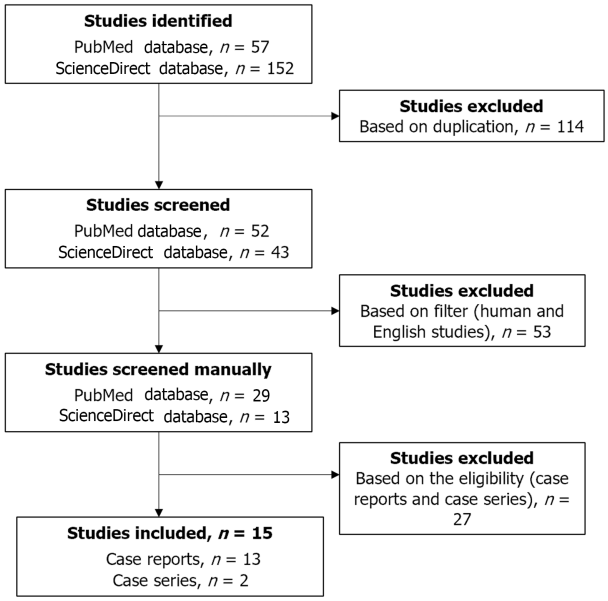
From the current study search, 27 cases of acute liver damage published between 2011 and 2021[11-25] following DOAC exposure were retrieved (Table 1). There was an equal distribution of males and females (48.1%) while most of the cases were reported from Europe (77.8%) and North America (18.5%). At the time of presentation, the age of the patients was in the range of 41 to 91 years. Out of the total population, 20 (74.1%) patients were aged ≥ 65 years or above. The time between the initiation of DOACs and the onset of liver injury ranged from 6 d to 6 mo, while one patient presented with acute condition after accidental ingestion. The major indications for DOACs were prevention of venous thromboembolism (VTE) in patients who were undergoing elective knee surgery and prevention of stroke and systemic embolism in patients with nonvalvular atrial fibrillation (AF). However, three

Table 1 The baseline patient parameters, *n* (%)

Variables	Number of patients (*n* = 27)
Age (± SD), yr	72.7 (± 11.4)
Sex	Females, 13 (48.1)
	Males, 13 (48.1)
	Not mentioned, 1 (3.7)
Country of origin	Switzerland, 12 (44.4)
	Italy, 4 (14.8)
	United States of America, 4 (14.8)
	France, 3 (11.1)
	Australia, 1 (3.7)
	Canada, 1 (3.7)
	Ireland, 1 (3.7)
	Spain, 1 (3.7)
Clinical presentation	Jaundice, 15 (55.6)
	Malaise, 9 (33.3)
	Vomiting, 9 (33.3)
	Abdominal pain, 3 (11.1)
	Itching, 3 (11.1)
	Anorexia, 2 (7.4)
	Fatigue, 2 (7.4)
	Dyspepsia, 1 (3.7)
	Breathlessness, 1 (3.7)
	Pruritus, 1 (3.7)
	Weight loss, 1 (3.7)
Comorbidities	Hypertension, 6 (22.2)
	Diabetes, 3 (11.1)
	Coronary artery disease, 3 (11.1)
	Others, 6 (22.2)
Other drugs causing liver injury	None, 11 (40.7)
	Statins, 4 (14.8)
	Not mentioned, 12 (44.4)
DOACs implicated	Rivaroxaban, 20 (74.1)
	Apixaban, 4 (14.8)
	Dabigatran, 3 (11.1)
Indications of DOACs	Atrial fibrillation, 13 (48.1)
	Pulmonary embolism, 2 (7.4)
	Transient ischemic attack, 1 (3.7)
	Anti-phospholipid antibody syndrome, 1 (3.7)
	Deep vein thrombosis, 1 (3.7)
	Major lower limb surgery, 1 (3.7)
	Sinus node dysfunction, 1 (3.7)
	Accidental, 1 (3.7)

Time to presentation after initiation of DOACs (d)	40.6 ± 42.8
Need for organ support	Renal replacement therapy, 1 (3.7)
	Vasopressors, 1 (3.7)
	Invasive mechanical ventilation, 1 (3.7)
Need for specific antidote	Idarucizumab, 1 (3.7)
Days in hospital (d)	8 ± 9.3
Days in ICU (d)	7 ± 12.1
Outcome	Alive, 26 (96.3)
	Death, 1 (3.7)

SD: Standard deviation; DOAC: Direct-acting oral anticoagulants; ICU: Intensive care unit.



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Figure 1 PRISMA flow diagram of the selected literature for the current meta summary.

patients received DOACs for the management of deep vein thrombosis (DVT) and pulmonary embolism (PE). A total of 20 patients (74.1%) received rivaroxaban, four (14.8%) received apixaban, and three (11.1%) received dabigatran. In four cases (14.8%), the consumption of other potential hepatotoxic medications (statins) was reported. The most reported symptom was jaundice in 15 (57.7%) patients, followed by malaise and vomiting in 9 (34.6%) patients. Extracorporeal therapy was initiated in one patient, whereas only one patient with dabigatran-induced DILI received idarucizumab therapy. Table 2 shows the liver function parameters at the time of presentation. Though most of the patients showed complete recovery of the liver function, only 3 (11.1%) patients were reported to have persistent liver dysfunction at the time of discharge, and only one (3.7%) death was reported.

DISCUSSION

Prolonged anticoagulant therapy translates to varied clinical conditions for which vitamin K antagonists are the only therapeutic option. However, the introduction of DOACs has provided the physicians, a safer and an effective alternative. They possess several inherent advantages, including predictable pharmacokinetics and immediate activity after the first dose. This pattern helps in using a fixed dose and obliterate the need for routine laboratory monitoring in most of the patients. However, their side effect profile needs to be fully understood. The first DOAC *i.e.*, ximelagatran was approved for clinical use based on the data from short-term trials. However, the long-term data found DALI in up to 8% of the patients, who received the drug[26]. The fact came into light after its prolonged clinical use. Hence, ximelagatran was withdrawn from the market because of its unfavourable side effect profile. Similarly,

Table 2 Liver function test parameters

Parameter	Mean	Standard deviation	Range
SGOT, at presentation (units/L)	575.5	672.2	90-2304
SGPT, at presentation (units/L)	800.2	1159.6	90-4000
Total bilirubin, at presentation (mg/dl)	6.3	6	0.64-21.8
Direct bilirubin, at presentation (mg/dl)	4.8	4.9	0.3-10.3
Alkaline phosphatase, at presentation (units/L)	269.2	279.5	60-1039

SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase.

significant hepatotoxicity was not reported in the initial trials of other DOACs. However, several case reports have been published in the recent years, describing their hepatotoxic potential.

It is challenging to diagnose DILI with the absence of any specific clinical or biochemical marker. The clinical features of DILI are usually non-specific and may vary from asymptomatic liver enzyme elevation to ALF. Even liver histology is not specific and may also be found in other liver disorders[27]. Hence, a high index of suspicion is warranted to enable early diagnosis. Even though jaundice was present in 55.6% of the study patients, all others presented with non-specific symptoms like malaise, vomiting, anorexia and fatigue. The latency from the exposure to DILI, especially in case of metabolic idiosyncrasies, varies from days to weeks and at times, even months too. The data, from the meta-analysis study that assessed the hepatotoxic potential of DOACs, also reported that the mean age of the patients was in the range of 55 to 71 years. Further, the time to develop DILI after the initiation of DOACs ranged from 2 wk to 2 years[10].

The diagnosis of DILI requires the exclusion of other liver dysfunction etiologies associated with a similar clinical and biochemical picture, along with the documentation of temporal association related to the exposure of the offending agent[28]. The accepted definition for hepatocellular liver injury has been given by an international DILI expert working group; fivefold or greater elevation above the upper limit of normal (ULN) for alanine aminotransferase/serum glutamic pyruvic transaminase (ALT/SGPT)[29]. The cholestatic picture is defined to be more than twofold elevation above the ULN for alkaline phosphatase with a concomitant elevation of the activity of 5'-nucleotidase or γ-glutamyltranspeptidase [29]. However, when the ratio of elevation, above the ULN for ALT/SGPT and alkaline phosphatase, is more than two-fold yet less than five-fold, it is defined as a mixed pattern of liver injury[29,30]. The use of DOACs has been associated with both hepatocellular and cholestatic patterns of liver injury[11].

As per the clinical studies, DOACs are rarely associated with the development of DILI with the frequency of developing DILI ranging between 0.1%-1%[10]. However, a higher incidence of 2.3% was reported when using rivaroxaban[31]. In the current study analysis, rivaroxaban was reported to be the offending agent in 74% of the cases. This may also be explained by the fact that it is arguably the most prescribed DOAC[32]. However, these clinical trials were neither designed nor powered to detect the risks of rare idiosyncratic adverse reactions like DILI. Moreover, the patients at high risk of developing these complications and with pre-existing liver disease were often not included in these clinical trials.

Several DOACs are approved and prescribed for both prevention and the treatment of DVT or PE. Further, they are also prescribed to prevent stroke and systemic embolism in adults with non-valvular AF and other risk factors. DOACs are also routinely used in the prevention of VTE after hip or knee surgeries. The current study analytical outcomes too depict a similar usage pattern. The patients, who developed DILI, were prescribed DOACs for similar indications, among which the non-valvular AF was the most common indication. The data from the systematic analysis has also shown that the most common indication for initiating DOACs was non-valvular AF[10].

Except for dabigatran and its glucuronide metabolite, which are predominantly excreted through kidneys, all the DOACs are metabolised in the liver. Moreover, dabigatran is also a substrate for P-glycoprotein (P-gp), and hence, dose adjustment needs to be done for patients with renal dysfunction and those on P-gp inhibitors like azole antifungals, amiodarone, macrolides, atorvastatin, digoxin *etc* [33]. Nearly one-third of the administered rivaroxaban is excreted unchanged in the urine, whereas the rest undergoes metabolic degradation. In this context too, half gets eliminated by the kidneys and the other half is eliminated through hepatobiliary route. In the liver, it is mainly metabolised *via* the cytochrome P450 (CYP) 3A4 and some CYP-independent mechanisms. It is also a substrate of P-gp. Apixaban is excreted through multiple pathways such as the renal (25%), intestinal (55%) and the rest through hepatobiliary route (CYP3A4-dependent mechanisms)[34]. Concomitant use of hepatotoxic drugs, or CYP3A4/P-gp inhibitors may increase the risk of hepatotoxicity with DOACs. Hence, these patients require a close monitoring of their liver function. Further, patients may develop hepatoxicity even in the absence of these drugs. As per the current study analysis, the concomitant use of potentially hepatotoxic drugs was reported in only 14.8% of the cases.

The current study has several strengths to its credit. The authors included DILI associated with all the currently prescribed DOACs and included all the latest case reports and series. However, there exists some limitations to the meta-summary. The studies included, were only case reports and case series without a control arm. Additionally, these studies were heterogeneous, with a high risk of bias and missing data. So, it may affect the generalisability of the results. Further, the authors might have missed some relevant cases too, because those case reports or series that did not have any individual biochemical data were excluded.

CONCLUSION

DOACs may rarely lead to DILI. Most of the cases have been reported among elderly patients, who may be at higher risk of developing DILI. It may develop without the concomitant use of other hepatotoxic drugs. The outcomes of these patients are generally favourable and liver dysfunction is mainly reversible if it is recognised early and the intake of offending agent is stopped. Hence, physicians prescribing these drugs must be aware of such rare complications to ensure early diagnosis and prompt management.

ARTICLE HIGHLIGHTS

Research background

Drug-induced liver injury (DILI) can be caused by any prescribed drug and is a significant reason for the withdrawal of newly launched drugs. Direct-acting oral anticoagulants (DOACs) are non-vitamin K-based antagonists recently introduced and increasingly used for various clinical conditions. It is challenging to predict the risk factors for DILI in individual patients with exclusion of patients with pre-existing liver disease from these studies.

Research motivation

To determine the risk factors and outcomes of DILI in patients taking DOACs and provide clinicians with essential information for the management of DILI secondary to DOACs.

Research objectives

To determine the incidence, probability, and risk factors for developing DILI secondary to DOACs and its outcomes in affected patients.

Research methods

The authors conducted a systematic search of multiple databases for the literature published in English using specific search terms. The results were filtered and analysed to determine the risk factors and outcomes of DILI in patients taking DOACs.

Research results

The analysis of recent case reports and series showed that DOACs can rarely cause DILI, and the incidence, probability, and risk factors for developing DILI varied among different DOACs.

Research conclusions

DOACs can cause DILI, and the incidence, probability, and risk factors for developing DILI vary among different DOACs. Clinicians should have a high index of suspicion for DILI in patients taking DOACs, especially those with multiple risk factors. Prompt cessation of the suspected drug is recommended as the first step in managing DILI.

Research perspectives

The findings of this study provide essential information for clinicians to manage DILI secondary to DOACs. However, further research is required to identify the true incidence of DILI and its risk factors, including genetic associations. Post-marketing pharmacovigilance reports can help to assess the risk of hepatoxicity associated with DOACs.

FOOTNOTES

Author contributions: Nasa P conceptualized and designed the article; Juneja D, Nasa P, and Jain R performed acquisition of data, analysis and interpretation of data, and drafted the article; Juneja D and Jain R revised the article; and all authors have read and approve the final manuscript.

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SYSTEMATIC REVIEWS

Management of sepsis in a cirrhotic patient admitted to the intensive care unit: A systematic literature review

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Abstract

BACKGROUND

Sepsis is a severe medical condition that occurs when the body's immune system overreacts to an infection, leading to life-threatening organ dysfunction. The "Third international consensus definitions for sepsis and septic shock (Sepsis-3)" defines sepsis as an increase in sequential organ failure assessment score of 2 points or more, with a mortality rate above 10%. Sepsis is a leading cause of intensive care unit (ICU) admissions, and patients with underlying conditions such as cirrhosis have a higher risk of poor outcomes. Therefore, it is critical to recognize and manage sepsis promptly by administering fluids, vasopressors, steroids, and antibiotics, and identifying and treating the source of infection.

AIM

To conduct a systematic review and meta-analysis of existing literature on the management of sepsis in cirrhotic patients admitted to the ICU and compare the management of sepsis between cirrhotic and non-cirrhotic patients in the ICU.

METHODS

This study is a systematic literature review that followed the PRISMA statement's standardized search method. The search for relevant studies was conducted across multiple databases, including PubMed, Embase, Base, and Cochrane, using predefined search terms. One reviewer conducted the initial search, and the eligibility criteria were applied to the titles and abstracts of the retrieved articles. The selected articles were then evaluated based on the research objectives to ensure relevance to the study's aims.

RESULTS

The study findings indicate that cirrhotic patients are more susceptible to infections, resulting in higher mortality rates ranging from 18% to 60%. Early identification of the infection source followed by timely administration of antibiotics, vasopressors, and corticosteroids has been shown to improve patient outcomes. Procalcitonin is a useful biomarker for diagnosing infections in

cirrhotic patients. Moreover, presepsin and resistin have been found to be reliable markers of bacterial infection in patients with decompensated liver cirrhosis, with similar diagnostic performance compared to procalcitonin.

CONCLUSION

This review highlights the importance of early detection and management of infections in cirrhosis patients to reduce mortality. Therefore, early detection of infection using procalcitonin test and other biomarker as presepsin and resistin, associated with early management with antibiotics, fluids, vasopressors and low dose corticosteroids might reduce the mortality associated with sepsis in cirrhotic patients.

Key Words: Sepsis; Septic shock; Cirrhosis; Sequential organ failure assessment score; Mean arterial pressure; Intensive care unit

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Core Tip: Sepsis is a severe condition encountered in the intensive care unit (ICU), and when it occurs in cirrhotic patients, it often leads to high mortality due to impaired immunity and multiorgan failure. To diagnose and monitor sepsis in cirrhotic patients, various scoring systems have been developed, including the Sequential Organ Failure Assessment (SOFA) score, the Chronic Liver Failure-Sequential Organ Failure Assessment (CLIF-SOFA) score, quick SOFA (qSOFA), Model for End-Stage Liver Disease (MELD), and MELD-Na score. Although the proposed current management of sepsis in cirrhotic patients might follow the guidelines proposed by the Surviving Sepsis Campaign, this approach has might not cause significant improvement in patient outcomes. Therefore, early recognition of infection and its source is critical, followed by timely initiation of antibiotic therapy, fluid resuscitation with albumin (5% or 20%), vasopressors, and low-dose corticosteroids such as hydrocortisone. Studies have shown that this approach reduces mortality in the ICU. In addition to pharmacological interventions, interventions to control the source of infection, such as surgical drainage, may also be necessary. Finally, procalcitonin levels can be used as a diagnostic biomarker in cirrhotic patients with sepsis, helping to guide antibiotic therapy and improve patient outcomes. In conclusion, timely recognition and management of sepsis in cirrhotic patients in the ICU is crucial, and early initiation of appropriate interventions, including antibiotics, fluids, and corticosteroids, may improve patient outcomes.

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INTRODUCTION

Physiologically, sepsis is viewed as a proinflammatory and procoagulant response to invading pathogens with three recognized stages in the inflammatory response, with a progressively increased risk of end-organ failure and death[1]. Evidence shows that sepsis in cirrhotic patients causes a marked imbalance of cytokine response, known as a "cytokine storm," which converts responses that are normally beneficial for fighting infections into excessive, damaging inflammation. Therefore, the three recognized stages are sepsis, severe sepsis, and septic shock, and cirrhotic patients are prone to developing sepsis-induced organ failure and death[1]. Severe sepsis in cirrhotic patients is associated with high production of proinflammatory cytokines that play a role in the worsening of liver function and development of organ or system failure such as shock, acute lung injury, acute respiratory distress syndrome, coagulopathy, renal failure, or hepatic encephalopathy[1]. Furthermore, cirrhotic patients with severe sepsis can develop sepsis-induced hyperglycemia, defective arginine-vasopressin secretion, adrenal insufficiency, or compartmental syndrome[2].

Sepsis is a severe condition characterized by a deregulation of the body's response to infection and can lead to life-threatening organ dysfunction. As one of the leading causes of admission to intensive care units (ICUs), sepsis has been found to have poorer outcomes in patients with comorbidities such as cirrhosis, as stated in the "Third International Consensus Definitions for Sepsis and Septic Shock"[1-3]. Organ dysfunction in sepsis is measured by an increase of two points or more in the Sequential Organ Failure Assessment (SOFA) score, which is associated with a mortality rate greater than 10%[4-5]. The SOFA score comprises six sub-scores, including liver failure, which has been found to be associated with

higher mortality. The other sub-scores include respiratory, coagulation, cardiovascular, central nervous system, and renal. Each sub-score is rated on a scale of 0 to 4 and summed up to a final score from 0 to 24. Systematic Inflammatory Response Syndrome occurs when two or more criteria, such as body temperature > 38 ℃ or < 36 ℃, tachycardia > 90/min, hyperventilation, and abnormal white blood cell count, are met[2].

Septic shock is a subset of sepsis that leads to profound circulatory and cellular metabolism abnormalities, resulting in substantially increased mortality[4]. To identify septic shock, one should look for hypotension that requires vasopressor therapy and a mean arterial pressure (MAP) of less than 65 mmHg despite adequate fluid resuscitation and systolic blood pressure. Additionally, signs of tissue hypoperfusion such as low urinary output, acidosis, and worsening mental status, along with evidence of systemic inflammatory response syndrome, including a body temperature above 38 or below 36 ºC, tachycardia, tachypnea, leukocytosis, and documented infection, are also considered[5].

Elevated lactate levels reflect cellular dysfunction in sepsis, and multiple factors contribute to their elevation, including insufficient tissue oxygen delivery, impaired aerobic respiration, acceleration of aerobic glycolysis, and reduced hepatic clearance[1]. However, defining sepsis and septic shock poses inherent challenges[3].

The acute change in total SOFA score of more than 2 points due to an infection is identified as organ dysfunction. In patients with a SOFA score of 2 or more, the overall mortality risk is approximately 10%, which is higher than the overall mortality rate of ST-segment elevation myocardial infarction. This score also identifies a 2-25 fold increased risk of dying compared to patients with a SOFA score less than 2. However, this score is not used as a tool for managing septic patients in the ICU but rather to characterize them clinically. SOFA has greater predictive validity in patients suspected of sepsis in an ICU[3,4].

There are several risk factors associated with sepsis, including patient factors such as immunosuppression, comorbidity, or therapy, microbe factors such as the presence of multi-resistant or virulent bacteria, and procedural risks such as surgery, indwelling catheters, or implantable devices[6]. Cirrhosis, which is the end-stage of most chronic liver diseases, has two clinical phases: Compensated and decompensated. The compensated phase is defined as the period between the onset of cirrhosis with minor or no symptoms and the first major complication, while the decompensated phase is when the patient first presents with ascites, variceal hemorrhage, hepatic encephalopathy, spontaneous bacterial peritonitis, and hepatorenal syndrome. This period is associated with a short survival time. Cirrhosis may be diagnosed by liver biopsy or by signs of chronic liver disease with documented portal hypertension. Cirrhotic patients have a reduced capacity of the reticuloendothelial system to clear bacteria from the gut, resulting in a higher rate of infections and a worse prognosis[7].

Cirrhosis is an irreversible condition caused by several factors or conditions, such as viral hepatitis, alcoholic liver disease, and non-alcoholic fatty liver disease. According to the World Health Organization, cirrhosis was the 9th leading cause of death in the west in 2015[8]. Studies have shown that mortality among cirrhotic patients with sepsis in the ICU ranges from 18%-66%, with mechanical ventilation being an independent predictor of mortality. The MELD and MELD-Na scores are used for the prediction of 90-day mortality and for organ allocation in liver transplantation. A cohort study by Baudry *et al*[9] found that mortality of cirrhotic patients with sepsis ranges from 18%-66%. WHO estimates cirrhosis as the 12th cause of mortality in the world, with deaths exceeding 1 million per year. ICUs provide specialized treatment and monitoring for critically ill patients.

The aim of the present paper is to determine the optimal current management of sepsis in cirrhotic patients admitted to the ICU.

MATERIALS AND METHODS

This systematic review follows the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA-P) protocol[10] and examines published papers on the management of sepsis in cirrhotic patients admitted to the ICU.

Inclusion criteria

Inclusion criteria were cirrhotic patients over 18 years old, admitted to the ICU with sepsis. The study analyzed the management and prognosis of cirrhotic patients with sepsis, as well as compared the management of sepsis in cirrhotic patients to those without cirrhosis. Only English-language randomized controlled trials (RCTs), retrospective cohort studies, and prospective cohort studies were included.

Outcomes

The analyzed outcomes include survival, length of ICU stay, and the overall prognosis of cirrhotic patients with sepsis admitted to the ICU.

Search strategies

Searches were conducted on PubMed, Google Scholar, Embase, and Cochrane databases. Retrieved papers were initially filtered based on their titles and abstracts, and the full text of selected papers were then retrieved and analyzed. Only papers that met the inclusion criteria were included and analyzed. The search strategy is described in Appendix 1 and the critical appraisal of the papers is presented in Appendix 2.

RESULTS

Study selection

Figure 1 illustrates the selection process following the PRISMA-P protocol. Initially, 351 search results were retrieved, out of which 284 were excluded after screening the titles, 46 were excluded after the abstract, and 3 were excluded after full articles. A total of 19 papers met the inclusion criteria and were included for full-text review. The primary outcome of all reviewed papers was the survival of cirrhosis patients with sepsis in the ICU. The review also analyzed the prognostic value of scores such as Child-Turcotte-Pugh, Model for End-Stage Liver Disease (MELD), Model for End-Stage Liver Disease Sodium (MELD-Na), and SOFA scores for cirrhotic patients with sepsis. The summarized data is available on Table 1 for randomized controlled trials, Table 2 for prospective cohort studies, Table 3 for retrospective cohort studies and Table 4 for selected studies.

Findings of the review

Albumin: Philips *et al*[11] found that 5% human albumin corrected hypotension in sepsis with cirrhosis (Table 1). Maimone *et al*[12] found that albumin 20% increased MAP above 65 mmHg 3 h after infusion compared to plasmolyte, but with a risk of inducing pulmonary edema (Table 3).

Corticosteroids: Arabi *et al*[13] concluded that corticosteroids improved the hemodynamic status of the patient but did not change mortality (Table 1). Rinaldi *et al*[5] and Piccolo Serafim *et al*[14] found similar results to Arabi *et al*[13] (Tables 2 and 3).

Infection diagnosis: Villarreal *et al*[15] concluded that procalcitonin as a biomarker helped with infection diagnosis in cirrhotic patients. Fischer *et al*[16] found that both presepsin and resistin may be reliable markers of bacterial infection in patients with decompensated liver cirrhosis and have similar diagnostic performance compared to procalcitonin (Table 3).

Prognosis: Baudry *et al*[9], Fischer *et al*[16], Chang *et al*[17], and Sauneuf *et al*[18], found that the prognosis is poor in ICU for cirrhosis patients with sepsis (Table 3). Sasso *et al*[19] found that mechanically ventilated cirrhotic patients with sepsis have an extremely poor prognosis, and vasopressor use was strongly a predictor of mortality (Table 3).

Vasopressors: Durst *et al*[20] found that norepinephrine is the best vasopressor to use in cirrhotic patients with sepsis to maintain MAP above 60 mmHg. Umgelter *et al*[21] concluded that terlipressin is effective as a vasopressor in septic cirrhotic patients in combination with norepinephrine to correct hypotension. Chebl *et al*[22] recommend starting vasopressors early to avoid aggressive fluid resuscitation and maintain MAP > 65 mmHg (Table 3).

Hyperdynamic syndrome: Thierry *et al*[23] found that echocardiography helps diagnose hyperdynamic syndrome with high LVEF in septic patients (Table 3).

Mortality: Bal *et al*[24] found 50-day mortality to be about 43.11%. Baudry *et al*[9] found that the mortality of cirrhotic patients with sepsis ranges from 18%-66%, which is close to the WHO finding that estimates cirrhosis as the 12th cause of mortality in the world, with death exceeding 1 million a year (Table 3).

Scoring system: Chen *et al*[25] concluded that the qSOFA (Quick SOFA) criteria, consisting of 3 variables, are a better predictor of adverse outcomes associated with sepsis. The presence of two or more abnormalities in patients with suspected infection identifies a higher risk of developing adverse outcomes.

Hemodynamic monitoring: Administer antibiotics within the first hour and monitor physiological parameters like urine output and lactate clearance to prevent end-organ dysfunction[7]. In advanced cirrhosis, elevated cardiac index, low systemic vascular resistance, low MAP, and higher central venous oxygen saturation may be present. Lactate levels should be carefully evaluated as they may take a while to lower down to normal levels. Serum lactate measurement is still recommended in these patients[7]. Skin mottling score and tissue oxygenation saturation assessed with laser Doppler can also be used as hypoxia of the tissue markers in cirrhosis[7].

Table 1 Summary of randomized controlled trials

Ref.	Purpose	Type of study	Sample size	Conclusion	Setting
Philips *et al*[11]	Assessed the use of 5% human fluid for the resuscitation of cirrhotic patients with sepsis		Three hundred-eight patients were divided into two groups	5% human albumin was safe and more beneficial in correcting hypotension than normal saline	ICU
Arabi *et al*[13]	Assess the use of a low dose of hydrocortisone in cirrhotic patients with sepsis	RCT	140 patients were 65 excluded, and 39 received hydrocortisone and 36 placeboes	That study did not find mortality improvement with corticosteroids despite hemodynamic improvement. The treatment proposed: Hemodynamic monitoring and management, laboratory test culture, stress ulcer prophylaxis as histamine H2 receptor antagonists, norepinephrine as vasopressors, and empiric antibiotic. The outcome was 28 days of all-cause mortality	ICU

RCT: Randomized controlled trial; ICU: Intensive care unit.

Table 2 Prospective cohort studies

Ref.	Purpose	Type of study	Sample size	Conclusion	Setting
Rinaldi *et al*[5]	The aim was to evaluate the effect of adherence to evidence-based guidelines of the Surviving Sepsis Campaign (SSC) on the outcome of cirrhotic patients with shock admitted to the ICU. Resuscitation of sepsis with hydrocortisone	Prospective cohort	38 patients		ICU
Thierry *et al*[23]	Assess the use of echocardiography in assessing the LVEF on cirrhotic patients with septic shock	The prospective cohort single-center study	34 patients compared	Echocardiography in a cirrhotic patient with septic shock show hyperdynamic syndrome with high LVEF	ICU

SSC: Surviving sepsis campaign; ICU: Intensive care unit; LVEF: Left ventricle ejection function.

Fluid resuscitation: Aggressive intravenous fluid resuscitation is recommended in any patient with hypotension or elevated serum lactate. However, the choice of fluid between crystalloid or colloid remains controversial[6,11,12]. The SAFE study concluded that albumin improves hemodynamic status and may reduce mortality, while the VISEP study found that pentastarch colloids can cause acute kidney injury in sepsis and increase 90-day mortality[6,11]. Human albumin is the fluid of choice in cirrhotic patients with sepsis, as it corrects hypotension more effectively than crystalloid[12]. Early goal-directed therapy can help reduce mortality, but the methodology of the Rivers study has been questioned. The recommended fluid should be one that sustains an increase in intravascular volume and contains a chemical composition similar to that of extracellular fluid[6]. Hydroxyethyl starch is not recommended in cirrhosis patients as it increases nephrotoxicity, while albumin is associated with dose-dependent acute kidney injury[6]. An albumin dose of 50-100 g/day is used over crystalloid for initial fluid resuscitation in cirrhosis patients, but no strong evidence exists[6].

Sepsis bundle protocol: According to the Surviving Sepsis Campaign (SSC) guidelines, the sepsis bundle protocol did not improve survival in cirrhotic patients with sepsis[7].

Vasopressors: Vasopressors are frequently indicated to maintain a MAP of at least 65 mmHg in persistently hypotensive patients. Norepinephrine is widely used in distributive shock for its predominantly alpha-adrenoceptor agonism and vasoconstrictive effect[21]. Cirrhotic patients with sepsis and cirrhosis needing vasopressors should have a goal of maintaining the MAP above 60 mmHg. Blood culture and antibiotics should be started as early as possible according to the SCC guidelines[20]. SSC international guideline does not recommend vasopressors as monotherapy or the first line for septic shock treatment, and a randomized trial shows the benefit of angiotensin II for refractory vasodilatory shock treatment[7].

Corticosteroids: Corticosteroids are commonly used for unsatisfactory responses to vasopressors. It helps hasten shock resolution, decreases the required dose of vasopressors, and improves the 90-day survival in septic shock patients, and it might increase shock recurrence. Nevertheless, its use in liver cirrhosis remains controversial[7]. Hydrocortisone improves the hemodynamic status of the patient without a relevant change in mortality[13,14,18]. Hydrocortisone is associated with better shock resolution, although without an impact on survival[5]. Low-dose corticosteroid is recommended to be administered early in patients with severe septic shock to patients who are not responding to

Table 3 Retrospective cohort studies

Ref.	Purpose	Type of study	Sample size	Conclusion	Setting
Guo *et al* [26]	Assessment of VCS parameter for evaluation of sepsis in cirrhotic patients	Retrospective analysis of prospective data	257 patients	Proposed management was collection of blood culture, white cell volume determination, procalcitonin, and interleukin -6, sCD163 laboratory tests. Conclusion VCS parameters have the potential to be used to evaluate and predict early infections in patients with cirrhosis, and VCS can increase sensitivity and specificity in the diagnosis of sepsis and cirrhosis patients	ICU
Villarreal *et al*[15]	Assessing the usefulness of procalcitonin for diagnosing infection in cirrhotic patients	Retrospective cohort study	66 patients of 255 admitted had procalcitonin tests. Patients with infection suspicion had a serum procal-citonin (PCT) test within the first 12 h	Septic patients with cirrhosis had elevated procalcitonin. As PCT has a sensitivity of 83% and specificity of 75% is an effective tool for diagnosing infection in patients with liver cirrhosis. Excellent tool for differentiating infectious disease in cirrhotic patients	ICU
Galbois *et al*[27]	Assess whether the mottling score and tissue oxygen saturation (StO_2) may be used as early death predictors on cirrhotic patients with septic shock. Hemodynamic parameters at 6 h in patients with liver cirrhosis according to their survival status at 14 days	42 out of 46 patients admitted with cirrhosis and septic shock were analyzed		There is systemic vasodilation and increased mortality in cirrhosis patients with sepsis. Patients with increased mottling died, and those with decreased survived. Mottling score and knee StO_2 measures 6 h after starting vasopressors are excellent predictors of 14-day mortality	ICU
Piccolo Serafim *et al*[14]	The study evaluates the use of steroids in a patient with septic shock and cirrhosis	A retrospective cohort study (2007-2017)	56 patients out of 179 admitted with septic shock received steroids during ICU	The use of steroids did not show significant differences in mortality. Vasopressor requirement and is not associated with decreased mortality	ICU
Chang *et al*[17]	aimed to determine whether septic patients with liver cirrhosis had worse survival than patients without liver cirrhosis	Retrospective cohort	776 patients, 64 had sepsis with cirrhosis, 712 sepsis without cirrhosis	Cirrhotic patients with sepsis had a poor outcome, and the survival of sepsis and cirrhosis after matching was not inferior to those without cirrhosis	ICU
Sauneuf *et al*[18]	Assess the use of albumin as an adjuvant to vasopressors in managing septic shock in cirrhotic patients	Retrospective cohort single center and observational overdone over 14 years studied done from 1997 to 2004 and 2005 to 2010	During the period 2005 to 2010, 42, cirrhotic patients with septic shock in ICU were included	In conclusion, the survival rate of septic shock in cirrhosis remains low, and current shock management could benefit cirrhotic patients. Treatment use is: Vasopressors used is norepinephrine, epinephrine, and dobutamine; mechanical ventilation was used in the case of ARDS, and a protective strategy with a low tidal volume of 6 m/kg of body weight, and the plateau was kept below 30 cmH2O, small -dose of corticosteroids (200 mg hydrocortisone per day, insulin therapy, The main sites of infections were: Pneumonia, spontaneous or secondary peritonitis, and urinary tract infection. There were gram-positive and negative. Septic shock represent a severe complication of cirrhosis with very low survival rates. Sepsis in a cirrhotic patient has a poor prognosis. Hydrocortisone did not reduce mortality and was associated with adverse effects such as shock relapse and gastrointestinal bleeding. Cirrhotic patients are commonly perceived as poor candidates for `ICU admission because of the very high mortality	ICU
Umgelter *et al*[21]	Assess the outcome of the continuous low dose of TP in a septic shock patient	Small cohort study	2004-2007: 12 patients, 8 males, and 4 females were included with sepsis due to spontaneous bacterial peritonitis, pneumonia, and cholangitis	TP is currently used in treating cirrhotic patients with hepatorenal syndrome and as an adjunct to NE in a cirrhotic patient with septic shock and kidney failure; TP dose 2 ug/kg if a patient was started NE in the first 24 h. 11 patients had RRT, TP increased SVR index and NE doses needed to obtain target MAP decreased while the CI remained stable. Despite hemodynamic improvement, 11 out of 12 patients died. The author concluded that TP was effective as a vasopressor in septic cirrhotic patients at a low dose in combination with NE, and there was no dramatic decrease in CI. TP has a role in the early treatment of septic shock, and the author recommends a controlled study with TP in a cirrhotic patient with sepsis	ICU

Durst *et al* [20]	The study aimed to evaluate the use of vasopressor in septic shock with cirrhosis and without cirrhosis	single-center, retrospective cohort, 18 years	122 patients included were 61 with cirrhosis and 61 non-cirrhosis with sepsis, and septic with cirrhosis		ICU
Maimone *et al*[12]	Compare the 20% albumin to plasmolytes in managing cirrhosis and sepsis in the intensive care unit	Retrospective cohort study	100 patients with cirrhosis and sepsis-induced hypotension		ICU
Bal *et al* [24]	The aim is to predict 50 days in hospital mortality in decomposed cirrhosis patients with SBP	A single-centre study prospective study	218 were admitted to ICU from 2013-2014 with cirrhosis and spontaneous bacterial peritonitis		ICU
Chebl *et al*[22]	Assess the outcome and mortality predictor of cirrhosis patients with sepsis	A single-center retrospective cohort study	200 patients	The study revealed an increased risk of sepsis in cirrhotic patients and sepsis-induced organ failure and related death in cirrhosis. The management of shock is to keep MAP above 65 mmgh with vasopressors; the aggressive fluid hydration may worsen the outcome as there is low oncotic pressure in a cirrhotic patient, which may lead to oedema with aggressive fluid hydration, so it is good to start with vasopressors early in the treatment of septic cirrhosis patients to avoid complications, a cirrhotic patient has higher lactate than the non-cirrhotic because of decreased lactate clearance by the liver	ICU
Chen *et al* [25]		A single-center, retrospective cohort study from 2015 to 2018	104 patients with cirrhosis and bacteremia were subdivided into afebrile (55) and febrile (49)	The cirrhotic patient is prone to infection. Cirrhotic patients with bacterial infections present with atypical manifest-ations such as normothermia. Scoring systems focused on organ dysfunction, such as quick sequential organ failure assessment (qSOFA) score or chronic liver failure sequential organ failure assessment (CLIF-SOFA) score, have better predictor ability	In the emergency department
Sasso *et al* [19]	Assess the prediction of mortality in a cirrhotic patient	Prospective cohort	113 patients mechanically ventilator cirrhotic from 2014-2018	Conclude that cirrhotic patients requiring mechanical ventilation have an extremely poor prognosis, and the vasopressor requirement was strongly a predictor of mortality in mechanical ventilation cirrhosis with sepsis	ICU
Fischer *et al*[12]	Assess the use of presepsin and resistin as markers of bacterial infections in cirrhotic patients with sepsis			Conclusion: Both presepsin and resistin may be reliable markers of bacterial infections in patients with decompensated liver cirrhosis and have similar diagnostic performance for bacterial infection and sepsis compared to C-reactive protein (CRP) and PCT. The best cut-off level of presepsin for diagnosis of sepsis was 1444 pg/mL. Conclusion PCT, CRP, Presepsin, and resistin had similar accuracy in diagnosing infection and sepsis in decompensated cirrhosis	ICU
Baudry *et al*[9]	Assess the prognosis of sepsis in cirrhotic patients	A Retrospective cohort study from 2002-2013	7644 patients were admitted, where 149 were		ICU

VCS: Value of volume conductivity and scattering; PCT: Procalcitonin; STO_2: Tissue oxygen saturation; ARDS: Acute respiratory distress syndrome; TP: Telipressin; NE: Norepinephrine; SVR: Systemic vascular resistance; RRT: Renal replacement therapy MAP: Mean arterial pressure; CI: Cardiac index, ATB: Antibiotic; MELD: Model for End-Stage Liver Disease; AKI: Acute kidney injury; SBP: Systolic blood pressure; qSOFA: Quick sequential organ failure assessment; ICU: Intensive care unit.

vasopressors, but this is still controversial[6].

Antibiotics: Broad-spectrum empirical antimicrobial therapy should be commenced early after obtaining blood for culture and microscopy. Many studies have shown mortality improvement when the antibiotic is administered within 1 h of the recognition of sepsis and hypotension[6]. The selection of the antimicrobial agents considers antifungal, antiviral, or antiparasitic agents that are directed by the clinical finding, knowledge of the common local pathogens and their antibiotic resistance profiles, and consideration of the patient's potential predisposition to a specific infection, for example, immunosuppression as for Cirrhosis[6]. Avoid prolonged therapy with broad-spectrum antimicrobials because it promotes the evolution of resistant organisms, which can lead to the failure of the treatment[6]. Sepsis in cirrhotic patients requires a high grade of suspicion so that empiric antibiotics might be started as early

Table 4 Summary of selected studies

Ref.	Year	Aim	Setting	Results	Conclusion
Philips *et al*[11]	2021	Assessed the use of 5% human fluid for the resuscitation of cirrhotic patients with sepsis	ICU	Found that primary, the two groups were different, with *P* values of less than 0.05, which is statistically significant. Study was done among 300 patients with sepsis with hypotension and cirrhosis 123 (n = 154, 79.8% receive albumin, and 131 (154, 85.1%) receive normal saline. Outcome related to MAP. measurement only 7.5 (n = 23) show reversal hypotension MAP > 65 at the end of the first hours of the resuscitation period; after 3 h, it was 11.7% (n = 18) and 32% (n = 5) in albumin and saline groups respectively (P = 0.008); Secondary outcome related to MAP, in the first hours while the study group, 62 patients (20.1%, n = 308) fluid resuscitation and sustained at 2 h in 42 (13.6%) patients improved MAP more than 65 mmHgf was seen in 25.3 (n = 39). In the albumin group at the end of the first hour compared to 14.9% (n = 23) patients in the saline group (P = 0.03). In second hour 17.5% (n = 27) in albumin group compare 9.7% (in = 15, P = 0.06) in saline group 5% albumin showed better improvement of MAP hemodynamic response compared to saline with P < 0.001 that is statistically significant. First hour, HA *vs* NS: 99.5 ± 7.9 *vs* 101.7 ± 8.8, P = 0.02; Second hour 97.9 ± 5.5 *vs* 103.4 ± 6.7, P < 0.001); Third hour 96.6 ± 3.6 *vs* 103.1 ± 5.9, P < 0.001	Found 5% human albumin correcting hypotension in sepsis with cirrhosis patients. Data were analyzed using IBM SPSS 22.0 statistic window. Quantitative variables were presented as mean ± SD and presented as number and percentage, the Chi-square or Fischer's exact test was used for categorical data, and continuous data were analyzed using the students' test or Mann-Whitney *U* test, Kaplan-Meier used for survival curves
Arabi *et al*[13]	2010	Assess the use of a low dose of hydrocortisone in cirrhotic patients with sepsis	ICU	140 patients screened 75 enrolled in the study. 60 (80%) with shock within 24 h and 71 (95%) with shock within 48 h. Twenty-eight days mortality with hydrocortisone treatment compared to its placebo 33 (85%) *vs* 26 (72%) relative risk (RR) 1.17, 95% confidence interval 0.92-1.49, P = 0.19). There was relative adrenal insufficiency in cirrhosis patients presenting with septic shock. Hydrocortisone show significant hemodynamic improvement. The 28-day mortality 33 (85) P = 0.19 and ICU mortality 24 (62) P = 0.64. Hemodynamic response was shock reversal 24 (62) P = 0.05 statistically significant	Found that corticosteroids improve hemodynamic status of the patient but do not change mortality
Rinaldi *et al*[5]	2013	The aim was to evaluate the effect of adherence to evidence-based guidelines of the surviving sepsis campaign (SSC) on the outcome of cirrhotic patients with shock admitted to the ICU	ICU	30 day-mortality of cirrhotic patients with septic shock in ICU is extremely high, and the application of SSC guidelines did not seem to improve the survival rate in this population. In addition, approximately 40% of cirrhotic patients developed an infection. 30 days mortality of 31 (81.6%) patients, 13 (86.6) with the bundle completed and 18 (78.2%) with the bundle not completed. This difference was not statistically significant	Hydrocortisone was associated with shock resolution but no survival modification. Chang *et al*[17], (2022) show that sepsis in cirrhotic patients has poor outcome than sepsis without Cirrhosis. And Sauneuf *et al*[18], (2013) found also that sepsis in cirrhotic patient survival remain low despite current management. Bal *et al*[24], 2016 found that mortality in 50 days in septic with cirrhosis patients was 43%
Thierry *et al*[23]	2007	Assess the use of echocardiography in assessing the LVEF on cirrhotic patients with septic shock	ICU	Show clinical and echocardiographic hemodynamic parameters between patients with Cirrhosis and without Cirrhosis; Cirrhosis had higher. Without Cirrhosis, Cirrhosis had higher values for the CI (3.69+/-1 *vs* 2.86+/-0.81/min/m2, P = 0.02. SI (37.5 ± 8 *vs* 32.4 ± 7 mL/m2, P = 0.04); LVEF (67 ± 7 *vs* 55.9 ± 12%, P = 0.005 and lower value for the SVR (1636.1 ± 523 *vs* 2136.6+/-633 dynes/cm5 m2, P = 0.04). The MELD score was not significantly correlated with the CI (R = 0.20, P = 0.49, or S (r = 0.15, P = 0.6). Mortality in ICU was 53% overall (64% *vs* 45%, P = 0.27), not statistically different from the patient without Cirrhosis	Show that echocardiography is of important help in the management of Cirrhosis with sepsis, showing hyperdynamic syndrome with high LVEF
Guo *et al*[26]	2019	Assessment of VCS parameter for evaluation of sepsis in cirrhotic patients	ICU	52% of positive culture in septic patients with Cirrhosis, with traditional infection markers (PCT, IL-6) and sCD163 between the two groups significantly different (P < 0.001). VCS parameters WBC range from 1.4 to 18.3 in sepsis, and leucocytes range from 1.6 to 19.2 in patients with infection no difference in the two groups for WBC. Test sensitivity was 75.9%, and a specificity of 73% was achieved	Reviewed the management of cirrhosis patients with sepsis and proposed: Blood vulture collection, white cell volume determination, procalcitonin and interleukin -6 and sCD163 test, and he concluded that VCS parameters predict the presence of infection early in cirrhotic patients
Villareal *et al*[15]	2016	Assessing the usefulness of procal-citonin for diagnosing infection in cirrhotic patients	ICU	Found that the mean scores as mean child-Pugh score 9.5 ± 2 and MELD score 23 ± 8 with P = 0.14 and P = 0.33, respectively, and there were not statistically significant for Cirrhosis with and without infection, and the mortality was high 62.9%. Procalcitonin (PCT) as biomarkers was found to be higher in a patient with infection than those without infection 4.20 (1.4-	Procalcitonin as biomarker might help with infection diagnosis in cirrhotic patients, and P. Fischer *et al*. (2019) found that both presepsin and resistin may be reliable markers of

				10.2) *vs* 0.16 (0.1-0.23) through statistically significant differences were not reached $P = 0.53$, severe sepsis or septic shock was associated with higher PCT	bacterial infection in patients with decompensated liver cirrhosis and have similar diagnostic performance compared to PCT
Chen *et al* [25]	2019		ICU	Find that the mean time of initiation of the antibiotic treatment was 3.5 h in the patient (afebrile: 4.3 h, febrile 2.8 h $P = 0.23$ high incidence of the afebrile group admitted in ICU (43.6% *vs* 20.4% $P = 0.01$) and higher 30 days mortality in afebrile group 40% *vs* 18.4%) $P = 0.02$) and endotracheal intubation 27.3% *vs* 10.2%, $P = 0.03$) infection	Found that the cirrhotic patient has an atypical presentation, and the qSOFA score or CLIF-SOFA score has a better predictor ability
Umgelter *et al*[21]	2008	Assess the outcome of the continuous low dose of terlipressin (TP) in a septic shock patient	ICU	Find that ICU admission patients had a mean age of 58 ± 85 mean Child-Pugh score of 13.8 ± 0.8, and a mean APACHE ii score of 31 ± 6 where TP decreases systemic vascular resistance index and norepinephrine (NE) doses needed to obtain the target MAP decreased, while cardiac index CI remained stable, median survival after initiating TP was ranging 5-15 days	Found that TP at a dose of 2 ug/kg can be used as an adjunct to NE in a cirrhotic patient with sepsis for hemodynamic improvement
Durst *et al*[20]	2021	The study aimed to evaluate the use of vasopressor in septic shock with cirrhosis and without cirrhosis	ICU	state that sepsis in cirrhosis was more likely to occur than in non-cirrhotic 34 (55.7%) versus 23(37.8%), $P = 0.046$, and received steroid 38.3% and 19.7%, respectively $P = 0.024$. The cirrhosis group requires increased median (IQR) total vasopressor dosage when compared to non-cirrhotic [71.5 (15.5-239.5)] *vs* 24.7 (5.3-77.9) mg NE equivalent, $P = 0.003$ and required a significantly higher total number of vasopressor agents 3 (1-4) *vs* 2 (1-3) agents $P = 0.03$. The length of ICU stays 7.0 (3.6-11.4) *vs* 5.0 (2.6-10.4) days $P = 0.146$ no statistically significant and MAP goal greater than baseline BP was 3 (4.9%)	Found that for sepsis and Cirrhosis needing vasopressors, MAP should be maintained above 60 mmgh, and blood culture and antibiotic should be started early as a survival campaign guideline
Galbois *et al*[27]	2015	Assessment of VCS parameter for evaluation of sepsis in cirrhotic patients	ICU	Found that cirrhosis patients with sepsis admitted to ICU were child-Pugh c without mottling, and mortality at 14 days was 71% (at day 28:78% in ICU: 76% in hospital: 82%). Hemodynamic parameters at 6 h were: MAP more than 65mmgh that was 88%, CVP more than 8mmgh: was 90%, $ScvO_2$ more than 81%, Urine output more than 0.5 mL/kg/h: 24%. Thenar and knee Sto2 at H6 to predict the outcome. Thenar Sto2 levels measured at H0 and H6 were not different in survivors and non-survivors. [H0: 77% (72-87) *vs* 84% (79-90), $P = 0.11$, H6:84% (79-89) *vs* 83% (71-92), $P = 0.89$]. Mottling score changes during the first 24 h of septic shock in a patient with and without Cirrhosis; in survivors, the proportion of patients with a mottling score of more than 2 decreased over time in both groups. in non-survivors, the proportion of patients with severe mottling score (4-5) increased over time in both groups. In non-survivors, the proportion of patients with a mottling score (0-1) was higher in patients with Cirrhosis than in patients without at H0 $P = 0.001$) and at H6 ($P = 0.02$), but was not significantly later	Described that mottling score and knee StO_2 measurement at 6 h after vasopressors have excellent 14 days mortality prediction
Piccolo Serafim *et al*[14]	2021	The study evaluates the use of steroids in a patient with septic shock and Cirrhosis	ICU	Found that patients who received steroids received a higher total of vasopressors (91.2 mg *vs* 39.1 mg, $P = 0.04$) and lower of lactate (1.8 mmol/L *vs* 2.6 mmol/L, $P = 0.007$)	Show that steroids did not improve mortality despite hemodynamic changes
Chebl *et al*[22]	2021	Assess the outcome and mortality predictor of cirrhosis patients with sepsis	ICU	found that cirrhotic patients were more likely to get intubated than non-cirrhotic patients (72.49% *vs* 61.62%, $P = 0.001$), and there was no statistically significant difference in mechanical ventilation duration or ICU LOS among survivors. Cirrhotic patients have higher hospital mortality than non-cirrhotic patients (64.79% *vs* 31.54% $P = 0.001$) and higher ICU mortality (47.47% *vs* 18.05% $P = 0.001$)	proposed as management of cirrhotic patient with sepsis to keep MAP > 65 mmgh with vasopressors, and start vasopressors early because of reduced oncotic pressure and risk of pulmonary oedema, avoid aggressive fluid resuscitation, cirrhotic patients have higher lactate levels
Maimone *et al*[12]	2022	Compare the 20% albumin to plasmolytes in managing Cirrhosis and sepsis in the intensive care unit.	ICU	Found that sepsis and septic shock in cirrhotic patient was the leading cause of acute decompensation or acute, chronic liver failure and had a poor prognosis and increased mortality	Show that albumin 20% increases MAP above 65 mmgh 3 h after infusion compared with plasmolyte and restores hemodynamic status rapidly but induce pulmonary oedema; why is it important to close monitoring with ultrasound so early detection of pulmonary oedema and management
Bal *et al* [24]	2013	The aim is to predict 50 days in hospital mortality in decomposed cirrhosis	ICU	Bal *et al*[24] study show that 50 days mortality in ICU was 43.11% of the patient admitted	Show that patients admitted to intensive care units with sepsis and Cirrhosis have poor prognoses and are a poor

		patients with SBP			candidate for ICU
Baudry *et al*[9]	2019	Assess the prognosis of sepsis in cirrhotic patients.	ICU		
Sauneuf *et al*[18]	2013	Assess the use of albumin as an adjuvant to vasopressors in managing septic shock in cirrhotic patients.	ICU	Find from 2005 to 2010, 40.5% were discharged from ICU, and 26% were alive six months after discharge. IV albumin was frequently given (57.1% *vs* 8.5%, $P < 0.001$), and crystalloid infusion was reduced at the same time [3 (1.7-4.5) L *vs* 6 (3-8,9) L, $P = 0.08$]. The ventilatory management with a smaller tidal volume 8.6 *vs* 7ml/kg, $P = 0.001$). Intensive insulin therapy and low-dose glucocorticoids were also used frequently during the second period, 83.3% *vs* 31.9% $P < 0.001$ and 81% *vs* 44.7, $P < 0.001$, respectively. Marked survival improvement in ICU as compared 1997-2004 period (40% *vs* 17%, $P = 0.02$, and 29% *vs* 6%, $P = 0.009$, respectively)	
Sasso *et al* [19]	2020	Assess the Prediction of mortality in a cirrhotic patient	ICU	Study shows changes in SOFA score median (IQR) in a cirrhotic patient. 24 h post admission 2.5 (0.75 to 5, $P = 0.122$ and 48 h post admission 1(0 to 4) $P = 0.269$. End of vasopressor therapy 0 (-3.5 to 21, $P = 0.963$, that is not statistically significant. But the duration of vasopressor in (hour) median (IQR) 86 (42.0-164.5) $P = 0.003$. MAP goal decreased during vasopressor course *n* (%) 13 (21.3) $P = 0.041$ statistically significant	Concluded that mechanically ventilated cirrhotic patients with sepsis have an extremely poor prognosis, and vasopressor use was strongly a predictor of mortality
Chang *et al*[17]	(2022)	The study aimed to determine whether septic patients with liver cirrhosis had worse survival than patients without liver cirrhosis	ICU	Found that liver cirrhosis was more common in male patients with 48% median range APACHE II was 25.5%, 27% of ICU mortality, sepsis with compensated liver cirrhosis mechanical ventilation 24% *P* value 0.179 and 4% ($P = 0.842$) needed for renal replacement therapy	
Fischer *et al*[16]	2019	Assess the use of presepsin and resistin as markers of bacterial infections in cirrhotic patients with sepsis	ICU	Found that 63% of the aetiology of Cirrhosis was alcoholism, 46% was bacterial infection (SBP), as infection markers presepsin, resistin, CRP, and PCT for predicting 28 days survival were AUROC = 0.74 (95% VI: 0.64-0.84) ($P < 0.001$), 0.68 (95%CI: 0.57-0.82) ($P = 0.006$, 0.74 (95%CI: 0.64-0.84)($P < 0.001$) and 0.70 (95%CI: 0.59-0.81) ($P = 0.001$) respectively	

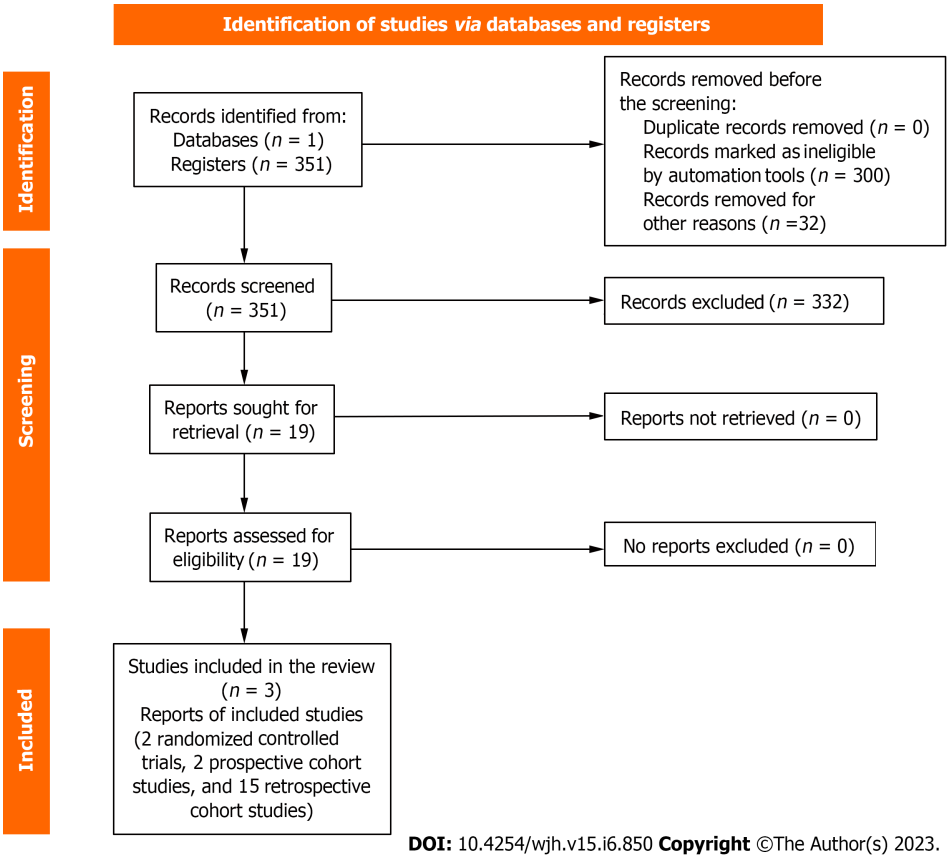
CI: Confidence interval; ICU: Intensive care unit; IL-6: Interleukin 6; LVEF: Left ventricle ejection function; MAP: Mean arterial pressure; MELD: Model for End-Stage Liver Disease; NE: Norepinephrine; PCT: Procalcitonin; qSOFA: Quick sequential organ failure assessment; RR: Relative risk; SBP: Systolic blood pressure; SSC: Surviving sepsis campaign; TP: Terlipressin; VCS: Value of volume conductivity and scattering; WBC: White blood cell.

as possible. Each hour delay in the starting the antimicrobial increases mortality by 1.86 times. Broad-spectrum antibiotics should be considered in patients at risk for resistant bacteria[6,7]. Early antibiotic start and intravenous administration of albumin 5% or 20% decrease the risk of renal failure development and improve survival in a cirrhotic patient with sepsis[2].

Procalcitonin: Procalcitonin is used as a biomarker for the risk of severe bacterial infection and for stopping antimicrobial therapy, but its role in cirrhotic patients has not been established yet[7]. In contrast, Villarreal *et al*[15] found that procalcitonin might be helpful in identifying bacterial infections in cirrhotic patients. Fischer *et al*[16] by Fischer *et al*[16] concludes that both presepsin and resistin are reliable markers of bacterial infection in patients with decompensated liver cirrhosis and have similar diagnostic performance to procalcitonin.

Liver transplantation: Liver transplantation is the definitive treatment for cirrhotic patients[7]. Cirrhotic patients are prone to bacterial infections and have higher mortality. Early therapeutic management of sepsis in the cirrhotic patient is crucial, and treatment should focus on correcting hypotension and avoiding aggressive fluid resuscitation[22]. Echocardiography can help diagnose hyperdynamic syndrome with high LVEF in cirrhotic patients with sepsis. Blood tests and VCS parameters can predict the presence of infection early in cirrhotic patients[23,26]. Mottling score and knee score and tissue oxygen saturation measurement six hours after vasopressors have an excellent 14-day mortality prediction[27]. Sepsis in cirrhotic patients has a poor outcome compared to sepsis without cirrhosis. Vasopressors, mechanical ventilation, and corticosteroids are suggested treatments, but mortality in 50 days in cirrhosis patients with sepsis was 43%. Mechanically ventilated cirrhotic patients with sepsis have an extremely poor prognosis, and vasopressor use was a predictor of mortality[17,18,19,24]. Cirrhotic patients have atypical presentations, and the qSOFA score or CLIF-SOFA score has better predictive ability[25].

Renal-replacement therapy and liver-support system: The use of hemofiltration in patients with sepsis



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Figure 1 PRISMA-P protocol for the systematic review.

has the potential benefit of alleviating the systemic inflammation of sepsis by removing circulating inflammatory mediators. However, two RCTs did not demonstrate significant reduction in inflammatory mediators nor patients' outcomes. Therefore, hemofiltration should not be recommended for routine management of patients with severe sepsis[1,6]. For liver support in the management of cirrhosis, it is recommended to treat the grade of ascites that are grade1 (mild) or grade 2 (moderate) where it is managed out of the ICU with restricted dietary sodium intake, start antidiuretic and monitor urea and electrolyte. For grade 3 that have a large volume of ascites with respiratory implication, paracentesis is recommended followed by dietary sodium restriction and diuretic therapy. Antibiotic prophylaxis should be used to prevent severe sepsis in a cirrhotic patient with ascites, gastrointestinal bleeding, or with more than one episode of spontaneous bacterial infection[4].

Glucose control: Hyperglycemia and insulin resistance are common in sepsis, and hyperglycemia may act as a procoagulant, impair neutrophil function, and increase the risk of death. Therefore, it is recommended to monitor and control glucose levels in patients with sepsis[1].

Infection source control: Source control in sepsis involves physical measures for removing the focus of infection. It is essential to identify and manage the source of infection promptly in the ICU[6].

DISCUSSION

The management of sepsis in cirrhosis patients is crucial to decrease the high mortality rate associated with this condition. In recent years, research has aimed to find the most effective therapeutic management for sepsis in cirrhosis patients. Interestingly, current therapeutic strategies for sepsis in cirrhosis patients are similar to the SSC international guidelines accepted for the general population.

Despite current management strategies, mortality remains high in cirrhosis patients with sepsis. Mortality rates are currently around 38%, with 30% of deaths due to infection[28]. Liver-specific scores, such as the CLIF-SOFA, CLIF-C Acute-on-Chronic Liver Failure (ACLF), and CLIF-C acute decompensation, have been developed to predict mortality in severely decompensated cirrhosis patients[29].

As the cirrhotic liver patient is prone to bacterial infection and impaired immunity status, which triggers complications related to cirrhosis such as hepatic encephalopathy, ascites, variceal bleeding, or hepatorenal syndrome[30-33] that further impaired prognosis[34]. The SSC guideline recommends early

detection of the source of infection, early initiation of antibiotics, fluid resuscitation, vasopressors, and corticosteroids[11,12].

Several studies have investigated the effectiveness of different therapeutic strategies for sepsis in cirrhosis patients. The use of human albumin 5% and 20% has been found to be beneficial for correcting hypotension and maintaining MAP above 65 instead of crystalloid[11,12]. Furthermore, norepinephrine has been found to be the best vasopressor for correcting hypotension in cirrhosis patients with sepsis, and combination therapy with terlipressin and norepinephrine has also been found to be effective[20, 22].

One interesting finding is that early vasopressor administration may be more beneficial than aggressive fluid administration in cirrhotic patients with sepsis. Chebl *et al*[22] found that early use of vasopressors was associated with better outcomes in cirrhosis patients. However, the use of corticosteroids did not show a decrease in mortality in cirrhotic patients with sepsis[9,18,19].

The management of sepsis in cirrhosis patients requires early detection and intervention with antibiotics, fluid resuscitation, vasopressors, and corticosteroids. While current management strategies are similar to those recommended in the SSC international guideline[36], studies have shown that the use of human albumin and norepinephrine or combination therapy with terlipressin and norepinephrine may be more effective. Choudhury *et al*[35] found that terlipressin is as effective as noradrenaline in increasing the MAP of more than 65 mmHg at 6 h and 48 h, and has a potential role in treating and preventing variceal bleeding as well as acute kidney injury[36]. Despite these efforts, mortality remains high, emphasizing the need for further research in this area to improve outcomes in cirrhosis patients with sepsis[37].

The use of EASL-CLIF criteria on ACLF and CLIF-SOFA for prognostication of sepsis in cirrhotic patients admitted to the ICU has gained significant attention[8,38]. These scoring systems have been developed to assess the severity of liver disease and predict mortality in severely decompensated cirrhosis patients[39-42]. By incorporating organ failure parameters, such as cardiovascular, renal, respiratory, neurological, hematological, and hepatic dysfunction, these criteria provide a comprehensive evaluation of the patient's condition. In the context of sepsis, the EASL-CLIF criteria can help identify cirrhotic patients at higher risk of poor outcomes and guide clinicians in making informed decisions regarding treatment strategies and resource allocation[8,38]. The CLIF-SOFA score, in particular, has shown promise in predicting short-term mortality and facilitating risk stratification in this vulnerable population[29,43-45]. By utilizing these criteria, healthcare professionals can enhance their ability to prognosticate sepsis in cirrhotic patients, thereby improving patient care and potentially reducing mortality rates. Further research and validation studies are warranted to optimize the use of EASL-CLIF criteria for prognostication and guide personalized interventions in this challenging clinical scenario[8,38].

The studies included in this systematic review provide valuable insights into the management of sepsis in patients with cirrhosis. However, these studies also have several limitations that need to be acknowledged. One of the major limitations of these studies is the absence of complete guidelines on the management of sepsis in patients with cirrhosis[37]. Although different therapeutic steps were proposed, these studies do not provide a comprehensive guide for managing these patients.

Moreover, most of the studies included in this systematic review were RCTs and cohort prospective and retrospective studies. While these studies provide strong and moderate evidence, they also have limitations in terms of generalizability. This is because most of these studies were conducted on single centers with small sample sizes. For instance, studies by Rinaldi *et al*[5], Philips *et al*[11], Maimone *et al*[12], Arabi *et al*[13], Sauneuf *et al*[18], Durst *et al*[20], Thierry *et al*[23] Bal *et al*[24], Chen *et al*[25], Galbois *et al*[27] were conducted on small sample sizes, which limits the generalizability of their findings.

Furthermore, the prospective nature of some studies can also affect the results due to missing information. For example, studies by Rinaldi *et al*[5], Baudry *et al*[9], Philips *et al*[11], Maimone *et al*[12], Arabi *et al*[13], Piccolo Serafim *et al*[14], Chang *et al*[17], Sauneuf *et al*[18] Sasso *et al*[19] Durst *et al*[20], Chebl *et al*[22], Thierry *et al*[23], Bal *et al*[24], and Chen *et al*[25], and Galbois *et al*[27] were conducted prospectively and some information was missing, which can affect the accuracy of the results.

Moreover, retrospective studies have their limitations as well, as not all information was present. For instance, Rinaldi *et al*[5], Baudry *et al*[9], Philips *et al*[11], Maimone *et al*[12], Arabi *et al*[13], Piccolo Serafim *et al*[14], Villarreal *et al*[15], Fischer *et al*[16] Chang *et al*[17], Sauneuf *et al*[18], Durst *et al*[20], Umgelter *et al* [21] Chebl *et al*[22], Thierry *et al*[23], Bal *et al*[24], Chen *et al*[25], Guo *et al*[26] and Galbois *et al*[27], all suffered from selection bias and missing information bias.

In addition, it is important to acknowledge that this review has certain limitations. Although we made efforts to gather relevant sources, we were unable to conduct an exhaustive search, leading to some sources remaining unexplored. This constraint resulted from the time limitations imposed during the review process. Consequently, the review may not encompass the full breadth and depth of available literature on the management of cirrhosis patients with sepsis admitted to the ICU. Furthermore, it is worth noting that a substantial proportion of the included research papers were retrospective studies with occasional missing information. To enhance the understanding and enhance outcomes in cirrhotic patients with sepsis, further research endeavors are warranted.

CONCLUSION

In conclusion, sepsis in cirrhotic patients is a complex and challenging clinical scenario. Our systematic review of the literature revealed that there is no standardized approach to the management of sepsis in cirrhotic patients admitted to the ICU. Although there is evidence to support early identification of infection, prompt administration of antibiotics, and aggressive resuscitation with fluids and vasopressors, the optimal management of these patients remains unclear. Furthermore, the studies included in this review were limited by small sample sizes, single-center designs, and missing data, highlighting the need for larger, multicenter trials to establish best practices for managing sepsis in cirrhotic patients.

Despite these limitations, our review suggests that using prognostic scores such as SOFA, MELD, and MELD-Na can help identify high-risk patients and guide clinical decision-making. Furthermore, improving outcomes in septic cirrhotic patients will require a multidisciplinary approach, including collaboration between intensivists, hepatologists, infectious disease specialists, and other healthcare providers. With the growing burden of cirrhosis and sepsis worldwide, further research is urgently needed to clarify the optimal management of this complex patient population and improve outcomes for these critically ill patients.

ARTICLE HIGHLIGHTS

Research background

The background of the study lies in the physiological response of sepsis, characterized by a dysregulated inflammatory reaction to infection, which can progress to organ failure and death. Cirrhotic patients are particularly susceptible to sepsis-induced organ failure and have higher mortality rates. The imbalance of cytokine response, known as a "cytokine storm," plays a significant role in the worsening of liver function and the development of organ/system failure in severe sepsis. The severity of sepsis in cirrhotic patients is associated with increased production of proinflammatory cytokines. Additionally, cirrhotic patients with severe sepsis can experience complications such as shock, acute lung injury, coagulopathy, renal failure, or hepatic encephalopathy. Understanding the background and significance of sepsis in cirrhosis is crucial for effective management and improved outcomes.

Research motivation

The motivation behind this research is to address the impact of sepsis in cirrhotic patients and the associated challenges in managing this complex condition. Sepsis is a major cause of admission to intensive care units (ICUs), and its outcomes are worse in patients with comorbidities like cirrhosis. Organ dysfunction in sepsis, measured by the Sequential Organ Failure Assessment (SOFA) score, including liver failure, is linked to higher mortality rates. Defining sepsis and septic shock accurately remains challenging. Given the high mortality and complexity of sepsis in cirrhosis, understanding the key problems and finding effective solutions is crucial. Solving these problems not only improves patient outcomes but also contributes to future research in this field by providing insights into personalized interventions, risk stratification, and resource allocation.

Research objectives

The main objectives of this study are to determine the optimal management of sepsis in cirrhotic patients admitted to the ICU and to explore strategies for improving outcomes in this population. Realizing these objectives has significant implications for future research in this field. By identifying effective management approaches, personalized interventions can be developed to address the specific needs of cirrhotic patients with sepsis. Furthermore, understanding the impact of different interventions on mortality and organ failure rates provides valuable insights for risk stratification and resource allocation. The successful realization of these objectives contributes to the advancement of knowledge and practices in managing sepsis in cirrhotic patients, ultimately improving patient care and outcomes in this challenging clinical scenario.

Research methods

This study utilized a systematic review methodology following the PRISMA-P protocol to investigate the management of sepsis in cirrhotic patients admitted to the ICU. The inclusion criteria comprised cirrhotic patients over 18 years old with sepsis in the ICU, and the analysis focused on sepsis management and prognosis in this population. English-language randomized controlled trials, retrospective cohort studies, and prospective cohort studies were considered. The outcomes assessed included survival, ICU length of stay, and overall prognosis. Searches were conducted on PubMed, Google Scholar, Embase, and Cochrane databases, with filtering based on titles and abstracts. Relevant papers underwent full-text analysis, and only those meeting the inclusion criteria were included. This systematic review offers valuable insights into sepsis management and prognosis in cirrhotic patients

admitted to the ICU, utilizing a comprehensive approach to assess existing literature.

Research results

The study conducted a systematic review to investigate the management of sepsis in cirrhotic patients admitted to the ICU. The researchers selected 19 papers that met the inclusion criteria, focusing on survival and prognostic factors for this patient population. The findings indicated that albumin administration corrected hypotension in sepsis with cirrhosis, while corticosteroids improved hemodynamic status without affecting mortality. Procalcitonin was found to be helpful in diagnosing bacterial infections in cirrhotic patients, and vasopressors such as norepinephrine and terlipressin were recommended to maintain mean arterial pressure above specific thresholds. The prognosis was generally poor for cirrhotic patients with sepsis, especially for mechanically ventilated patients or those requiring vasopressors. The use of fluid resuscitation, particularly with human albumin, was recommended, and early antibiotic administration within the first hour showed improved outcomes. The qSOFA criteria were identified as a better predictor of adverse outcomes in sepsis, and echocardiography aided in diagnosing hyperdynamic syndrome. Liver transplantation was highlighted as the definitive treatment for cirrhotic patients. The study also mentioned the potential benefits and limitations of renal replacement therapy and liver support systems in sepsis management. Source control and glucose control were emphasized as essential aspects of sepsis management.

Research conclusions

The study proposes that the current therapeutic strategies for sepsis in cirrhosis patients, which are similar to the Surviving Sepsis Campaign guidelines for the general population, may not be sufficient in reducing mortality rates in this specific patient group. It highlights the need for further research and development of comprehensive management guidelines for sepsis in cirrhosis patients. The study suggests that the use of human albumin and norepinephrine, as well as combination therapy with terlipressin and norepinephrine, may be effective in correcting hypotension and improving outcomes in cirrhosis patients with sepsis. Additionally, it indicates that early administration of vasopressors could be more beneficial than aggressive fluid administration in this patient population. However, the use of corticosteroids did not show a decrease in mortality.

Research perspectives

Future research should focus on developing standardized management guidelines specifically tailored for sepsis in cirrhosis patients. These guidelines should encompass early detection of infection, appropriate antibiotic therapy, fluid resuscitation, vasopressor selection, and corticosteroid use. There is a need for larger, multicenter trials to validate the findings of existing studies and establish best practices for managing sepsis in cirrhosis patients. These studies should have larger sample sizes and address the limitations of previous research, such as single-center designs and missing data. Prognostic scores, such as SOFA, Model for End-Stage Liver Disease (MELD), and MELD-Na, should be further evaluated and incorporated into the management of sepsis in cirrhosis patients to identify high-risk individuals and guide treatment decisions. A multidisciplinary approach involving intensivists, hepatologists, infectious disease specialists, and other healthcare providers is essential for improving outcomes in septic cirrhotic patients. Collaboration and coordination among these specialties should be emphasized in future research and clinical practice.

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REVIEW

Gut microbiome and metabolic-associated fatty liver disease: Current status and potential applications

Gong-Jing Guo, Fei Yao, Wei-Peng Lu, Hao-Ming Xu

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Abstract

Metabolic-associated fatty liver disease (MAFLD) is one of the most common chronic liver diseases worldwide. In recent years, the occurrence rate of MAFLD has been on the rise, mainly due to lifestyle changes, high-calorie diets, and imbalanced dietary structures, thereby posing a threat to human health and creating heavy social and economic burdens. With the development of 16S sequencing and integrated multi-omics analysis, the role of the gut microbiota (GM) and its metabolites in MAFLD has been further recognized. The GM plays a role in digestion, energy metabolism, vitamin synthesis, the prevention of pathogenic bacteria colonisation, and immunoregulation. The gut-liver axis is one of the vital links between the GM and the liver. Toxic substances in the intestine can enter the liver through the portal vascular system when the intestinal barrier is severely damaged. The liver also influences the GM in various ways, such as bile acid circulation. The gut-liver axis is essential in maintaining the body's normal physiological state and plays a role in the onset and prognosis of many diseases, including MAFLD. This article reviews the status of the GM and MAFLD and summarizes the GM characteristics in MAFLD. The relationship between the GM and MAFLD is discussed in terms of bile acid circulation, energy metabolism, micronutrients, and signalling pathways. Current MAFLD treat-

ments targeting the GM are also listed.

Key Words: Metabolic-associated fatty liver disease; Gut microbiota; Current status; Application

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Core Tip: Metabolic-associated fatty liver disease (MAFLD) is a highly prevalent metabolic disease worldwide. In this review, we provide an overview of the current status and potential applications of the gut microbiota (GM) in MAFLD, focusing on key aspects such as bile acid circulation, energy metabolism, and microelement disorder, as well as signal pathways and GM metabolites implicated in MAFLD development and treatments, with a particular emphasis on targeting the microbiome.

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INTRODUCTION

Metabolic-associated fatty liver disease (MAFLD), originally known as non-alcoholic fatty liver disease (NAFLD), is one of the most common chronic liver diseases worldwide. Liver inflammation and fibrosis are the pathological processes implicated in MAFLD. MAFLD can develop into non-alcoholic steatohepatitis (NASH), which can then progress to liver cirrhosis, hepatic failure, and liver cancer[1]. The rate of MAFLD has been on the rise, with a global rate of 25%-30%, due to lifestyle changes, excessive calorie intake, and unbalanced diet structures. In certain groups, such as patients with Type 2 diabetes, the rate of MAFLD even exceeds 70%[2,3]. MAFLD poses a threat to human health and leads to substantial social and economic burdens. The gut microbiota (GM) lives in the human intestinal tract. In the past 10 years, there has been an exponential growth of studies on the relationship between GM and human health and disease in databases such as PubMed. The GM consists of over 10^{14} microorganisms[4], and its genome comprises of over 3 million genes, whereas the human genome consists of approximately 23000 genes[5]. Therefore, the GM is considered as one of the "new organs" in human beings. Steady-state GM plays a role in digestion, energy metabolism, vitamin synthesis, the prevention of pathogenic bacteria colonisation, and immunoregulation[6,7]. The gut-liver axis is one of the vital links between the GM and the liver. The intestine and liver both originate from the ventral foregut endoderm. When the intestinal barrier is severely damaged, toxic substances in the intestinal tract enter the portal vein through the superior and inferior mesenteric veins and then flow into the liver. Meanwhile, the liver influences the intestinal microecology in various ways. For example, the liver secretes bile acids, which enter in the enterohepatic circulation to alter the intestinal microecology [8]. In recent years, the development of 16S sequencing and integrated multi-omics analysis has helped to further understand the role of the GM and its metabolites in MAFLD. It is reported that *Akkermansia muciniphila* can improve liver function, reduce oxidative stress, inhibit inflammation, and reverse the metabolic disorder caused by high-fat diets [9]. Some secondary bile acid-producing bacteria, such as *Lactobacillaceae* and *Lachnospiracea*, have cholesterol-reducing potential[10]. Specific bile acids produced by bacteria can regulate GM structure and restore GM balance[11]. Numerous studies have confirmed that GM metabolites play a significant role in the onset and progression of MAFLD when they are present in the intestine or enter the circulation. GM can mediate the fermentation of dietary fibres, leading to the production of short-chain fatty acids (SCFAs) as the primary metabolites of this process. Among the SCFAs, butyric acid can improve the MAFLD induced by high-fat diets *via* activating peroxisome proliferator-activated receptor α to inhibit liver inflammation and enhance the expression of glucagon-like peptide-1 receptor[12]. Furthermore, SCFAs can also activate G-protein-coupled receptor and induce the release of glucagon-like peptide-1 (GLP-1) and peptide YY, resulting in metabolically balanced feedback regulation[13]. SCFAs can not only regulate glycolipid metabolism, inhibit fat synthesis, and reduce liver fat content but also escalate intestinal barrier function, thereby improving MAFLD[14]. Therefore, the main aims of this research field are to observe and characterize the GM in MAFLD, investigate the impact of GM and its metabolites on the onset and progression of MAFLD, and explore the potential of targeting the GM for MAFLD treatment.

This article primary focuses on elucidating the impact of the gut-liver axis on MAFLD. It provides an overview of the existing clinical MAFLD cases and commonly utilized animal models. The review involves the important aspects such as bile acid circulation, energy metabolism, microelement disorder, and other relevant factors such as signal pathways and GM metabolites implicated the development of MAFLD. Then, current MAFLD treatments utilizing GM as the target are presented. Table 1 summarizes the key characteristics of GM in both clinical MAFLD cases and commonly used MAFLD animal models.

Table 1 Studies presenting gut dysbiosis in non-alcoholic fatty liver disease/non-alcoholic steatohepatitis

			Alterations of GM (↑/↓)			
Country	Subjects	Year	Phylum	Family	Genus/Species	Ref.
United States	Obese patients without NASH (n = 25), NASH (n = 22), Controls (n = 16)	2013	↑*Bacteroidetes*; ↑ *Proteobacteria*; ↓ *Firmicutes*; ↓*Actinobacteria*	↓*Bifidobacteriaceae*; ↑*Alcaligenaceae*; ↓ *Clostridiales family XI*; ↑*Campylobacteraceae*; ↓*Lachnospiraceae*; ↑*Enterobacteriaceae*	↑*Prevotella*; ↑*Escherichia*	[69]
Canada	NAFLD (n = 33) *vs* Controls (n = 17)	2013	↓*Bacteroidetes*	/	↑*Clostridium coccoides*	[124]
Canada	NAFLD (n = 30) *vs* Controls (n = 30)	2013	↑*Proteobacteria*; ↑ *Firmicutes*; ↓ *Bacteroidetes*	↑*Kiloniellaceae*; ↑*Pasteurellaceae*; ↑ *Lactobacillaceae*; ↑*Lachnospiraceae*; ↑ *Veillonellaceae*; ↓*Ruminococcaceae*; ↓ *Porphyromonadaceae*	↑*Lactobacillu*; ↑*Robinsoniella*; ↑*Roseburia*; ↑ *Dorea*; ↓*Oscillibacter*	[125]
Hong Kong	NASH (n = 16) *vs* Controls (n = 22)	2013	↓*Firmicutes*	/	↓*Faecalibacterium*; ↓*Anaerosporobacter*; ↑ *Parabacteroides*; ↑*Allisonella*	[126]
China	NAFLD (n = 53) *vs* Controls (n = 32)	2015	↑*Firmicutes*; ↑ *Proteobacteria*	↑*Peptostreptococcaceae*; ↑*Lactobacillaceae*; ↓*Ruminococcaceae*; ↓ *Porphyromonadaceae*	↑*Escherichia*; ↑*Lactobacillus*; ↑*Streptococcus*; ↑*Anaerobacter*; ↓*Prevotella*	[127]
United States	Controls (n = 26), obese (n = 11), NAFLD (n = 13)	2015	↑*Actinobacteria*	/	↓*Erysipelotrichia*; ↑*Prevotella*; ↓*Alphaproteobacteria*; ↑*Clostridia*; ↓*Verrucomicrobia*; ↑ *Fusobacteria*; ↑*Epsilonproteobacteria*; ↑ *Gammaproteobacteria*	[67]
France	NASH (n = 35) *vs* Controls (n = 22)	2016	↑*Proteobacteria*	↑*Enterobacteriaceae*; ↓*Ruminococcaceae*	↑*Ruminococcus*; ↓*Prevotella*; ↑*Escherichia*; ↓ *Anaerospacter*; ↓*Coprococcus*; ↓*Eubacterium*; ↓*Faecalibacterium*; ↑*Bacteroides*	[128]
Italy	NAFLD (n = 61) *vs* Controls (n = 54)	2017	↑*Actinobacteria*; ↓ *Bacteroidetes*	↓*Rikenellaceae*	↑*Bradyrhizobium*; ↑*Anaerococcus*; ↑*Peptoniphilus*; ↑*Ruminococcus*; ↓*Oscillopira*; ↑*Dorea* ; ↑*Blautia*; ↑*Propionibacterium acnes*	[129]
China	NAFLD (n = 43) *vs* Controls (n = 83)	2016	↑*Bacteroidetes*; ↓ *Firmicutes*	↑*Bacteroidaceae*; ↓*Lachnospiraceae*; ↑ *Prevotellaceae*; ↓*Ruminococcaceae*; ↓ *Lactobacillaceae*; ↓*Peptostreptococcaceae*	↓*Coprococcus*; ↓*Anaerosporobacter*; ↓ *Anaerotruncus*; ↓*Ruminococcus*; ↓*Lactobacillus*	[130]
China	NAFLD (n = 25) *vs* Controls (n = 22)	2017	↑*Proteobacteria*; ↓ *Bacteroidetes*	↑*Lachnospiraceae*; ↑*Enterobacteriaceae*; ↓*Prevotellaceae*; ↓*Ruminococcaceae*; ↑ *Erysipelotrichaceae*; ↑*Streptococcaceae*	↑*Fusobacteria*; ↓*Prevotella*; ↑*Blautia*; ↑ *Escherichia*; ↑*Shigella*; ↑*Fusobacteria*; ↑ *Escherichia Shigella*	[131]
Canada	NAFLD (n = 39) *vs* Controls (n = 28)	2018	↓*Firmicutes*; ↓ *Bacteroidetes*	↑*Lactobacillaceae*	↓*Ruminococcus*; ↓*Faecalibacterium*; ↓ *Coprococcus*	[132]
Italy	Obese, NAFL and NASH (n = 61) and Controls (n = 54)	2016	/	/	↑*Lactobacilli*; ↓*Bifidobacteria*; ↑*Lactobacilli mucosae*; ↓*Bifidobacteria longum*; ↓ *Bifidobacteria adolescent*; ↓*Bifidobacteria bifidum*	[133]
Brazil	NASH (n = 13) *vs* Controls (n = 10)	2017	/	/	↑*Bacteroides*; ↑*Lactobacilli*; ↓*Ruminococcu*; ↓ *Bifidobacterium*; ↑*Prevotella*; ↓*Faecalibacterium*	[134]
China	NAFLD (n = 30) *vs* Controls (n = 37)	2018	/	↑*Lactobacillaceae*; ↑*Veillonellaceae*; ↑ *Peptostreptococcaceae*; ↑*Coprobacillaceae*; ↑*Erysipelotrichaceae*; ↓*Parapre-votellaceae*; ↓*Victivallaceae*	↑*Porphyromonas*; ↑*Clostridium*; ↑*Blautia*; ↑ *Dorea*; ↑*Peptococcus*; ↑*Peptococcaceae_rc4-4*; ↑*Mitsuokella*; ↑*Slackia*; ↑*Succinivibrio*; ↓ *Odoribacter*; ↓*Coprococcus*; ↓*Proteus*	[135]
Germany	NAFLD (n = 90) *vs* Controls (n = 21)	2020	↓*Bacteroidetes*	↓*Ruminococcaceae*; ↑*Lactobacillaceae*; ↑ *Veillonellaceae*	↑*Dorea*	[136]
United States	NAFLD (n = 44) *vs* Controls (n = 29)	2020	↓*Bacteroidetes*	/	↓*Prevotella*; ↓*Gemmiger*; ↓*Oscillospira*	[137]

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

GM AND METABOLIC DISORDERS IN MAFLD

Bile acid metabolism disorders

Primary bile acids are synthesized in the liver before being secreted into the gall bladder and released into the duodenum after a meal. Bacteria metabolize primary bile acids in the intestinal tract into secondary bile acids, which are then reabsorbed into the portal vein. While most bile acid molecules are captured by the liver and undergo recirculation, a small fraction of them persists in the blood as signalling molecules. Bile acid synthesis in hepatocytes involves the oxidation of cholesterol mediated by cytochromes P450 enzymes. The synthesis mainly occurs through the classic and

alternative pathways, producing cholic and chenodeoxycholic acids, which are subsequently conjugated to taurine and glycine, respectively, to form conjugated bile acids. Synthesized primary bile acids are deposited into the gallbladder *via* the bile salt export pump. Gall bladder contraction triggered by eating promotes bile acid secretion into the intestinal tract [15]. Primary bile acids increase the permeability of the intestinal mucosa, resulting in endotoxemia and aggravating MAFLD. Thus, the bile acid level is elevated in the liver tissue, serum, and urine of MAFLD patients. Meanwhile, there is a significantly higher proportion of hydrophobic and cytotoxic bile acids[16]. Patients with NASH exhibit increased synthesis of bile acids compared to other conditions. The ratio of primary bile acids to secondary bile acids is also higher than in healthy individuals[17]. Bile salt hydrolases produced by *Bacteroides*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*, and *Listeria* in the GM can deconjugate conjugated bile acids to form free bile acids. *Clostridium*, *Fusobacterium*, *Peptococcus*, and *Pseudomonas* species have the ability to catalyze the desulfuration of bile acids. *Bacteroides*, *Eubacterium*, *Clostridium*, *Escherichia*, *Eggerthella*, *Peptostreptococcus*, and *Ruminococcus* are implicated in the dehydroxylation of primary bile acids to produce secondary bile acids[18]. Moreover, it was found that *Clostridium leptum* is positively related to taurocholic acid and negatively related to cholic acid and chenodeoxycholic acid. This indicates that *Clostridium leptum* may promote the transformation from primary bile acids to secondary bile acids, thereby reducing the damage caused by primary bile acids to the liver[19].

About 95% of primary and secondary bile acids can be reabsorbed in the intestine and transported back into the liver through the portal veins. However, lithocholic acids present in secondary bile acids are primarily excreted with the feces. Hepatocytes synthesize new bile acids to compensate for the bile acids lost in the enterohepatic circulation. After reabsorption, bile acids are conjugated to the farnesoid X receptor (FXR) and G protein-coupled bile acid receptor (GPBAR; also named TGR5), thereby promoting the secretion of fibroblast growth factor 19 (FGF19) by intestinal cells. FGF19 conjugates to fibroblast growth factor receptor 4 (FGFR4), activating c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). These two signaling pathways decrease the genetic expression of cholesterol 7 α-hydroxylase (CYP7A1), which inhibits bile acid synthesis through negative feedback[20]. The effect of choline on lipid metabolism may be mediated by activating FXR to participate in liver lipid metabolism, thereby reducing the synthesis of cholesterol and triglycerides. The effect of bile acids on glucose metabolism has been established, as evidenced by the presence of insulin resistance and hyperglycemia in FXR gene-deficient mice, and the administration of oral dietary cholic acid to activate FXR can inhibit the expression of the gluconeogenesis gene in mice, reduce fasting glucose, and increase insulin sensitivity. Choline alleviates metabolic inflammation induced by tumor necrosis factor-alpha (TNF-α) and lipopolysaccharides (LPS), while bile acids such as cholic acid, deoxycholic acid, and chenodeoxycholic acid can inhibit the release of monocyte chemotactic protein-1 induced by TNF and LPS, suggesting that bile acids have anti-inflammatory effects[21]. Choline is a pleiotropic hormone-like signaling molecule with both metabolic and endocrine functions. It plays an important role in regulating cholesterol and triglyceride metabolism, insulin resistance, metabolic inflammation, and liver steatosis *via* activation of choline-specific receptors that are widely distributed in the body.

GM can regulate the synthesis and reabsorption of bile acids. Germ-free animals exhibit reduced excretion of bile acids in feces, accompanied by a significant increase in the total bile acid content in the gall bladder and small intestine[22,23]. Probiotics supplements or fecal microbiota transplantation can distinctly reduce the total bile acid content in the liver, gall bladder, and cecum germ-free animals[24]. In aseptic conditions, tauro-β-muricholic acid, a primary bile acid, accumulates due to its inability to undergo further metabolism. It can be used as an antagonist to inhibit intestinal FXR expression, thereby downregulating FXR expression. The expression of liver CYP7A1 promotes liver bile acid synthesis and is regulated *via* the enterohepatic circulation[23]. There is an increased expression of bile acid transporters in the ileum and colon of germ-free animals. This leads to decreased bile acid excretion in the feces, resulting in highly efficient bile acid reabsorption. The key enzymes in bile acid synthesis, such as CYP7A1, CYP7B1, and CYP27A1, can all be regulated by the GM, mainly *via* induction of the FXR signaling pathway[25]. Moreover, bile acids also have ab effect on the GM. Amphipathic bile acids directly perform the anti-bacterial function by breaking the cell membrane of bacteria, which is critical to maintaining the steady state of the bacterial flora. Studies revealed that bile duct ligation could arouse bacterial translocation in rat's mesenteric lymph nodes as early as after one week. Three weeks after bile duct ligation, the translocation was found to be expanded to tissue such as the liver, spleen, and lung. In addition, gram-negative bacteria in the animal cecum and endotoxin levels in the blood were significantly elevated. The villi of the distal ileum were flattened, and Peyer's patches increased in size[26]. Bacteria overgrowth in the small intestine, bacterial translocation, and endotoxemia can be effectively inhibited by taking bile acids orally[27]. Secondary bile acids can inhibit the growth of *Clostridium difficile*[28]. In addition, bile acids can enrich the bacteria that utilize bile acids. For example, bacteria with bile salt hydrolase activity, such as *Lactobacillus reuteri*, can resist cytotoxicity resulting from bile salts[29]. An in vitro culture experiment revealed that bile acids were required for the growth of *Bilophila wadsworthia*[30]. Studies have shown that diets rich in milk fat altered the bile acid profile, mainly by increasing the total amount of bile acid, and the abundance of *Bilophila wadsworthia* also increased with the increase in bile acids.

Choline metabolism disorders

Choline is a quaternary amine rich in methyl groups that exists in tissues in either free or esterified forms. In the liver, choline exists in the form of phosphatidylcholine (PC). Choline has been recognized as an essential nutrient by the National Academy of Sciences (NAS) since 1998. The biological functions of choline mainly include neurotransmitter synthesis, lipid metabolism, and cell membrane signal transduction. Choline can also serve as a methyl donor for the synthesis of PC in the liver[31]. PC, in turn, is indispensable for the synthesis and secretion of very low-density lipoprotein (VLDL). Moreover, choline also prevents abnormal lipid accumulation by mediating liver lipid transport. Therefore, the lack of choline may lead to hepatic steatosis[32]. For over 50 years, researchers have recognized the association between choline deficiency and accumulation of fat in the liver. Choline-deficient diets are often used in animal experiments to induce MAFLD. The administration of diets deficient in choline and vitamin B12 to weanling rats

induces fatty liver and renal cortical necrosis, resulting in high deaths rate within 10 d[33,34]. Research has demonstrated that patients with MAFLD exhibit varying degrees of decreased choline levels in their plasma, which is associated with the degree of liver damage[31,35].

Choline has two primary sources. Approximately 70% of choline is obtained from dietary sources, while the remaining 30% is synthesized by the GM. Among 79 gut microbiota strains screened from the human gastrointestinal tract, eight strains have been identified to significantly affect choline metabolism (*Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Edwardsiella tarda*, *Escherichia fergusonii*, *Proteus penneri*, and *Providencia rettgeri*). However, genetic analysis has revealed that the gene set responsible for anaerobic choline metabolism is widely distributed among the three main bacterial groups present in the human gut, including *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. This metabolic pathway contributes to the bioavailability of choline in the human body and subsequently affects serum choline concentration. On the other hand, choline levels in the diet may also affect the gut microbiota. In patients with choline deficiency, choline supplementation has been shown to decrease the abundance of *Gammaproteobacteria*. This reduction in *Gammaproteobacteria* can alleviate the inhibition of key enzymes in choline metabolism, thereby reducing the occurrence of MAFLD[36-38]. As a result, GM disruption can alter choline metabolism and reduce the host's capacity to efficiently utilize choline, leading to a relative deficiency of choline and increased production of substances such as trimethylamine N-oxide (TMAO). In turn, this can lead to the occurrence of hepatic steatosis[32,39,40]. Bacterial species such as *Escherichia coli* and *Desulfovibrio desulfuricans* within in GM have the capability to utilize choline and convert it into methylamine. When there is a disruption in the GM involved in the metabolism of choline, it can lead to a deficiency of choline and potentially contribute to the development of MAFLD[40]. In the presence of abundant MAFLD-associated intestinal bacteria, there is an increase demand for choline by these bacteria. This leads to choline deficiency in the host, exacerbating the risk of MAFLD and potentially progressing to NASH[41]. Reduced choline utilization may lead to decreased PC synthesis in the body, thereby inducing fatty acid synthesis and increasing triglyceride (TG) production. Meanwhile, it decreases the surface activity of lipid droplets lacking PC. Large lipid droplets are easier to form. Therefore, it is difficult for lipoprotein lipase (LPL) to decompose lipid droplets[9,42]. GM disruption can alter the body's reservoir of choline, thereby inducing choline deficiency and decreasing VLDL secretion, which leads to the accumulation of fat in the liver[43]. Moreover, TMAO and GM are closely associated with choline metabolism. GM can produce enzymes that catalyze the transformation of dietary choline into methylamine. After metabolism, methylamine is transformed into TMAO by GM-produced trimethylamine-lyase. TMAO can regulate protein activity and stability, increase foam cell production, inhibit cholesterol transport, aggravate liver fat deposition, and even induce liver inflammation[31,32,44]. Trimethylamine lyases in GM can decompose dietary choline into TMA, which enters the liver through the portal vein and is oxidated into TMAO. TMAO can upregulate the expression of sterol regulatory element-binding protein-1c (SREBP-1c). SREBP-1c is a critical transcription factor in the regulation of liver lipid metabolism, which promotes TG synthesis, aggravating hepatic steatosis[45]. TMAO also upregulates glucose metabolism and increases serum inflammatory factors for insulin resistance (IR) promotion[46,47]. It affects lipid metabolism and cholesterol's steady state by reducing the transformation of cholesterols into bile acids[35]. TMAO contributes to the progression of MAFLD through various mechanisms. GM metabolites also include secondary bile acids and ethanol, which have been discussed in the previous section.

Lipid metabolism disorders

The GM has the ability to generate energy from indigestible substances (*e.g.*, *Firmicutes* can ferment resistant starch to provide energy for intestinal epithelial cells)[48]. Therefore, GM is critical in the development of obesity, and it disruption can lead to obesity-related MAFLD[49]. Obesity can increase the level of proinflammatory cytokines secreted by macrophages and promote adipose tissue infiltration, leading to the development of hepatic steatosis[50]. The intestines of obese people are rich in *Firmicutes*. *Firmicutes* can ferment indigestible dietary fiber (polysaccharide) and produce additional energy from the intestine content, which promotes the progression of obesity and MAFLD. The fecal microbiota of obese mice caused by high-fat fodder was transplanted to mice fed with regular fodder, and it was found that mice transplanted with fecal microbiota on a high-fat diet had more fat deposition than mice transplanted with fecal microbiota on a regular diet. A further study found that structural changes in the GM could lead to greater lipid absorption by the body, promoting the biosynthesis of fatty acids. However, it was proven that some probiotics, such as *Lactobacillus*, could reduce liver fat deposition by reducing fatty acid absorption in the host intestine[51,52].

Fasting-induced adipocyte factor (FIAF) is a lipoprotein lipase inhibitor, and inhibition of the FIAF gene can significantly reduce body fat deposition. LPL is the key regulatory factor of fatty acid released from lipoprotein in skeletal muscles, the heart, and adipocytes. Under physiological conditions, GM can inhibit FIAF gene expression, promote LPL expression, and decrease TG accumulation in the cells[50,53,54]. Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a regulatory factor that serves in maintaining energy balance in the cells, playing a vital part in energy balance. AMPK can be directly phosphorylated by acetyl-CoA carboxylase, which promotes fatty acid oxidation in the tissue and further reduces fat deposition. GM disruption can lead to a reduction in the levels of AMPK in skeletal muscles and the liver, which subsequently leads to inhibition of fatty acid oxidation and excessive accumulation of fat in the liver [50]. Besides, the GM can induce or inhibit angiopoietin-like protein 4 (AGTPL-4) through bile acids to influence LPL activity, thereby affecting fat deposition inside the liver and steatosis outside the liver[42]. A study[55] revealed that giving mice fodders rich in saturated fatty acids, cholesterols, and sugar could increase lipid accumulation in their livers and cause a significant rise in the relative abundance of *Firmicutes* in mice's intestines. *Firmicutes* are important in the fermentation of resistant starch and dietary fibers and for energy use. The fermentation products are present in the form of SCFAs. On the one hand, SCFAs are essential energy substances of intestinal epithelial cells, which can enhance energy production by promoting sugar and fat synthesis[56]. On the other, SCFAs can alter fatty acid oxidation by inhibiting AMPK, leading to the accumulation of fatty acids in the liver[57]. Moreover, SCFAs are the ligands of G protein-coupled

receptors 41 (GPR41). After conjugating to GPR41, SCFAs can mediate leptin production by stimulating GRP41 in mouse adipocytes to regulate energy metabolism[58].

IR

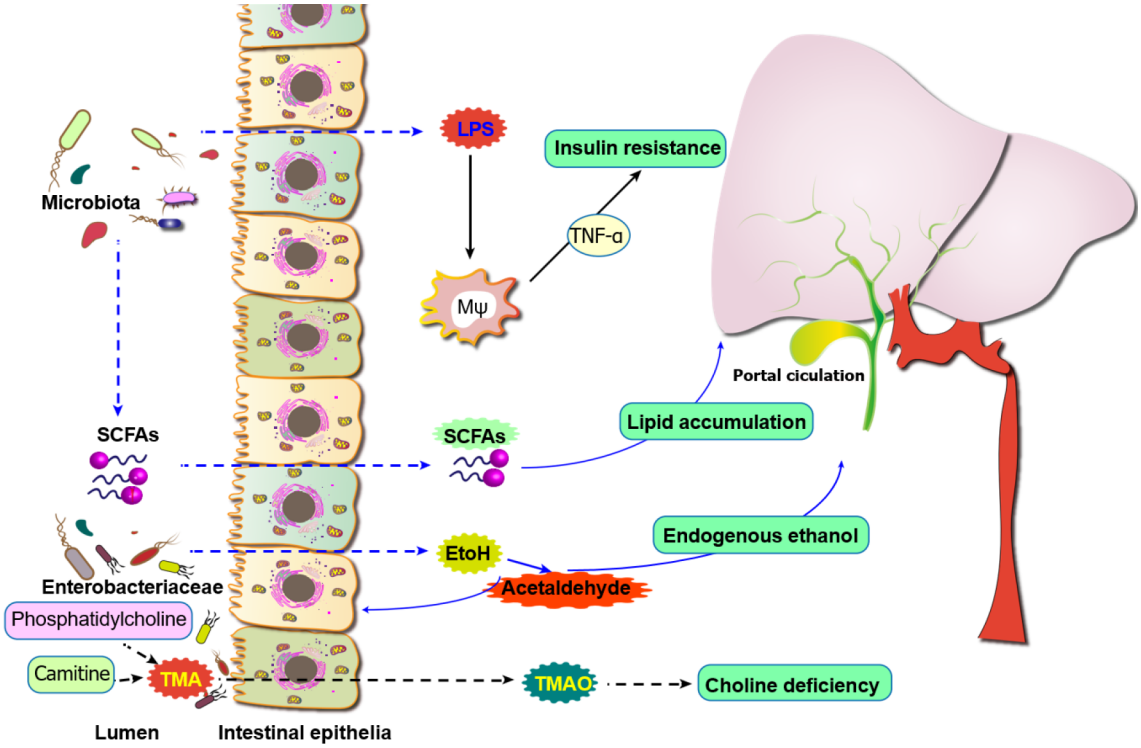
Insulin is an important hormone that regulates the steady state of glucose levels in the body. The activation of insulin receptors through phosphorylation initiates the body's biological response, inclusing increased glucose transport in skeletal muscles and adipose tissues, glycogen synthesis, and lipogenesis. IR reduces the biological effect of insulin, causing hyperinsulinemia (HIS). In turn, HIS leads to abnormal glucose transport and increased glycogenolysis and fatty acid synthesis, promoting MAFLD. Therefore, IR is one of the vital causes of MAFLD[59]. The composition and relative abundance of GM bacteria differ between obese individuals and those who are slim. The GM of obese individuals is mainly composed of *Firmicutes* and *Actinobacteriota, while Bacteroidete are* also present but less predominant. As mentioned above, *Firmicutes* with high relative abundance can enhance energy consumption by utilizing more indigestible substances in the intestinal content[60]. An imbalanced ratio of gut bacteria can also disrupt intestinal permeability, leading to increased absorption of LPS. Administration of antibiotics in high-fat diet (HFD) mice has been shown to decrease blood LPS concentration. Furthermore, this reduction in blood LPS concentration contributes to a decrease in adipose tissue inflammation and oxidative stress, which helps prevent adipose tissue hypertrophy and improves glycolipid metabolism parameters of HFD mice[61].

LPS are cell wall components found in gram-negative bacteria and are considered to be a key trigger of IR. Studies[62, 63] have shown that IR caused by intestinal endotoxins is mainly mediated by Toll-like receptor 4 (TLR4). In other words, LPS can activate the TLR4 located on the surface of insulin target cells. TLR4 can stimulate hepatocytes to produce inflammatory factors. Stimulating the production of proinflammatory kinase (*e.g.*, JNK) can inhibit the phosphorylation of insulin receptor substrates, thereby inhibiting the insulin signal transduction pathway. Moreover, GM disruption can accelerate the above processes to trigger IR. Reduced insulin sensitivity leads to a decrease in the rate of blood glucose utilization. HIS occurs when islet β cells are in the compensatory hypersecretory state. Further, HIS can disrupt the islet signaling pathway in the liver, forming a vicious circle. IR can alter the regulation of fat by insulin, enhancing steatosis and increasing free fatty acids in the serum. Fatty acids can cause hepatotoxicity. On one side, they can trigger MAFLD *via* various mechanisms, such as causing mitochondrion swelling to increase their permeability, inflammatory invasion, hepatocyte degeneration and necrosis, and induction of cell apoptosis[54]. On the other hand, the liver can transform free fatty acids into TG. Excessive fat will be deposited in the liver when the fat synthesized in the liver exceeds the hepatocyte's ability for oxidative utilization and synthetic lipoprotein transport, thereby promoting the development of MAFLD[61].

Increased endogenous ethanol

The GM can produce and metabolize ethanol. In the relatively hypoxic environment of the intestine, pyruvic acids produced through carbohydrate decomposition can be metabolized by the GM into acetaldehyde, which is further reduced into ethanol[64]. When there is intestinal bacteria overgrowth (Small intestinal bacteria overgrowth often exists in MAFLD) or excessive carbohydrate intake, ethanol metabolism mediated by the GM becomes active[65]. A current study[66] suggested that the primary product of *Enterobacteriaceae* (*e.g. Escherichia*) metabolism is ethanol. Other GM, such as *Bacteroides*, *Bifidobacterium,* and *Clostridium*, may also produce ethanol. The ethanol metabolized by GM is also called endogenous ethanol. Under normal conditions, the liver efficiently eliminates endogenous ethanol from the bloodstream of the portal vein through the action of liver alcohol dehydrogenase, catalase, and the ethanol oxidation system. However, in the intestines of MAFLD patients, the abnormal increase of ethanol-producing bacteria promotes and increased production of ethanol. Some patients with MAFLD have a preference for carbohydrates. Due to these two factors, reactive oxygen species are constantly provided to the liver, inducing liver oxidation and triggering inflammation, which is the "second strike" to the liver[66]. A study on children with MAFLD[67] revealed that the relative abundances of *Gammaproteobacteria* and *Prevotella* in these children were significantly higher than in healthy children. For this reason, the production of endogenous ethanol was also distinctly enhanced. Besides, an animal experiment[68] proved that administering antibiotics to alter the GM could reduce the ethanol concentration of the air exhaled by obese mice. A similar conclusion was also confirmed in NASH patients[69], as the air exhaled by NASH patients also had higher ethanol concentrations than healthy individuals. Besides, it was observed that the relative abundance of *Escherichia* in the GM increased significantly, which also confirmed that the increase in endogenous ethanol caused by GM disruption is related to MAFLD.

GM disruption leads to an increase in the relative abundance of bacteria producing ethanol, thereby increasing the ethanol content in the intestines. Ethanol activates various cytokines in the intestinal epithelial cells to increase intestinal wall permeability. Meanwhile, ethanol and acetaldehyde, which are metabolized products, enter the liver through the portal vein. These products can either directly stimulate hepatocytes or activate liver TLR to produce multiple cytokines and inflammatory mediators, resulting in inflammatory liver injury. In addition, the acetaldehyde produced by ethanol through intestinal metabolism can damage the expression of tight-junction proteins between intestinal epithelial cells to alter the intestinal barrier function, leading to bacterial translocation and endotoxemia (Figure 1).



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Figure 1 Gut microflora can affect several factors related to the development of metabolic-associated fatty liver disease. These effects lead to the production of free fatty acids, insulin resistance, and impaired bile secretion in the liver, respectively. In addition, changes in intestinal microflora may lead to increased intestinal permeability, and microbial-derived compounds are transferred from the intestine to the liver through the portal vein, resulting in changes in pro-inflammatory signals, metabolism, and toxicity. Finally, ethanol and its toxic derivative acetaldehyde aggravated hyperoxidative stress and choline deficiency in hepatocytes. EtOH: Ethanol; LPS: Lipopolysaccharides; SCFAs: Short chain fatty acids; TNF-α: Tumor-necrosis factor; TMAO: Trimethylamine N-oxide.

THE INFLUENCE OF GM ON RELEVANT SIGNALING PATHWAYS

FXR signaling pathway

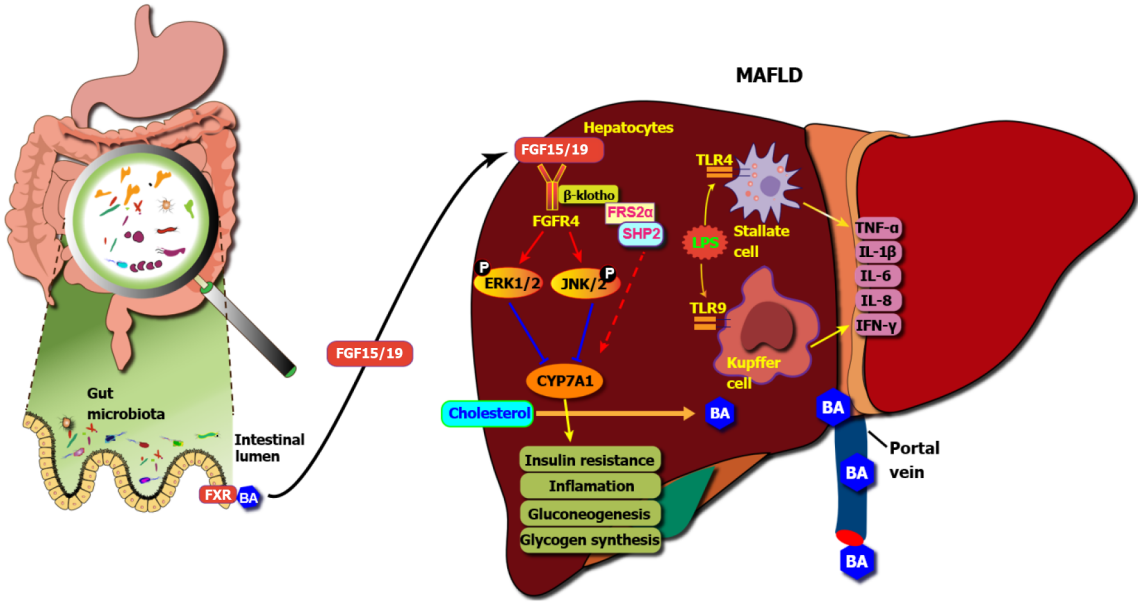
The FXR signaling pathway is one of the members of the nuclear receptor superfamily, and FXR's primary function is to regulate bile acid metabolism and enterohepatic circulation. The synthesis, metabolism, and reabsorption of bile acids are regulated by the negative feedback of FXR-relevant signaling pathways in the liver and ileum. Activating FXR can adjust the metabolic state of blood fat, blood glucose, and cholesterol and improve IR[70,71]. Obeticholic acid is an FXR agonist, which inhibits bile acid synthesis and enhances bile salt excretion through the FXR/FGF15/19 signaling pathways, thereby reducing bile acid reabsorption by the liver. Meanwhile, obeticholic acid can regulate the GM, improve intestinal mucosa barrier function, reduce inflammation, decrease the production and translocation of intestinal endotoxin, maintain gut-liver axis balance, and alleviate liver inflammation[70]. GM can also activate FXR in various ways (*e.g.*, *via* increasing fatty acid oxidation). Activated FXR improves glucose metabolism by inhibiting gluconeogenesis and glycogenolysis, reducing fat synthesis, and enhancing skeletal muscle insulin sensitivity. GM disruption can inhibit the transduction of the FXR signaling pathway, leading to an escalation of fatty acid synthesis and the generation of lipid toxicity. This, in turn, further deteriorates hepatic steatosis and promotes the occurrence and progression of MAFLD[72-74].

GPBAR signaling pathway

The mechanism of the GPBAR signaling pathway is similar that of FXR, and these two pathways are closely related to each other[75]. After reabsorption, bile acids induce ileal cells to secrete FGF19 by conjugating to the FXR and GPBAR of ileal cells. FGF19 further conjugates to FGFR4, which reduces CYP7A1 gene expression by activating the JNK and ERK signaling pathways. Therefore, bile acid synthesis is inhibited in negative feedback[23]. GM disruption can alter FXR expression and the transduction of the GPBAR signaling pathway, leading to the production of proinflammatory factors. For example, GPBAR can activate cyclic adenosine monophosphate and epidermal growth factor receptor kinase pathways, resulting in the activation of protein kinase C, which leads to the activation of nuclear factor- κB (NF-κB). Activated NF-κB enhances the expression of numerous proinflammatory factors, such as Il-1β, IL-6, and TNF-α. As a result, this activates the inflammatory immune response of the liver, promoting the occurrence and progression of MAFLD[76,77].

TLR signaling pathway

TLR is essential in the gut-liver axis, especially in maintaining the intestinal mucosa barrier. A damaged intestinal mucosa



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Figure 2 Mechanisms showing the role of gut microbiota in metabolic-associated fatty liver disease. FXR: Farnesoid X receptor; TGR5: Takeda G protein-coupled; MAFLD: Metabolic-associated fatty liver disease; BA: Bile acid; LPS: Lipopolysaccharides; TNF-α: Tumor-necrosis factor alpha; TLR: Toll like receptor.

barrier leads to increased permeability, which induces GM transposition. Therefore, a growing number of bacterial metabolites, bacterial substances, and other compounds can enter the liver through the portal vein, causing inflammation, oxidative stress, and lipid deposition. This eventually leads to fat liver injury, which can progress rapidly to liver fibrosis, also called "intestinal leakage"[78]. Bacterial flora translocation increases the endotoxin level in the portal vein or the liver. Pathogen-associated molecular patterns accumulate in the portal vein circulation, promoting the development of liver inflammation[79]. Besides, the increased abundance of pathogenic bacteria caused by GM disruption (or distinct abnormal relative abundance of opportunistic pathogens) lead to the excessive production of LPS. Subsequently, LPS stimulates endothelial cell TLR4 and dendritic cell TLR9 and induces the production of inflammasomes (*e.g.*, NLRP3) and proinflammatory factors (*e.g.*, IL-1β). This further damages intestinal mucosa permeability and reduces liver insulin sensitivity, thereby increasing visceral and subcutaneous fat and promoting the occurrence and progression of MAFLD [80] (Figure 2).

Immunoregulation

Liver inflammation is the critical driving factor of MAFLD development, and the gut-liver immune axis plays a vital role in the process. LPS, peptidoglycan (PGN), and bacterial deoxyribonucleic acid (DNA) can be translocated into the liver through the injured intestinal barrier, causing immune cell hyperactivation. LPS can tale advantage of the injured intestinal barrier to enter the liver *via* portal blood flow and induce the activation of inflammasomes[81]. PGN is one of the components of the bacterial cell wall. It is a macromolecule polymerized by acetylglucosamine, acetylmuramic acid, and amino acid short-chain peptide, which plays a role in insulin tolerance[82]. PGN and TLR2 can activate relevant NF-κB and TNF-α signaling pathways after conjugating to nucleotide oligomerization domain (NOD) 1 or NOD2, resulting in liver inflammation. NOD1 can also detect nutrient overload by sensing changing in bacterial microorganisms and promoting the translocation of PGN to regulate the energy metabolism in the gut-liver axis[83]. Bacterial DNA can directly activate immune cells such as macrophages, natural killer cells, B lymphocytes, and dendritic cells. It can also conjugate to TLR9 inside lysosomes *via* endocytosis, activating the NF-κB pathway and secreting inflammatory factors, such as IL-1β, IL-6, and TNF-α[84].

The bacterial flora therapy of MAFLD

Antibiotics: Research[85] has shown that short-term use of antibiotics can reduce circulating endotoxins and serum transaminases, improving the clinical symptoms of MAFLD patients. Among the antibiotics, the application of rifaximin has received the greatest attention. After rifaximin treatment, the BMI index, transaminase level, and hepatic steatosis degree of MAFLD patients decreased significantly. Even clinical research revealed that rifaximin could reduce the fermentation of carbohydrates and sterols by altering the GM structure, lowering serum inflammatory factors, and improving IR. Antibiotics cocktail (ampicillin, neomycin, metronidazole and vancomycin) can regulate free and conjugated secondary bile acid levels to decrease liver inflammation[86], inhibit intestinal FXR to reduce hepatic steatosis [73], and inhibit the activation of liver macrophage to lower liver inflammation[87]. However, antibiotics play a dual role. Short-term antibiotic treatment can exert therapeutic effects, while long-term application may result in bacterial drug resistance and increase the risk of secondary infection.

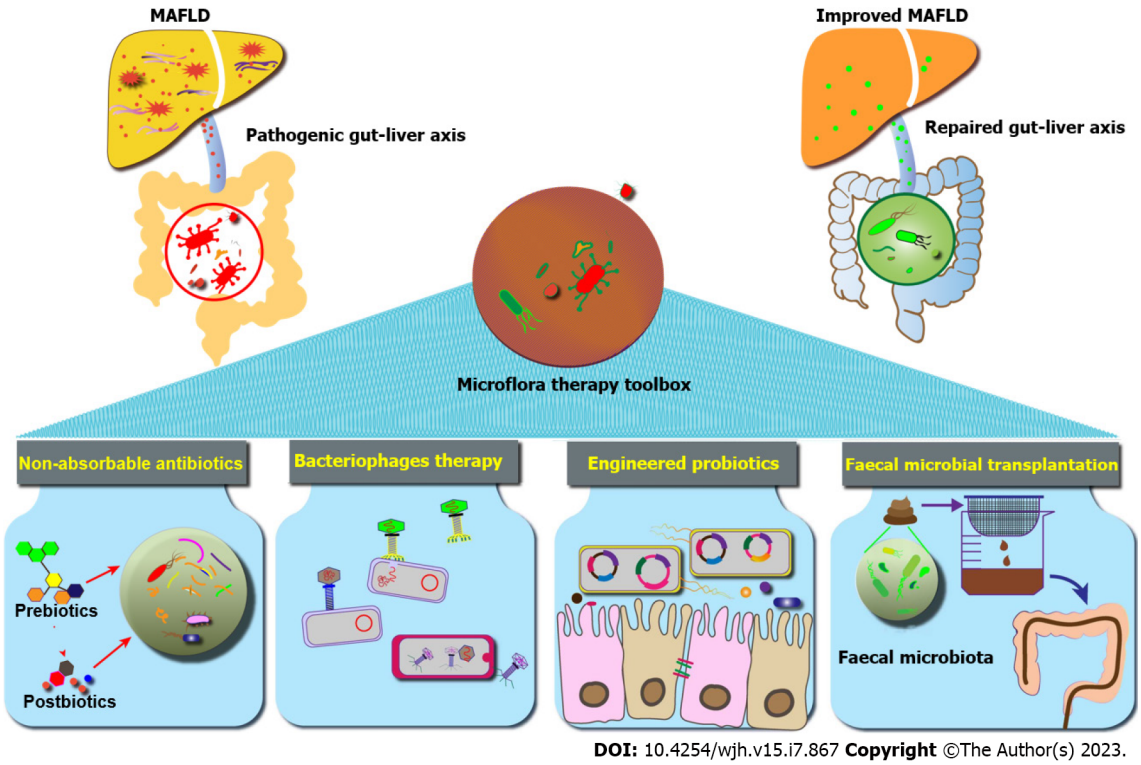


Figure 3 Therapeutic interventions for metabolic-associated fatty liver disease based on microbiota. Intestinal-centered therapy including antibiotics, bacterial metabolites, probiotics, engineered bacteria, bacteriophages, and fecal microbial transplantation can specifically interfere with intestinal microflora to re-establish the interface between the liver and the microbiome. MAFLD: Metabolic-associated fatty liver disease.

Probiotics: Clinical experiments on MAFLD patients[88] demonstrated that *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* could significantly reduce the patient's serum transaminase level. After taking *Lactobacillus bulgaricus* and *Streptococcus thermophilus* for three months, the transaminase level of MAFLD patients improved[89]. The application of *Clostridium butyricum* in clinic settings and MAFLD animal models has shown promising potential in preventing hepatic steatosis[90, 91]. In addition, multiple probiotic formulations present better therapeutic effects than one specific bacterial strain. For example, VSL3 (consisting of eight probiotic bacterial strains: *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus bulgaricus*) has better therapeutic effects than any single bacterial strain[90,92,93]. Studies targeting children with MAFLD [68,94,95] revealed that after VSL3 treatment, patients' fatty liver disease condition and BMI were distinctly improved. Follow-up research showed that the total quantity and activity of GLP-1 increased after VSL3 treatment. Meanwhile, VSL3 has been found to regulate plasmic peroxide, such as malondialdehyde and 4-hydroxynonenal, leading to therapeutic effects and relieving chronic liver disease. It achieves this by protecting the intestinal barrier and reducing endotoxemia and oxidative/nitroso stress. An animal experiment also verified[96] that probiotics can lower the weight of mice and improve GM disruption. Probiotic intervention can increase the adundance of intestinal anaerobic bacteria (*e.g.*, *Actobacillus* and *Bifidobacterium*). However, this decreases the abundance of *Escherichia* and *Enterococcus*, enhancing the integrity of the intestinal mucosa barrier. Highly expressed TLR4 in the liver improves serum inflammatory factors, liver histology, serum liver enzyme, metabolic index, and glucose metabolism. Evidence indicates that probiotics can decrease liver and systematic inflammation by inhibiting the LPS/TLR4 signal transduction inflammatory cascade.

Prebiotics: Fructo-oligosaccharides (FOS) is an indigestible fermentable dietary fiber compound that lowers liver oxidative stress and inflammation by improving intestinal permeability and the integrity of close junctions[97]. Lactulose is another prebiotic that enhances the growth of Bifidobacteria and Lactobacillus and inhibits endotoxic gram-negative bacteria. After taking lactulose for six weeks, the inflammation and liver injury of HFD obese mice was reduced, which was related to lowered LPS level in the circulation[98]. Clinical research[99] has shown that the serum ALT and AST levels of NASH patients decreased significantly after receiving Bifidobacteria and FOS treatments. Combining multiple probiotic bacterial strains (Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacteria breve, Bifidobacteria longum, and Streptococcus thermophilus) and FOS in combination with lifestyle interventions were more beneficial than lifestyle changes alone for MAFLD patients[100]. Combining Bifidobacteria longum and FOS with lifestyle interventions can significantly decrease NASH activity index and liver fat accumulation[101]. The result of a meta-analysis revealed that combining prebiotics could distinctly lower hepatic steatosis and the levels of ALT, AST, LDL, TG, and TC. Moreover, it was also helpful for reducing levels of inflammatory factors such as TNF-α and IR[102]. However, the administration of inulin diet, a soluble fiber, to mice with TLR5 gene knock-out led to an increase in the mice's bilirubin level, indicating that excessive inulin intake may cause liver injury and

even liver cancer[103]. Research has also highlighted that acetate, the fermentation product of inulin in the colon, can provide excess substrate for fat synthesis in the liver, escalating the production of lipids in the liver[104].

Fecal microbiota transplantation

The earliest fecal microbiota transplantation (FMT) treatment can be traced back to the book called *A Handbook of Formulas for Emergencies (Zhou Hou Bei Ji Fang)* written by Ge Hong from the Eastern Jin Dynasty (266 A.D. - 317 A.D.) in China. In the book, FMT treatment, also called "Huang Long Soup," for food poisoning and severe diarrhea was first recorded. Later, Li Shizheng also recorded in *the Compendium of Materia Medica (Bencao Gangmu)* that FMT by oral administration can treat severe diarrhea, fever, vomiting, and constipation[105]. FMT in modern medicine started in the year 1985. Ben Eiseman performed FMT by enema for patients with severe pseudomembranous colitis using feces from the patient's family member, and three out of the four patients were cured[106]. FMT was officially written into the clinical guidance for recurrent *Clostridium difficile* infection treatment in 2013[107]. As research progresses, numerous pieces of evidence support the potential efficacy of FMT in treating GM-related liver disease and metabolic disorders such as MAFLD. Studies have shown[108,109] that transplanting the GM of slim or obese mice can induce the recipient to have a phenotype similar to that of the host. The bacterial flora from slim mice can make obese mice lose weight. Six weeks after overweight patients with metabolic syndrome received bacterial flora from the slim individuals, the sensitivity of their liver and peripheral insulin was significantly enhanced[110]. Several studies have demonstrated[111-114] that the therapeutic effects of FMT on patients with T2DM and ulcerative colitis were related to GM steady state, normal blood fat level, and IR improvement. Feces from HFD-responsive and non-responsive mice were transplanted into germ-free mice. Mice receiving bacterial flora from the responsive group developed steatosis and exhibited increased relative abundance of *Barnesiella* and *Roseburia*. In contrast, the non-responsive group showed an increased relative abundance of *Allobaculum* in their bacterial flora[55]. In addition, FMT could significantly restore the GM disruption in NASH mice models induced by HFD by increasing the relative abundance of probiotics (*e.g.*, *Christensen* and *Lactobacillus*) and mitigate endotoxemia, hepatic steatosis, and inflammation[115]. In a RCT admitting 75 MAFLD patients, Xue *et al*[116] divided the patients into an FMT group (47 individuals) and a non-FMT group (28 individuals). The patients from the non-FMT group took oral probiotics, while the FMT group received three FMT enemas within three days. Both groups received a healthy diet and conducted exercise regularly for over 40 min. After treatment for one month, it was found that FMT lead to a reduction in liver fat deposition by improving the GM disruption, lowering the incidence of fatty liver disease. Moreover, the GM reconstruction effect of FMT on thin-type MAFLD patients was better compared to obese MAFLD patients. FMT can be administered through various methods to meet the requirements of different patients, including *via* nasogastric tubes, nasojejunal feeding tubes, gastroscopes, coloscopes, colonic catheters, retention enema, and capsules. However, FMT may pose certains risks. For example, the GM condition of different providers may affect the therapeutic effect, infection may occur during the transplantation, and it is uncertain how the GM can be effectively colonized in the patient's intestine. All these problems require further exploration.

Phage therapy

Phages are viruses that specifically infect and kill bacteria. They have the ability to adapt and evolve, enabling them to overcome the developing defensive mechanism of bacteria. Phages do not have the same mechanism as antibiotics. Thus, antibiotic resistance does not affect phages, and bacteria with high antibiotic resistance can still be inhibited by phages [117]. By studying the changes in bacterial composition and relative abundance, bacteria can be targeted for eradication using phages specific to that bacteria, after determining the mechanism by which a specific bacterium affects the onset or progression of MAFLD or whether the two are causally related. Its adverse effects on MAFLD can be eliminated without affecting the normal function of other bacteria[118]. For example, using phages for the targeted eradication of high-alcohol-producing *Klebsiella pneumoniae* (HiAlc-Kpn, found in over 60% of MAFLD patients, can produce enormous amounts of alcohol and is the leading cause of the bacterial auto-brewery syndrome) can effectively mitigate the bacterial auto-brewery syndrome of MAFLD model mice[119]. An analysis was conducted on feces samples of NASH patients and healthy people. The *Enterococcus faecalis* level in the feces samples of alcoholic hepatitis patients is 300 higher than in healthy subjects. The relative abundance of *Enterococcus faecalis* significantly increased in approximately 80% of feces samples from patients with alcoholic hepatitis. Further analysis showed that a gene which can encode cytolysin existed in approximately 30% of *Enterococcus faecalis* species[118]. Alcoholic hepatitis mouse models were built using a high-alcohol diet. After being transplanted with a feces sample containing cytolysin, these alcoholic hepatitis mice developed specific hepatocyte injury and died. However, alcoholic hepatitis mice transplanted with samples without cytolysin did not develop liver injury. Targeting the phages specific to *Enterococcus faecalis* can effectively reduce the abundance of this bacterium, especially strains producing cytolysin. This targeted approach has shown promising results in lowering the degree of liver injury in alcoholic hepatitis mice, serving as a protective measure[118]. This is a critical attempt at phage therapy in the gut-liver axis, indicating the potential application value of phage therapy in MAFLD treatment (Figure 3).

In addition to the antibiotics mentioned above, probiotics, FMT, and other therapies that directly target GM, there is growing evidence that modifying dietary habits and increasing physicial exercise both improve MAFLD and the GM disorder in MAFLD. High-fat and high-sugar diets can change the GM structure in different ways[120]. HFD mice have a greater abundance of *Firmicutes,* while mice with a high-sugar diet have a lower relative abundance of *Firmicutes* and *Bacteroides*, which is closely related to the onset and development of MAFLD. High-fructose intake will up-regulate the re-synthesis of fat and inhibit the oxidation of fatty acid β. It can cause hepatic steatosis, induce inflammation through the TLR signaling pathway, and release inflammatory factors. High-fructose intake can also reduce insulin sensitivity. HFD decreases the number of intestinal probiotics *Bifidobacteria* and bacteria that produce butyric acid. It also enhances intestinal permeability, LPS translocation, and chronic systemic inflammation. High saturated fatty acid intake can lower

the GM diversity and increase the ratio of *Firmicutes* to *Bacteroides*, result in weight gain, and increase plasmic insulin and TG content[121]. In MAFLD mice induced by HFD, six-week HFD increases *Firmicutes* abundance and decreases *Bacteroides* abundance. The ratio of *Firmicutes* to *Bacteroides was* significantly enhanced, and the ratio was maitained till the end of the experiment. However, exercise can improve GM disorder resulting from a HFD. This is achieved by changing the ratio of the two bacteria *via* reducing *Firmicutes* abundance and increasing *Bacteroides* abundance[122]. Exercise distinctly alleviates GM disruption caused by HFD and restores the intestinal mucosa barrier function to a certain extent. The relative abundance of some GM reaches a level similar to normal rats. Meanwhile, exercise also significantly downregulated the expression of FXR and CD36 in the liver, indicating improvements in liver lipid metabolism[123]. Research combining exercise and lycium barbarum polysaccharide also found that aerobic exercise restores the close junction of the colon and ileum and improves intestinal mucosa permeability by enhancing ZO-1 expression. Relevant indicators such as intestinal LPS, liver LPS binding protein, and inflammatory factors are also downregulated[123]. However, research on the effects of exercise on GM in MAFLD patients is relatively sparse. Although animal experiments have demonstrated that exercise can improve MAFLD symptoms by restoring GM, more clinical experiments are needed.

CONCLUSION

Generally, with the increase in metabolic diseases such as obesity, the incidence of MADLF also increases yearly. Many clinical studies and animal experiments have demonstrated that the GM is vital in the onset and development of MAFLD, but most studies remain at a phenomenal level. There is no definitive conclusion regarding the "cause" and the "effect" of GM disorder and MAFLD. However, the efficacy of treatments targeting GM, such as probiotics and FMT, has been confirmed in both clinical applications and basic research without reporting severe adverse events. There is also a wide range of opinions on how many bacterial flora therapies are used to improve MAFLD. A healthy lifestyle and good dietary habits are still the foundation of MAFLD treatments. Comprehensive therapies combing bacterial flora therapy certainly have good prospects, but continuous efforts are still needed to design high-quality long-term clinical experiments. Meanwhile, histological studies combining multi-omics, such as metagenome, metabolome, and proteome, are needed. In the future, it is expected that GM can better help in the diagnosis, treatment, and prognosis evaluation.

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FOOTNOTES

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REVIEW

Shifting perspectives in liver diseases after kidney transplantation

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Abstract

Liver diseases after kidney transplantation range from mild biochemical abnormalities to severe hepatitis or cirrhosis. The causes are diverse and mainly associated with hepatotropic viruses, drug toxicity and metabolic disorders. Over the past decade, the aetiology of liver disease in kidney recipients has changed significantly. These relates to the use of direct-acting antiviral agents against hepatitis C virus, the increasing availability of vaccination against hepatitis B and a better understanding of drug-induced hepatotoxicity. In addition, the emergence of the severe acute respiratory syndrome coronavirus 2 pandemic has brought new challenges to kidney recipients. This review aims to provide healthcare professionals with a comprehensive understanding of recent advances in the management of liver complications in kidney recipients and to enable them to make informed decisions regarding the risks and impact of liver disease in this population.

Key Words: Kidney transplantation; Viral hepatitis; Non-alcoholic fatty liver disease; Drug-induced liver injury; COVID-19

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Core Tip: Liver disease is a common complication after kidney transplantation and can present in a variety of forms, from asymptomatic biochemical abnormalities to fibrosis/cirrhosis/decompensation/malignancy. Early recognition and referral to a hepatologist are crucial for effective treatment, as they can otherwise lead to impaired quality of life and increased morbidity. Screening of kidney transplant recipients for liver disease, including viral disease, metabolic disorders and drug toxicity, should be prioritised by healthcare providers.

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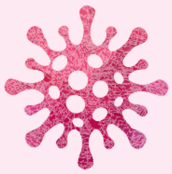
INTRODUCTION

The efficacy of kidney transplantation (KT) in the treatment of end-stage renal disease (ESRD) has been demonstrated by numerous studies, which have shown that KT is associated with significantly higher survival rates and better quality of life compared to dialysis. As such, KT is now considered the gold standard in the treatment of ESRD[1,2]. Prevalence of chronic kidney disease (CKD) is estimated at 13.4% (11.7%-15.1%) and is on the rise due to increasing rates of diabetes, arterial hypertension, obesity and ageing. This is reflected in the increasing rates of KT; 92532 KT performed in 2021, which is an estimated 40% increase from 2008[2,3]. Advances in organ procurement, surgical techniques, immunosuppression regimens targeting rejection, and prophylactic antibiotic therapies have enabled excellent short-term survival rates after KT, and long-term outcomes are steadily improving over time[4]. This has led to a prolongation of life expectancy of both the graft and the recipient, which is now appreciable well beyond the first year following transplantation[4]. Survival rates of KT recipients may be affected by allograft nephropathy and subsequent failure, as well as a variety of morbidities, including cardiovascular disease (CVD) and infections associated with immunosuppressive drugs [1]. In addition, the incidence of liver abnormalities after KT, which ranges from 20% to 50%, was recently demonstrated by Vieira *et al*[5] to have a significant impact on the survival and quality of life of these patients[5] .

Liver disease after KT occurs in a variety of forms ranging from asymptomatic biochemical abnormalities to severe hepatitis, cirrhosis or malignancy[6]. Causes are heterogeneous and mainly related to hepatotropic viruses, drug toxicity and metabolic disorders[6]. Genetic conditions, such as polycystic kidney disease, which lead to the need for KT are also associated with polycystic liver disease[6]. Significant advances in understanding and treating liver disease in kidney transplant recipients have been made in the last decade. The development of direct-acting antiviral drugs (DAAs) for the treatment of hepatitis C virus (HCV) infection has led to a marked improvement in the treatment of HCV-related liver disease. Hepatitis E has been identified as a causative agent of chronic hepatitis and cirrhosis following solid organ transplantation (SOT). In addition to these advances, the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its impact on SOT has highlighted the need for further research in this field. The impact of coronavirus disease 2019 (COVID-19) on liver function in kidney transplant recipients is not yet fully understood, and the long-term consequences of the virus on liver health in this population are still the subject of ongoing investigation. In addition, the prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing worldwide and it is also taking its toll in transplant population due to changes in eating habits, sedentary lifestyle and unavoidable effect of life-long immunosuppression[7]. This review aims to provide healthcare professionals with a comprehensive understanding of recent advances in the management of liver complications in KT recipients, allowing them to make informed decisions about the risks and impact of liver disease in this population (Figure 1).

NON-ALCOHOLIC FATTY LIVER DISEASE

NAFLD is a complex and multifactorial metabolic disorder influenced by a variety of genetic, environmental and lifestyle factors. The pathogenesis of NAFLD is characterised by a cascade of events that result in the accumulation of fat in the liver (hepatic steatosis), which can trigger an inflammatory response that further contributes to liver damage and leads to more severe liver diseases such as cirrhosis and hepatocellular carcinoma (HCC). However, the mechanisms underlying this progression remain unclear and are currently the subject of intense research efforts. Recent studies have highlighted the importance of gut microbiota dysbiosis, oxidative stress and immune system dysfunction in the development and progression of NAFLD[8]. Dysbiosis in the gut microbiota, characterised by a decrease in beneficial bacteria and an increase in harmful bacteria, can lead to increased gut permeability, endotoxaemia and inflammation, contributing to the development of NAFLD. Oxidative stress, an imbalance between reactive oxygen species and antioxidant defences, can also promote the progression of NAFLD by damaging hepatocytes and increasing the release of pro-inflammatory cytokines. Immune dysfunction, characterised by an imbalance between pro-inflammatory and anti-inflammatory metabolic pathways, can lead to an accumulation of immune cells in the liver, thus promoting the progression of NAFLD. In addition, genetic factors have been identified as important contributors to the development and progression of NAFLD. Variants in genes involved in lipid metabolism, insulin resistance and inflammation have been linked to an increased risk of NAFLD[8]. However, the interactions between genetic and environmental factors are complex and not

	NAFLD	Viral disease	DILI	ADPKD
				
Progress achieved to date	Diagnostic tools: Non-invasive methods - transient elastography and magnetic resonance elastography These methods can be used without the need for a liver biopsy, which can be risky for patients Risk stratification: Factors that increase the risk of NAFLD progression; obesity, insulin resistance and immunosuppressants (steroids and CNIs) Patient outcomes: Early detection and treatment of NAFLD improve outcomes	DAAs are highly effective drugs with SVR rates of over 97% of patients and good safety profiles HCV D+/R- kidney transplantation Entecavir and tenofovir suppress HBV replication and reduce the risk of liver disease progression HEV may lead to chronic hepatitis and cirrhosis in SOT COVID-19 can lead to various forms of liver injury	Mechanisms of injury: Progress in understanding the mechanisms by which drugs can cause liver damage, *e.g.* mitochondrial dysfunction or oxidative stress- leading to liver cell damage Risk factors: Age, gender, pre-existing liver disease	Disease-modifying therapies: Tolvaptan, a V2R antagonist
High priority topics	Management: Unmet need for effective and safe treatment strategies, including complex medical history, immunosuppressive medications and renal function	Safe and effective HEV vaccine A more equitable distribution of HCV- positive donor organs, combined with increased availability and affordability of DAAs, is essential to expand the donor pool and improve access to life- saving treatments for patients on the waiting list	Accurate and reliable biomarkers for diagnosis and monitoring	Tolvaptan safety, efficacy, and cost- effectiveness in KT recipients Optimal timing and appropriate selection criteria for transplantation

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Figure 1 Progress achieved to date and high priority topics in four main types of liver diseases affecting patients after kidney transplantation. NAFLD: Non-alcoholic fatty liver disease; CNIs: Calcineurin inhibitors; DAAs: Direct acting antivirals; HCV D+/R-: Hepatitis C virus donor positive/recipient negative; COVID-19: Coronavirus disease 2019; HEV: Hepatitis E virus; DILI: Drug induced liver injury; V2R: Vasopresin receptor 2; KT: Kidney transplantation; ADPKD: Autosomal dominant polycystic kidney disease.

fully understood, and more research is needed to fully elucidate the role of genetics in NAFLD.

NAFLD is now considered the most frequent chronic liver disease worldwide, with an estimated 25%-30%of the general population affected, particularly due to the rising prevalence of insulin resistance, obesity and hypertension[7]. Because type 2 diabetes mellitus (T2DM), obesity and dyslipidaemia often coexist with NAFLD, it is considered a hepatic manifestation of the metabolic syndrome[9]. While some experts suggest replacing the term NAFLD with metabolic-associated liver disease (MAFLD) to emphasise the importance of metabolic dysfunction in development and progression of the disease, the term is not yet universally accepted, as it can occur in patients without metabolic disorders[10]. In KT recipients, the high disease burden of NAFLD carries an increased risk of graft dysfunction and patient mortality, making early detection and treatment essential to reducing its impact on transplant outcomes. The increasing prevalence of NAFLD worldwide has led to a growing need for effective diagnostic and therapeutic strategies. Non-invasive diagnostic tools such as imaging and serum biomarkers have been developed to assess the severity of NAFLD and monitor disease progression. Various pharmacological and lifestyle interventions have also been investigated for the treatment of NAFLD, including weight loss, exercise, insulin-sensitising agents and antioxidants[11]. However, the optimal treatment for NAFLD remains uncertain, and more research is needed to find effective treatments that can prevent or reverse disease progression.

NAFLD and CKD

Patients with NAFLD and/or CKD share the same risk factors, including visceral obesity, arterial hypertension, prediabetes or T2DM, systemic insulin resistance, dyslipidaemia and low-grade inflammatory states[12]. Recently, several studies have shown that NAFLD is significantly associated with an increased prevalence of CKD. For example, the prevalence of CKD in patients with NAFLD ranged from 20% to 55%, while the prevalence of CKD in patients without NAFLD was 5% to 30%[13]. In addition, several retrospective and prospective cohort studies have found that the association between NAFLD and an increased incidence of CKD persists after adjustment for age, sex, obesity, hypertension and T2DM[14,15].

Impact of NAFLD on KT recipients

CVD is the main cause of mortality and graft loss after KT[16]. A meta-analysis of 16 studies found that patients with NAFLD had a higher risk of fatal and non-fatal cardiovascular events than patients without NAFLD (OR 1.64, 1.26-2.13) and that patients with severe NAFLD had a higher risk of fatal and non-fatal cardiovascular events (OR 2.58, 1.78-3.75) [17]. In addition, traditional risk factors (T2DM, obesity, hypertension, dyslipidaemia) for CVD and NAFLD may be exacerbated by immunosuppressive drugs, including calcineurin inhibitor and steroids in renal recipients[18]. In 2017, Kemmer and Buggs[19] reported the presence of NAFLD on ultrasound in 22% of KT candidates. Grupper *et al*[20] conducted a study of 341 consecutive KT recipients and showed that 36.4% of kidney recipients had sonographic evidence of NAFLD before transplantation. In the same study, recipients with NAFLD had a higher prevalence of new-onset diabetes before transplantation, and NAFLD was independently associated with cardiovascular mortality after KT (HR 4.4), even after adjustment for known CVD risk factors. It is still unclear whether NAFLD is associated with renal graft dysfunction. Grupper *et al*[20] found no correlation between graft function and NAFLD. However, from the studies already mentioned, NAFLD is significantly associated with a higher incidence of CKD in non-transplanted patients. This means that NAFLD and reduced graft function may also be the case in KT recipients[20]. The results of the above studies emphasise the need to diagnose NAFLD in KT recipients and to undertake aggressive risk factor modification early. The treatment of NAFLD in KT recipients is particularly challenging due to the complex interplay of metabolic disorders, immunosuppression and other comorbidities. Immunosuppressants used to prevent allograft rejection may exacerbate the metabolic disorder and contribute to the development and progression of NAFLD. Therefore, individualised treatment strategies that take into account each patient's unique needs are critical to achieving optimal outcomes according to KT. In addition, physicians caring for patients before and after KT should refer patients to a hepatologist as soon as NAFLD is diagnosed.

VIRAL DISEASES

Hepatitis B virus

The natural course of hepatitis B virus (HBV) infection is a dynamic process that reflects the interaction between HBV replication and the host immune response[21]. In immunocompetent adults, HBV infection is usually acute and self-limiting with more than 90% of patients achieving hepatitis B surface antigen (HBsAg) seroconversion within 6 mo, indicating natural resolution of the infection; however, infection in infancy or childhood often leads to chronicity[21]. Today, an estimated 240 million people are affected by chronic HBV infection[21]. Patients with detectable HBV DNA, especially those over 30 years of age and with a family history of HCC and cirrhosis, are typically considered for antiviral therapyand require lifelong treatment after KT[22,23]. Although modern antivirals such as entecavir or tenofovir result in viral suppression in almost all patients, treatment is long-term, possibly lifelong, and minimises the possibility of seroconversion[24]. Therefore, in chronically infected patients without signs of hepatitis, liver fibrosis or cirrhosis, without extrahepatic manifestations of HBV and with low viral replication rates (as determined by HBsAg and HBV DNA), treatment is not usually recommended by professional society guidelines[22,23]. In immunosuppressive states, *e.g.* due to chemotherapy or after organ transplantation, patients with cleared or inactive HBV may experience an abrupt increase in viral replication, which is called HBV reactivation.

Symptoms of HBV reactivation can range from asymptomatic to severe acute hepatitis, progression to chronic inflammation and fibrosis or HCC, and even acute liver failure. Where HBV is endemic, reported HBV reactivation rates with immunosuppression are as high as 41.5% (resolved HBV) and 70% (chronic HBV infection)[25,26]. As HBV prevalence in KT recipients ranges from 2.2 to 20.9% it is crucial that healthcare professionals understand their patient's status to determine appropriate monitoring or antiviral prophylaxis measures to prevent reactivation[27].

The patient with resolved HBV infection (HBsAg-negative, anti-HBc IgG positive recipient): Up to 30% of KT candidates may have resolved HBV infection [defined as HBsAg-negative and hepatitis B core antibody (anti-HBc) - positive], and 1.4%-9.6% may experience HBV reactivation after KT[27]. The American Association for the Study of Liver Diseases guidelines recommend monitoring every three months for the first year (alanine transaminase and HBV DNA) and antiviral prophylaxis only as an alternative[24]. However, since the risk factors for HBV reactivation are not precisely known, it is not clear which patients should be treated and which should be monitored. In a study by Mei *et al*[28] 52 patients with resolved HBV infection were retrospectively analysed. Five (9.6%) cases of HBV reactivation occurred, and anti-HBcAg titre (P = 0.042) and age (P = 0.037) were identified as risk factors for HBV reactivation. Interestingly, ATG treatment, steroid pulse doses and low-dose rituximab were not associated with HBV reactivation[28]. Shaikh *et al*[27] found that only 3.4% of KT recipients who did not receive antiviral prophylaxis experienced HBV reactivation, and delayed graft function was identified as a significant risk factor. Still, there were no significant adverse graft-related outcomes among those who experienced reactivation[27].

The patient with chronic HBV infection (HBsAg-positive recipient): Chronic HBV is defined by persistent HBsAg in serum for at least 6 mo. Although current guidelines recommend prophylactic treatment for HBsAg-positive patients if they require immunosuppressive therapy for transplantation, most evidence is based on patients undergoing chemotherapy[22,23]. A systematic review and meta-analysis by Thongprayoon *et al*[29] included a total of 87623 KT patients and found significant association between HBsAg-positive status and poor outcomes, including mortality (pooled OR = 2.48; 95%CI: 1.61-3.83) and allograft failure (pooled OR = 1.46; 95%CI: 1.08-1.96). There was also a significant negative correlation between study year and risk of allograft failure, suggesting a possible improvement in

patient and graft survival in HBsAg-positive recipients over time[29]. A recent study by Mo *et al*[30] confirmed high rates of viral reactivation in a real-world study of HBsAg-positive KT recipients, with inappropriate antiviral agents (all other than lifelong prophylaxis) (HR = 7.34, 95%CI 1.51-35.69, P = 0.01) and high levels of HBV DNA (≥ 1000 IU/mL) pre-transplant being the main risk factors (HR = 4.39, 95%CI 1.08-17.81, P = 0.04). However, the study found no difference in patients' or graft outcomes between patients who experienced reactivation and those who did not[30].

The kidney graft from donors with resolved HBV infection (HBsAg-negative, anti-HBc IgG positive donor): Donors with resolved HBV infection may alleviate organ shortage in KT. A study by Yamada *et al*[31] found a low risk of reactivation - 45 cases of KT from donors with resolved HBV infection to HBV-naive recipients were analysed, and one patient (2.2%) became seropositive for anti-HBc, and one patient (2.2%) had detectable HBV-DNA levels after transplantation. In the same study, the presence of covalently closed circular DNA in transplanted organs from donors with resolved HBV infection and the capability of HBV replication in kidney cell lines was demonstrated[31]. Accordingly, further research is needed to fully evaluate the safety and efficacy of this approach.

Hepatitis C virus

HCV infection is a global health problem affecting an estimated 71 million people worldwide[32]. The prevalence of HCV tends to be higher in patients with ESRD and in KT recipients than in the general population[33]. Although transplantation of HCV-infected patients offers a clear survival advantage over dialysis, HCV positivity has often been a barrier to KT in the past. Possible reasons for this include concerns about impaired survival of grafts and patients associated with certain HCV-related causes, such as increased rates of liver fibrosis and glomerulonephritis[34]. Highly effective, all-oral DAAs have revolutionised HCV treatment with cure rates of > 95%[35]. DAAs not only improve outcomes in HCV-positive KT patients, but also open new opportunities to increase scarce graft availability by safely transplanting HCV-positive organs into HCV-negative recipients[36]. The focus of interest today is the efficacy and safety of DAAs after KT and the challenges of using HCV-positive grafts in HCV-negative recipients.

DAA therapy for KT recipients: Sofosbuvir, a second-generation DAA approved in 2013, paved the way for efficient interferon/ribavirin-free treatment of HCV. Today, DAAs have proven their efficacy and safety both in clinical trials and in practice[37-39]. Chute *et al*[37] reported sustained viral response (SVR) rates of 97% in 418 of KT recipients, most of whom were treated with sofosbuvir-based therapies[37]. In the phase-3, open-label, single arm MAGELLAN-2 study, which evaluated a 12-wk course of the pangenotypic regimen of glecaprevir/pibrentasvir SVR was achieved in 98% of patients[38]. In addition to confirming the efficacy of the different DAA regimens used (23 patients, 100% SVR rate), Alkadi *et al*[40] found that there were no significant changes in renal function or calcineurin inhibitor levels during or after therapy[40]. In another study, DAAs were found to reduce HCV prevalence from 1.97% to 0.43% among recipients of KT when used over a five-year period[41]. Current DAA regimen guidelines for KT recipients are readily available through the American Association for the Study of Liver Diseases/Infectious Diseases Society of America (AASLD/IDSA) and the European Association for the Study of the Liver (EASL) guidelines[35,42].

ESRD and advanced liver disease: The prevalence and clinical-epidemiological profile of HCV infection in ESRD have changed over time. In particular, patients today tend to be older than in the past; more deceased donors are used for transplantation; there are fewer co-infections with HBV, but a higher percentage of cirrhotic patients are treated. Additionally, decompensation was found to be more frequent in recent years as well as patient survival rate being lower than before[32]. Since cirrhosis is an important predictor of poor survival after KT, it is advisable sess the stage of liver fibrosis in all KT candidates. In patients with established cirrhosis and portal hypertension in whom antiviral HCV treatment fails or is not an option, combined liver and KT must be considered[43].

Utilisation of HCV-positive donors: One of the most important advances in the last decade to increase transplantation rates has been the practise of KT from HCV-positive donors to HCV-negative patients (HCV D+/R-), followed by DAA therapy. After the pioneering studies THINKER and EXPANDER, which demonstrated good graft function and found little evidence of adverse outcomes after HCV D+/R- KT, this practice steadily increased in the United States[44,45]. United Network for Organ Sharing (UNOS) data showed and found increasing rates (0.3% to 6.9%) of HCV D+/R- from 1/1/2017 to 12/12/2020[46]. A growing number of real-world studies confirm the safety and efficacy of the HCV D+/R- approach and highlight its advantages, including shorter waiting times, access to younger donors with excellent allograft function, and similar survival compared to HCV D-/R- KT[46,47]. Substantial additional administrative work may be required outside of clinical trials for insurance approval of DAA therapy. A study by Edmonds *et al*[47] found that the median time from KT to the first dose of DAA was 45 d[47]. However, even delayed initiation of DAA treatment, with a median of 70 d after KT, had no negative impact on SVR rates or liver histology[48].

Understanding gaps and benefits/risks of HCV D+/R- KT may lead to greater acceptance. The majority of patients are willing to accept HCV-positive organs after being educated, citing shorter waiting time, DAA efficacy and faster return to higher functional status[49]. However, Nguyen *et al*[50] revealed disparities in HCV D+/R- KT access based on race/ethnicity, gender, and education level; minorities were 15%-60%, women over 20%, and those with elementary school degrees or less half as likely to receive a HCV nucleic acid amplification technique (NAT) positive kidney compared to those with bachelor's degrees. These findings underscore unequal distribution of breakthrough treatments, which can take years before recognition or reporting occurs[50]. In conclusion, use of HCV-positive kidney grafts for HCV-negative recipients followed by DAA therapy is a potential solution to organ shortage that can improve quality of life of ESRD patients.

Hepatitis E virus

The understanding of hepatitis E virus (HEV) has evolved over the past decade. Genotypes (gt) 1 and 2 are restricted to developing countries and cause large epidemics *via* the faecal-oral route, while gt 3 and 4 are endemic zoonoses in high-income countries. Although HEV usually causes an acute and mild form of hepatitis in imunocompetent hosts, it can lead to more severe and long-lasting infections in individuals who have undergone a SOT[51]. This has become an increasingly important issue because HEV is now considered one of the primary causes of acute viral hepatitis and cirrhosis in transplant recipients. Data on the seroprevalence of HEV in haemodialysis patients are inconsistent, ranging from 0 to 44%, possibly due to significant differences between the geographical regions studied[52].

In 2008, the first report of chronic hepatitis due to gt 3 and 4 was published in SOT[53]. Subsequent studies showed that organ recipients are at increased risk of HEV infection, with prevalence ranging from 0.44% to 24% depending on the organ transplanted and the geographic region[54]. Interestingly, HEV seropositivity was significantly higher in transplant recipients compared to patients on the waiting list (24% *vs* 16.4%, $P = 0.042$)[55]. Still, it is possible that HEV infection is underdiagnosed in SOT recipients, as serological assays with low sensitivity are often used[56]. Clinical course is inconspicuous in most cases with mild but often persistent abnormalities observed in liver function tests[53]. To properly diagnose chronic HEV infection, serum or plasma samples and, if possible, stool samples must be examined using NATs [51]. High clinical suspicion of chronic HEV is important because undetected infection, especially with gt 3, can lead to rapid progression of liver fibrosis and in some cases decompensation and death[57].

KT recipients are particularly susceptible to chronic HEV infections, which can lead to serious complications such as earlier graft rejection. KT recipients with positive ELISA tests or PCR results for HEV are more likely than controls to experience earlier rejection[58]. It is known that HEV infection can cause hepatic and extrahepatic symptoms. A case study of a KT recipient with HEV and membranous nephropathy (MN) illustrates the possible causal relationship between the two conditions. Treatment with ribavirin (RV) as monotherapy proved effective in treating HEV infection and resulted in complete remission of the nephrotic syndrome. This suggests that RV may also have beneficial effects on MN *via* non-specific immunomodulatory mechanisms[59].

Although chronic HEV was originally defined as viral replication lasting longer than 6 mo, in an observational study of SOT recipients, clearance did not occur 3-6 mo after infection, but only within the first 3 mo. These results suggest that SOT patients who are viraemic for longer than 3 mo should be considered for treatment[60]. A recent study that investigated the efficacy of dose reduction of mycophenolic acid in eight KT patients diagnosed with chronic HEV infection showed that only one patient achieved HEV clearance, while most patients required antiviral treatment with RV. The study provided the clinical evidence that reducing mycophenolic acid therapy alone is not sufficient to control viral replication in transplant patients. In addition, rituximab has been identified as a risk factor for chronic HEV that is complicated to treat[61].

Meta-analysis data show that RV is a safe and effective treatment for chronic HEV infection in SOT recipients. RV induces a sustained virological response in 76% of patients and represents a first-line therapy for this patient group[62]. However, it is important to note that the guidelines recommend monitoring HEV RNA for at least six months after completion of RV therapy, as reinfection or relapse can occur even if the virus has been eliminated by therapy. This is especially true for immunosuppressed transplant patients, including those with positive anti-HEV IgG.

Trials in China have shown the efficacy of recombinant HEV genotype 1 vaccine (Hecolin®, Xiamen Innovax Biotech). However, the vaccine is not available outside China and its efficacy against other genotypes is unknown. Further studies in developed countries are needed to assess its efficacy, durability of immune response and cost-effectiveness before it can be recommended[63]. For now, avoiding contaminated food/water and thorough cooking are the only preventive measures.

COVID-19

Since identified in December 2019, SARS-CoV-2 has infected more than 660 million people to date[64]. SARS-CoV-2 causes COVID -19, which typically presents as an upper respiratory tract infection but can lead to severe pneumonia with acute respiratory distress syndrome and multiorgan failure[65]. The virus infects target cells by binding the spike surface glycoprotein (S) to angiotensin-converting enzyme 2 (ACE2), which is mainly expressed in cells of the alveolar epithelium type II[66]. However, it was also found in the heart, ileum, kidney, bladder and liver, with cholangiocytes being the major ACE2-expressing cell population, with a 20-fold higher expression rate than in hepatocytes[67]. This could potentially make the liver susceptible to SARS-CoV-2 virus, but evidence for specific viral hepatotropism is limited.

Abnormalities in liver function tests have been observed in up to 78% of hospitalized COVID-19 patients, with some individuals developing acute liver injury (ALI) and reports of liver failure due to SARS-CoV-2 infection[68,69]. Given the significantly higher expression of ACE2 receptors on cholangiocytes, one might generally expect a cholestatic laboratory pattern to predominate in patients with COVID-19, but this is not the case. Therefore, it has been hypothesised that liver injury is not primarily due to direct virus-induced damage to hepatocytes, but rather to indirect causes such as hepatotoxic drugs, systemic inflammatory responses and respiratory distress syndrome-induced hypoxia[70]. Some cases of acute de novo autoimmune hepatitis have also been reported following COVID-19, suggesting possible immunogenicity due to molecular mimicry of SARS-CoV-2[71]. Cholangiopathy resembling secondary biliary cholangitis, a possible chronic complication of COVID-19, has also been reported in critically ill patients[72]. In addition, the presence of cirrhosis among patients infected with SARS-CoV-2 is associated with a higher risk of death[73]. Major hepatic histopathological findings in patients with COVID-19 are nonspecific and mainly consist of hepatic steatosis, congestion of hepatic sinuses, vascular thrombosis, and portal/periportal tract lymphocytic infiltrates[74].

COVID-19 and Kidney transplant recipients: KT recipients are often older, have multiple comorbidities and require chronic immunosuppression, and as such represent a high risk group for more serious COVID-19 disease. Since the onset

of the pandemic, a large amount of data on recipients of SOT and KT have been reported, mainly from case series, small cohorts and larger registries. However, large multicentre studies with appropriate control groups are lacking. So, it remains a challenge how to appropriately treat KT recipients with COVID-19 and to understand what factors influence the outcomes. Clinical presentation, including abnormalities in liver function tests, has been shown to be similar in SARS-CoV-2 infected KT patients and in the general population[75].

In a multicentre cohort study the incidence of hepatitis was 20.2% among KT recipients, who received some form of antiviral therapy for SARS-SoV-2[76]. As in immunocompetent patients, the liver disease in KT recipients is usually mild and transient, characterised mainly by elevated levels of AST and ALT[76]. AST abnormality is more pronounced in patients with a severe form of the disease, and higher AST values have been shown to be a marker of poor outcome[77,78]. Early results from national and international registries and multicentre studies found a variable mortality rate of 18% to 32% among KT recipients hospitalised for COVID-19[77,79].

One distinctive feature that must be considered when assessing the possible aetiology of liver damage in patients with COVID-19 is the possibility of drug-induced liver injury (DILI) associated with certain treatments. Data on adverse events are sparse and often cannot be attributed with certainty exclusively to drugs for COVID-19, as a variety of other medications such as antibiotics and antipyretics are widely used in the treatment of such patients. To date various medications have been tried in treatment of SARS-CoV-2 infection – glucocorticoids, antimalarials, immunomodulatory agents (JAK2 inhibitors, IL-6 and IL-1 inhibitors), monoclonal antibodies, antiviral drugs and all of them may theoretically exhibit hepatotoxicity[80,81]. Table 1 Lists the hepatic contraindications and risk of drug induced liver injury (DILI) for drugs approved for the treatment of SARS-CoV-2 infection in the European Union and the United States of America [80-82].

DRUG-INDUCED LIVER INJURY

DILI, defined as liver injury caused by various drugs, herbs or other xenobiotics, results in abnormalities in liver tests or liver dysfunction, where other causes can be reasonably excluded[83]. The incidence of DILI is increasing worldwide due to the development of new drugs in the West and herbal preparations in Asia[83]. The classes of drugs most commonly associated with DILI include antibiotics, anticonvulsants and psychotropic drugs[84]. Polypharmacy, a common occurrence in KT recipients, increases the risk of drug interactions that may contribute to a higher incidence of DILI[84].

The severity of DILI can be associated with the dose of the consumed substance, *e.g.*, acetaminophen overdose/poisoning. However, most medications cause DILI in a non-dose dependent way, *i.e.*, idiosyncratically. Recent research proposed a 3-step model of DILI in which direct cell stress, direct mitochondrial inhibition and/or specific immune reactions can lead to mitochondrial permeability transition. Various environmental (age-related changes of pharmacokinetics, induction/inhibition of CYP450 enzymes, impaired antioxidant defence, immunological sensitisation, pre-existing liver disease, concomitant infections (*e.g.* HBV, HCV), mitochondrial dysfunction) and genetic factors may influence each sequence of the proposed model. In KT recipients, there are few potential environmental risk factors, *e.g.* drug interactions (antibiotics, antifungal and antiviral prophylaxis, immunosuppressive therapy), immunological sensitisation, malnutrition, infections and mitochondrial dysfunction due to diabetes, as well as all possible genetic risk factors that make them more susceptible to developing DILI than the general population. Previous research reported the occurrence of suspected DILI in 23% of KT recipients with possible causality in 57% of cases, and the associated drugs were antimicrobials, immunosuppressants and diuretics[84].

Assessing causality of DILI remains a challenge due to the lack of reliable biomarkers for hepatotoxicity. Several scoring systems are available, but there are discrepancies between scales. The best agreement seems to be between the Roussel-Uclaf Causality Assessment Method (RUCAM) scale and the Digestive Disease Week-Japan (DDW-J) scale, which includes an *in vitro* drug lymphocyte stimulation test (DLST) and is based on the RUCAM scale. Newer methods such as bile acid metabolomics could be used to assess the severity of DILI[85].

Despite some advances in the diagnosis and prognosis of DILI, the mainstay of treatment is to assess the need for immediate discontinuation of the suspect drug. N-acetylcysteine is currently a worldwide accepted treatment for acetaminophen hepatotoxicity. Bicyclol is a substance that may attenuate ALI by several mechanisms, including induction of autophagy, inhibition of oxidative stress and inactivation of the NLRP3 inflammasome. The results of the research in China show a satisfactory safety profile of the substance with a potentially favourable clinical outcome[86,87].

AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASES

Autosomal dominant polycystic kidney disease (ADPKD) is a prevalent genetic disorder causing uninhibited cyst growth in the kidneys, liver, and pancreas. It is one of the top causes of ESRD with an estimated occurrence rate ranging from 1:400 to 1000 live births due to mutations in PKD1 and PKD2 genes[88]. In addition, patients may present with other abnormalities, such as cerebral aneurysms, cardiac valve malformations, colonic diverticulosis, hernias of the abdominal wall and inguinal region, and cysts of the seminal vesicles[88]. Given the frequency of the disease, patients with ADPKD make up a large percentage of KT recipients. In rare cases, patients with ADPKD are referred for simultaneous liver and KT due to an enlarged liver leading to early satiety, abdominal pain and cachexia. In the majority of cases, however, synthetic liver function remains intact, so that most patients receive only a KT. After transplantation, liver cysts may continue to grow but rarely cause problems. Nevertheless, cyst complications must be included in the differential

Table 1 Hepatic contraindications and risk of drug-induced liver injury among SARS-CoV-2 treatment drugs

Drug	Liver Contraindication	Risk of DILI
Systemic corticosteroids	Caution in liver failure	+
Remdesivir	ALT > 5 × ULN	++
Tocilizumab	ALT > 5 × ULN	++
Sarilumab	ALT > 1.5 × ULN	++
Anakinra	Efficacy and safety in patients with AST/ALT ≥ 1.5 × ULN not been evaluated.	+
	Not recommended with severe hepatic impairment (Child-Pugh C)	
Baricitinib	Not recommended with severe hepatic impairment (Child-Pugh C)	+
Tofacitinib	Not recommended with severe hepatic impairment (Child-Pugh C)	+
Nirmatrelvir/ritonavir	Not recommended with severe hepatic impairment (Child-Pugh C)	+
Lopinavir/ritonavir	Not recommended with severe hepatic impairment (Child-Pugh C)	++
Molnupiravir	Limited experience of the use with any degree of hepatic impairment.	+
Casirivimab/imdevimab	Not recommended with severe hepatic impairment (Child-Pugh C)	+
Sotrovimab	No data in patients with ALT 5 to < 10 × ULN).	+
Tixagevimab/cilgavimab	Not been evaluated in patients with hepatic impairment	+
Regdanvimab	Not been evaluated in patients with hepatic impairment	+

ALT: Alanine transaminase; AST: Aspartate aminotransferase; ULN: Upper limit normal; +: Uncommon; ++: Common; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

diagnosis of both fever of unknown origin in KT recipients with ADPKD (due to cyst inflammation) and abdominal pain (*e.g.* rupture).

For patients with symptoms of liver enlargement that persist after KT, there are several treatment options: surgical resection, fenestration or sclerotherapy of cysts, or drug treatment[89,90]. Liver transplantation and combined liver/KT have been performed in patients with severe, symptomatic disease. Sirolimus appears to reduce the volume of the polycystic liver, possibly through an antiproliferative effect. Liver volume was significantly lower in seven KT patients receiving sirolimus-mycophenolate-prednisone than in nine recipients receiving tacrolimus-mycophenolate-prednisone [91].

Recently, tolvaptan, an antidiuretic antagonist, was approved as the first therapy for ADPKD after the TEMPO and TEMPO-R trials demonstrated significantly less eGFR loss compared to the placebo group[92]. It is expected that more and more patients with ADPKD will be treated with tolvaptan in the future. Tolvaptan is a V2R antagonist that blocks vasopressin signalling, which plays an important role in cyst growth in ADPKD due to the resulting intracellular increase in cyclic adenosine monophosphate[92]. The main adverse effect of tolvaptan is liver toxicity, requiring frequent monitoring, and polyuria is a logical consequence of V2R blockade. However, adherence to tolvaptan appears to be well-feasible in the majority of patients[93]. Additional ongoing studies will determine whether the benefits are long-term, whether they can be observed in patients with advanced kidney disease and whether they can be translated into quality of life and cost/effectiveness parameters[94]. Tolvaptan may also be considered for patients after KT according to preliminary reports, taking into account the possible adverse effects, including hepatotoxicity, hypernatremia and alteration of serum tolvaptan concentration when used concomitantly with cyclosporine[95].

Other, rare cystic diseases, such as Autosomal Recessive Polycystic Kidney Disease, Autosomal Dominant Tubulointerstitial Kidney Disease and Carolli disease should also be considered in KT recipients[96].

OTHER LIVER DISEASES

Iron overload

Iron overload is a common problem in ESRD patients and transplant recipients, often resulting from the use of iron supplements to treat anaemia, the use of iron-based phosphate binders, and disruption of iron utilization due to chronic inflammation associated with CKD. Anaemia, defined as a hemoglobin concentration < 130 g/L in men and < 120 g/L in women, is a frequent complication of CKD[97]. The prevalence is 50%-70% in ESRD patients before transplantation and decreases to 51% at 6 mo and 37% at 2 years after KT[98]. Erythropoietin deficiency and impaired iron homeostasis, including absolute and functional iron deficiency, are major contributors to CKD-associated anaemia. Standard therapies for the treatment of anemia include exogenous substitution of erythropoietin, iron supplementation, and blood transfusion. The availability of erythropoiesis-stimulating agents has reduced the need for blood products and the risk of

blood-borne infections, iron overload, and allosensitization. Recently, inhibitors of the hypoxia-inducible factor prolyl hydroxylase (HIF-PHI) have emerged as a new therapeutic option for anaemia. HIF-PHIs mediate both the erythropoietin and iron metabolism pathways. HIF-PHIs stabilize hypoxia-inducible factor (HIF), which stimulates endogenous erythropoietin production and affect the transcription of several iron metabolism and transport genes, leading to a decrease in ferritin levels and hepcidin levels[99-102]. HIF-PHIs can reduce the need for iron supplements by mobilizing stored iron[101]. Several randomized control trials in CKD patients regardless of dialysis status of roxadustat, vadadustat, and daprodustat showed no inferiority compared to ESA therapy with a similar safety profile[99,102-105]. These promising results could have significant clinical relevance as iron supplementation could be reduced.

Adequate iron stores are necessary for normal functioning of ESA and maintenance of stable hemoglobin levels. However, iron overload can lead to accumulation of iron in various organs. Iron-related side effects, mostly mediated by the formation of reactive oxygen species, can affect the liver (secondary hemochromatosis, cirrhosis), the heart (heart failure and arrhythmias), and endocrine organs (hypogonadism, diabetes mellitus). Excess iron can aggravate inflammation and shift the immunoregulatory balance, negatively affecting the immune system.

Serum ferritin and serum transferrin saturation (TSAT) are commonly used tests for iron status in CKD patients, including transplant recipients. Hepcidin measurement is not clinically more useful or better than TSAT and ferritin. Serum ferritin levels are affected by inflammation as it acts as an acute phase reactant. Serum ferritin levels ≤ 30 μg/L indicate iron deficiency. However, the ferritin value at which the iron stores in the bone marrow are filled is controversial. Currently, the 2012 KDIGO guidelines recommend iron supplementation therapy based on the combination of TSAT (≤ 30%) and ferritin (≤ 500 μg/L)[106].

CONCLUSION

Liver disease is a frequent occurrence following KT, estimated to affect 20%-50% of cases, and has a substantial impact on the survival and quality of life of these patients. Symptoms vary from asymptomatic, transient elevations of liver enzymes to the development of fibrosis and chronic liver failure. In severe cases, liver failure can have an acute onset or, in the chronic form, progress to cirrhosis with clinical indications of decompensation, or even the development of malignant disease such as HCC. Vigilant monitoring of KT recipients is crucial for the early detection of liver disease, allowing for timely intervention and improved outcomes. The incidence of viral diseases such as HBV and HCV has declined due to the effectiveness of antiviral medications and HBV vaccination programs. Globally, the prevalence of metabolic diseases such as NAFLD is rising, especially in transplant patients who are vulnerable due to lifelong immunosuppressive therapy. HEV and, more recently, SARS-CoV-2 have also been implicated as potential causes of liver disease. Additionally, healthcare professionals should be aware of the possibility of DILI, as many immunosuppressive and adjunct drugs can cause liver damage. Patients should be monitored closely for signs of drug toxicity, and medications should be adjusted accordingly. Screening KT recipients for liver disease is a priority for healthcare professionals. Early diagnosis and referral to a hepatologist are critical for managing underlying liver problems, which can adversely impact quality of life and general health.

FOOTNOTES

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MINIREVIEWS

Solid-Tubulocystic carcinoma: A new variant of intrahepatic cholangiocarcinoma

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Abstract

A new variant of intrahepatic cholangiocarcinoma (iCCA) has been recognized in recent years presenting predominantly as a large hepatic mass in young woman with the characteristic expression of inhibin by immunohistochemistry. This variant iCCA was originally termed as cholangioblastic variant of iCCA, and subsequently proposed to be renamed as inhibin-positive hepatic carcinoma or solid-tubulocystic variant of iCCA to better reflect its immunohistochemical profile or morphologic spectrum. The tumor histologically is composed of small to medium sized cells with scant to moderate amount of eosinophilic cytoplasm heterogeneously organized in solid, tubular, and cystic growth patterns. The tumor cells are positive for biliary markers, inhibin and albumin, and have a novel recurrent gene fusion, *NIPBL::NACC1*. Awareness of this new iCCA variant and its clinicopathologic features will aid in the diagnostic work-up and avoid confusion with other primary and metastatic hepatic neoplasms.

Key Words: Cholangiocarcinoma; Intrahepatic; Solid-tubulocystic; Cholangioblastic; Inhibin

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Core Tip: Solid-tubulocystic variant of intrahepatic cholangiocarcinoma (iCCA) is a recently recognized iCCA variant, which previously was termed as cholangioblastic iCCA. This new variant iCCA predominantly presents in young woman characterized by a heterogenous microscopic appearance with small to medium sized tumor cells with eosinophilic cytoplasm organized in solid, tubular, and cystic growth patterns. One of the defining features is the diffuse expression of inhibin. Recurrent *NIPBL::NACC1* gene fusion has been identified in this iCCA variant. Compared to typical iCCAs, patients with this variant iCCA may have a better prognosis with 25% of the cases reported died of disease in 5 years.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a highly aggressive adenocarcinoma arising from the biliary tree and can be divided into intrahepatic CCA (iCCA), perihilar CCA and distal CCA. Perihilar CCA arises from the second-order bile ducts to the insertion of the cystic duct whereas distal CCA is confined to the common bile duct below this insertion[1,2]. Collectively perihilar and distal CCA are referred as extrahepatic CCA. The most recent WHO classification of the digestive system tumours classified iCCA into two main subtypes based on their histologic features, small duct and large duct[3]. Other recognized rare variants of iCCA include adenosquamous carcinoma, squamous carcinoma, mucinous carcinoma, mucoepidermoid carcinoma, signet-ring cell carcinoma, clear cell carcinoma, ductal plate malformation-like pattern carcinoma, lymphoepthelioma-like carcinoma and sarcomatous carcinoma[3,4]. Recently, a new variant of inhibin-positive iCCA has been reported, which was termed as solid-tubulocystic or cholangioblastic variant iCCA[5-10]. This novel variant has a characteristic gene fusion, *NIPBL::NACC1*, which was first recognized by Argani *et al*[7], and subsequently confirmed in another case report by González *et al*[5].

This mini review summarized the unique clinical, morphologic, and molecular features of this newly identified and underrecognized inhibin-positive solid-tubulocystic/cholangioblastic variant iCCA. Awareness of this new variant would aid practicing physicians to recognize this rare variant iCCA.

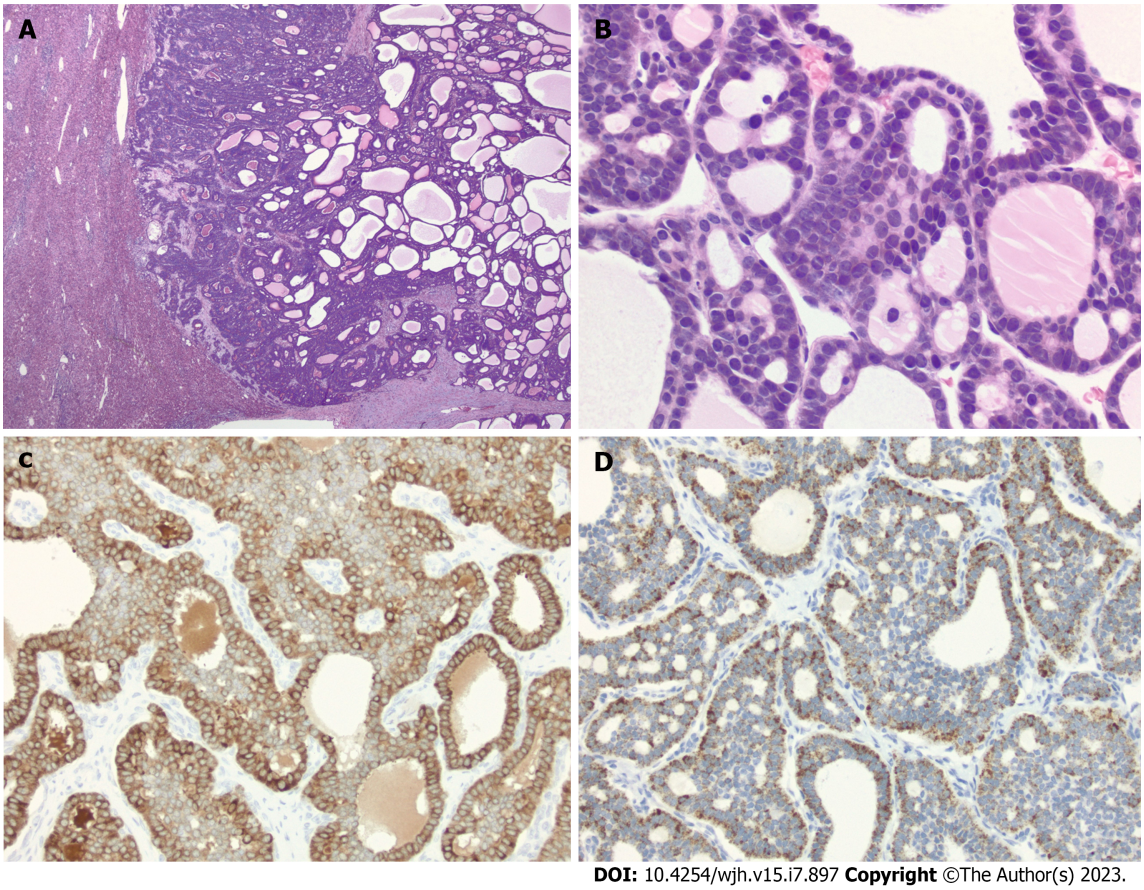
CLINICAL FEATURES

The inhibin-positive solid-tubulocystic variant iCCA presents with a median age of 28 years (range: 15-54 years) and predominantly occurs in woman (82%)[5-9,11-13]. The majority of the patients presents with non-specific abdominal distention and pain as well as nausea, vomiting and discomfort. On imaging the tumor is characterized by a heterogenous appearance with both solid and cystic areas and tends to have a well-demarcated border[5]. The median size at presentation is 16 cm (range: 6.9-32 cm). Of the patients reported with available follow-up information, half of the patients developed recurrence or metastasis during follow-up which were treated with adjuvant chemotherapy, and 25% (4 cases) died of disease all within 5 years of diagnosis.

MORPHOLOGIC FEATURES

The inhibin-positive solid-tubulocystic/cholangioblastic variant iCCA has distinct and characteristic morphologic features with multiple different patterns recognized within each tumor. Grossly, the tumors are well-circumscribed and the cut-surfaces vary from a solid tan-white to tan-yellow surface with areas of hemorrhage and degeneration[5]. Tumor necrosis is often seen particularly in larger tumors. Areas with a multicystic surface creating a spongiform appearance in some cases can be seen[5]. These cystic structures often have smooth inner surface and are filled with clear to tan-yellow fluid. In some cases, fibrous bands can be seen within the tumor.

As one could expect from the heterogenous gross appearance, the histologic features of this tumor vary in different areas within the same tumor which is both useful in their diagnosis but can also create confusion and a potential misdiagnosis. The solid areas are characterized by tumor cells with scant to moderate amount of eosinophilic focally granular cytoplasm with round to oval nuclei with a finely granular chromatin and occasional small nucleoli (Figures 1A and B). These tumor cells are organized in solid sheets to trabecular growth patterns and tubular/pseudoglandular structures. Both the cytologic morphology and architectural configuration are reminiscent of well-differentiated neuroendocrine tumors (WD-NET) and acinar cell carcinomas (ACC) which are differential diagnoses of these tumors. In other areas a tubular architecture is seen with cystic dilatation. The cysts are lined by tumor cells with similar cytologic features as the solid areas, and pink colloid-like secretions can be seen within the lumen in some cases mimicking thyroid follicular neoplasm. In some cases, the compact solid areas have a more crowded pattern with tumor cells showing only scant cytoplasm. Given this appearance these tumor cells are considered as a more "primitive" appearance and referred as



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Figure 1 Solid-tubulocystic variant of intrahepatic cholangiocarcinoma. A: Representative picture of a resected solid-tubulocystic variant of intrahepatic cholangiocarcinoma (right) in a non-cirrhotic liver (left). The tumor is well demarcated from the background liver (20x, Hematoxylin-eosin stain); B: Representative picture showing the tumor with solid, tubular, and cystic growth patterns (200x, Hematoxylin-eosin stain); C: Representative picture showing the tumor cells are positive for inhibin (200x, immunohistochemical stain); D: Representative picture showing tumor cells are positive for albumin (200x, in situ hybridization).

blastema-like areas. Therefore, these lesions have been termed as "cholangioblastic variant of iCCA" given the presence of blastema-like areas and the expression of neuroendocrine markers, a common feature of other primitive tumors[6]. However, in our opinion these tumors do not have the characteristics of other primitive epithelial tumors, nor recapitulate the developing ductal plate[5]. In light of this and the histologic features, we prefer to use the term of solid-tubulocystic variant of iCCA as proposed by Wen KW and colleagues[8], instead of cholangioblastic variant of iCCA.

IMMUNOPHENOTYPE

Perhaps given the rarity of this variant iCCA, an extensive immunophenotype has been reported in the case report and small case series in the literature with the most characteristic finding being the diffuse and strong expression of inhibin (Table 1) (Figure 1C)[5-9,11,14]. As expected, the tumor is diffusely positive for cytokeratin (CK) AE1/AE3, CAM5.2, CK7 and CK19, whereas being negative for CK20 and CDX2, consistent with a biliary phenotype. It is also negative for hepatocellular markers, such as HepPar1 and arginase-1. Of note, albumin by in situ hybridization is positive in tumor cells (Figure 1D), which is consistent with primary hepatic carcinoma - iCCA. One of the pitfalls is the focal to diffuse expression of multiple neuroendocrine markers including CD56, synaptophysin and chromogranin, which varies from a weak to strong positivity, but INSM1, a more specific neuroendocrine marker is so far reported to be negative.

MOLECULAR FEATURES

Recently, a novel fusion in this variant of iCCA involving the exon 8 of *NIPBL* and exon 2 of *NACC1, NIPBL::NACC1* has been identified in 3 cases[7]. An additional case was identified by the authors in the Cancer Genome Atlas harboring an identical fusion (case TCGA-ZH-A8Y6). The digital slide of this case available online (www.cbioportal.org) shows the characteristic morphologic features of solid-tubulocystic variant of iCCA. Most recently, a subsequent study reported one case to harbor this fusion[5]; hence to date 5 cases have been identified with this novel fusion. However, it remains unclear if this fusion is specific for this tumor type. To date this exact fusion has only been reported in 2 other malignancies both reported as "noncolorectal and nonpancreas gastrointestinal primary[15]." Unfortunately, no

Table 1 Aggregated selected immunophenotype[5,6,7,8,11], *n* (%)

Antibody	Positive	Negative
Inhibin	14 (100)	0
Cytokeratin AE1/AE3	6 (100)	0
CAM5.2	4 (100)	0
Cytokeratin-7	14 (100)	0
Cytokeratin-19	12 (100)	0
Cytokeratin-20	0	6 (100)
CDX2	0	3 (100)
Arginase-1	0	5 (100)
HepPar1	0	13 (100)
Glypican-3	1 (12.5)	7 (87.5)
Glutamine synthetase	3 (100)	0
Albumin by *in situ* hybridization	6 (100)	0
Synaptophysin	10 (76.9)	3 (23.1)
Chromogranin	9 (64.3)	5 (35.7)
CD56	5 (71.4)	2 (28.6)
INSM1	4 (100)	0
Beta-catenin	0	7 (100)
CD10	0	3 (100)
Pax8	0	2 (100)
Gata3	0	1 (100)
Mammaglobin	0	1 (100)
GCDFP-15	0	1 (100)
ER	0	2 (100)
PR	0	2 (100)
S100	0	3 (100)

CDX2: Caudal-related homeobox gene 2; INSM1: Insulinoma associated protein 1; GCDFP-15: Gross cystic disease fluid protein 15; ER: Estrogen receptor; PR: Progesterone receptor; HMB45: Human melanoma black 45; pCEA: Polyclonal carcinoembryonic antigen; mCEA: Monoclonal carcinoembryonic antigen; SF-1: Steroidogenic factor 1; WT-1: Wilms tumor 1; TTF-1: Thyroid transcription factor 1; AFP: Alpha fetoprotein; CA125: Cancer antigen 125; CA19.9: Cancer antigen 19.9; SALL4: Sal-like 4; Oct3/4: Octamer binding transcription factor 3/4; PLAP: Placental-like alkaline phosphatase; hCG: Human chorionic gonadotrophin; RCC: Renal cell carcinoma; SOX9: SRY-box transcription factor 9.

description of the morphology of these two tumors is available. Although this fusion appears to be specific for this tumor at this time, given its characteristic morphology and immunohistochemical profile, molecular testing may not be necessarily needed in our opinion for diagnostic purposes.

The *NIPBL* (Nipped-b homolog) gene codes the protein delangin[16], a cohesin loading factor, which is essential for the chromatin loading of cohesin, an important ring-shaped protein complex for the structural organization of chromosomes and for the repair of DNA double-strand breaks[17]. Delangin also plays a role in translocating cohesin along the chromatin fibers which is thought to be the underlying pathogenesis of Cornelia de Lange syndrome[18,19]. Additionally, *NIPBL* plays a crucial role in myeloid differentiation[20]. Loss-of-function of *NIPBL* has been associated with an increased number of myeloid progenitors and a decrease of mature myeloid cells, and has been reported in acute megakaryoblastic leukemia[21,22]. In solid tumors, variant in *NIPBL* occurs in tenosynovial giant cell tumor, specifically a *NIPBL::ERG* fusion[23]. Interestingly, NIPBL overexpression has been shown to correlate with poor prognosis and chemotherapy resistance in non–small cell lung cancer, and knockdown of NIPBL in non–small cell lung cancer cell or breast cancer cell lines induces impaired proliferation, cell cycle arrest, apoptosis and autophagy[24-26]. NAC1 coded by *NACC1* is a member of the Bric-a-Brac Tramtrack Broad (BRB) family of protein complex which is also known as pox virus and zinc finger (POZ). This protein complex has multiple cellular functions involving in cellular proliferation, apoptosis, protein degradation, transcription and cellular morphology, among others[27-29]. NAC1 is associated with development of endometrial carcinomas[29], and tumor recurrence[28], and the development of chemoresistance in ovarian cancers[30-

32]. Although both *NIPBL* and *NAC1* genes have a crucial role in cellular proliferation and DNA repair, it is unclear to date what is the mechanistic function of their fusion protein. Future studies are needed to understand its biological function of this fusion which could lead potentially to the development of targeted therapies.

DIFFERENTIAL DIAGNOSIS

Metastatic or primary hepatic WD-NETs often share similar histologic pattern, cytologic morphology and positivity of neuroendocrine markers as this solid-tubulocystic variant of iCCA. One of the main clues for a solid-tubulocystic variant of iCCA is the multiple growth patterns present within each case in contrast to WD-NET which tends to have a more homogenous growth pattern. However, immunohistochemistry work-up is essential in their distinction. Although both WD-NET and solid-tubulocystic variant of iCCA can express neuroendocrine markers (synaptophysin and chromogranin), the expression of CK7 and CK19, and albumin by in-situ hybridization in solid-tubulocystic variant of iCCA rules out a diagnosis of WD-NET. Since the morphologic similarity with metastatic ACC, a possibility of ACC needs to be excluded. The negative expression of neuroendocrine markers (synaptophysin and chromogranin) and the positive expression of acinar markers (BCL10, trypsin and chymotrypsin) can help to rule out solid-tubulocystic variant of iCCA. Of note, about 25% of the pancreatic ACCs are positive for albumin by in-situ hybridization[33]. The cystic and glandular architecture containing colloid-like secretions is reminiscent of thyroid follicular neoplasm. The negative expression of TTF-1 or thyroglobulin can help to exclude the possibility of metastatic follicular neoplasm. Although uncommon, a metastatic sex-cord stromal tumor may enter the differential diagnosis given the expression of inhibin and similarity of some of the morphologic features. However, the expression of CK7, CK19, neuroendocrine markers and albumin by in-situ hybridization by solid-tubulocystic variant of iCCA can help to rule out this possibility.

TREATMENT CONSIDERATIONS

Given the rarity of these cases it remains unclear what is the optimal management plan for patients with this new variant iCCA. The majority of the cases reported with available information underwent a complete surgical resection ranging from segmentectomies to lobectomies, and one case was deemed not a surgical candidate given the presence of widely metastatic disease[5-9]. Two patients were treated before resection, one with neoadjuvant chemotherapy and one with transarterial chemoembolization[6]. The non-surgical candidate was treated with cisplatin and gemcitabine[9]. Most of the patients were treated with adjuvant chemotherapy after resection with a similar regimen, cisplatin and gemcitabine [7]. The previously reported pediatric patient was treated with a different regimen which included capecitabine[5].

CONCLUSION

Inhibin-positive hepatic carcinoma with solid-tubulocystic features (solid-tubulocystic carcinoma) is a recently recognized iCCA variant which predominantly presents in young woman with a large size at presentation. They have a characteristic morphology of round to oval cells with scant to moderate amount of eosinophilic cytoplasm which are organized in multiple different growth patterns including solid, tubular, and cystic. This novel variant is associated with a characteristic fusion gene, *NIPBL::NAC1*, being the first iCCA with a described recurrent gene fusion. Although much remains unknown of this variant based on the reported cases to date, they are associated with relatively favorable outcome with a 25% mortality rate compared to the 7%-20% 5-year survival rate of typical iCCAs[2]. Further studies are needed to fully explore the biological function of this fusion gene and its role in the tumorigenesis and to continue to characterize this rare tumor.

FOOTNOTES

Author contributions: González IA reviewed the literature and drafted the manuscript; Luo W provided some of the histological images and edited the manuscript; Zhang X provided overall intellectual input, reviewed the literature, and edited the final version of the manuscript; all authors approved the final version to be published.

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ORIGINAL ARTICLE

Case Control Study

Liver stiffness in pregnant women with intrahepatic cholestasis of pregnancy: A case control study

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Abstract

BACKGROUND

Intrahepatic cholestasis of pregnancy (ICP) is a rare but severe complication for both the mother and the unborn child. The diagnosis is primarily based on elevated serum levels of bile acids. In a large ICP cohort, we here study in detail liver stiffness (LS) using transient elastography (TE), now widely used to non-invasively screen for liver cirrhosis within minutes.

AIM

To specifically explore LS in a large cohort of women with ICP compared to a control group with uncomplicated pregnancy.

METHODS

LS and hepatic steatosis marker controlled attenuation parameter (CAP) were measured in 100 pregnant women with ICP using TE (Fibroscan, Echosens, Paris, France) between 2010 and 2020. In 17 cases, LS could be measured postpartum. 450 women before and 38 women after delivery with uncomplicated pregnancy served as control group. Routine laboratory, levels of bile acids and apoptosis marker caspase-cleaved cytokeratin 18 fragment (M30) were also measured.

RESULTS

Women with ICP had significantly elevated transaminases but normal gamma-glutamyl transferase (GGT). Mean LS was significantly increased at 7.3 ± 3.0 kPa compared to the control group at 6.2 ± 2.3 kPa ($P < 0.0001$). Postpartum LS decreased significantly in both groups but was still higher in ICP (5.8 ± 1.7 kPa *vs*

4.2 ± 0.9 kPa, $P < 0.0001$), respectively. In ICP, LS was highly significantly correlated with levels of bile acids and M30 but not transaminases. No correlation was seen with GGT that even increased significantly after delivery in the ICP group. Bile acids were mostly correlated with the liver apoptosis marker M30, LS and levels of alanine aminotransferase, aspartate aminotransferase, and bilirubin. In multivariate analysis, LS remained the sole parameter that was independently associated with elevated bile acids.

CONCLUSION

In conclusion, LS is significantly elevated in ICP which is most likely due to toxic bile acid accumulation and hepatocyte apoptosis. In association with conventional laboratory markers, LS provides additional non-invasive information to rapidly identify women at risk for ICP.

Key Words: Intrahepatic cholestasis of pregnancy; Transient elastography; Bile acids; Liver stiffness; High risk pregnancy

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Core Tip: Intrahepatic cholestasis of pregnancy (ICP) is a rare but severe complication in both mothers and unborn children. In a large ICP cohort, we studied liver stiffness (LS) in detail using transient elastography, which is now widely used for non-invasive screening of liver cirrhosis within minutes. LS is significantly elevated in pregnancies with ICP, most likely owing to toxic bile acid accumulation and hepatocyte apoptosis. Interestingly, no correlation was observed with γ-glutamyl transferase. In association with conventional laboratory markers, LS provides a novel non-invasive tool to rapidly identify women at risk for pregnancy complications.

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INTRODUCTION

Approximately 3% of pregnant women have liver disorders that can cause severe problems for the mother and unborn child, *e.g.*, liver failure, preterm labor, and stillbirth[1-3]. Despite intensive research on pregnancy-related liver complications in recent decades, treatment options are still insufficient, and no effective screening tests for early assessment have been established[4-6]. Intrahepatic cholestasis of pregnancy (ICP) with elevated serum bile acid levels higher than 20 μmol/L is the most common pregnancy-specific liver disease. Its etiology is complex and consists of genetic, endocrine (circulating estrogen and progesterone), and environmental factors (reduced vitamin D and selenium levels in winter). Severe forms with bile acids levels higher than > 40 μmol/L are associated with abnormal fetal echocardiography, meconium-stained amniotic fluid, spontaneous preterm labor and fetal asphyxia. Moreover, women with a total serum bile acid level of 100 μmol/L have an increased risk of stillbirth. The incidence varied between 0.05% and 27.6% for all pregnancies[7-12].

ICP typically presents in the third trimester as nocturnal pruritus of the soles and palms[13]. ICP also increases the risk of gestational diabetes and pre-eclampsia[14]. Long-term consequences of ICP include a higher risk of cancer of the liver and biliary tree, diabetes mellitus, thyroid disease, autoimmune diseases (psoriasis, inflammatory polyarthropathies, and Crohn's disease), and cardiovascular disease[15]. Treatment with ursodeoxycholic acid has been proven to significantly improve itching, blood levels, and fetal outcomes in numerous studies; however, pregnancy termination remains the only causal therapy[2,7]. A rapid diagnosis of ICP is essential to protect mothers and children from (long-term) complications [16]. The diagnosis is normally based on elevated serum levels of bile acids. Unfortunately, these blood tests usually take several hours, even at maximum care facilities and are not available at any time. To date, no screening tests are available for liver disease during pregnancy, except serological testing for viral hepatitis in the third trimester.

Despite the scarcity of ICP data, enormous progress has been made in the molecular understanding of cholestatic liver disease in recent decades[10]. Hepatocytes and cholangiocytes cooperatively produce bile, which is a mixture of organic and inorganic compounds[17]. Cholestasis usually describes the impairment of bile flow caused by defects in hepatocytes, which form and secrete bile, and/or defects in the secretory machinery of cholangiocytes[17]. The detergent properties of bile render it highly toxic to cells and tissues[17]. In addition to drugs, inflammation, liver disease, and hormones, several gene mutations have been discovered that can cause cholestasis and ICP[8-10,18-21].

Measurement of liver stiffness (LS) using elastographic techniques has become the gold standard for the noninvasive diagnosis of liver fibrosis and cirrhosis and it often avoids invasive liver biopsies[22]. Transient elastography (TE) (FibroScan, Echosens, Paris, France), the first elastographic technique, is an ultrasound-based technique that uses a transducer probe to create an elastic shear wave[23]. Pulse-echo ultrasound is used to measure shear wave velocity, which is directly associated with LS expressed in kilopascals (kPa). TE requires only a few minutes, is highly accurate, and has a lower sampling error than biopsy, thus allowing for repetitive measurements[24]. LS values below 6 kPa are

considered normal, while the generally accepted cutoff values for liver fibrosis (F3) and cirrhosis (F4) are 8 and 12.5 kPa [25]. However, LS is not only elevated by the fibrosis stage but also by other important confounding factors, including physiological conditions such as food and alcohol intake, or pathological confounders such as inflammation or pressure elevation[22]. Of note, all these confounders and artifacts will always increase LS but never decrease it, which is the most important reason, while normal LS has a very high negative predictive value in excluding liver pathologies[24].

Therefore, liver elastography is an ideal diagnostic tool to address hepatic complications during pregnancy. In the first elastography study of > 500 pregnant women without liver disease, we recently demonstrated that LS increased significantly in the third trimester and was an independent predictive factor for pre-eclampsia[26]. These findings were independently confirmed in a smaller study in Denmark[27]. Moreover, in pregnant women with cirrhosis, LS predicts hepatic decompensation after delivery[28]. The aim of the present study was to specifically explore LS in a large cohort of women with ICP compared with a control group with uncomplicated pregnancies.

MATERIALS AND METHODS

Study design and patient cohort

The study protocol (435/2006 and S201/2015) of this observational, prospective, case-control study was approved by the Ethics Committee of the University of Heidelberg and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The study design is shown in Figure 1. Briefly, between February 2010 and March 2020, 652 women were recruited at the Department of Gynecology at the University of Heidelberg or Salem Medical Center in Heidelberg during prenatal ultrasound or who presented to the prenatal outpatient department with prenatal complications or in the ward. Postpartum examinations occurred 24 h after delivery. Inclusion criteria were age ≥ 18 years and an intact pregnancy at weeks 9 to 42 or status postpartum. The healthy control cohort was obtained from our previous study[26]. ICP was diagnosed based on typical clinical symptoms, such as pruritus, and laboratory markers, such as elevated transaminase and serum bile acid levels > 20 μmol/L. The exclusion criteria were as follows: No signed informed consent; other pregnancy complications, such as preeclampsia or HELLP syndrome; and no valid LS measurements.

Laboratory parameters

Routine blood parameters were measured at the General Laboratory of University Hospital Heidelberg and Limbach Laboratory in Heidelberg. In 100 patients, serologically detected caspase-cleaved (M30) and total (M65) cytokeratin 18 levels as markers of liver apoptosis was measured as described previously using ELISA (Peviva, Bromma, Sweden)[29]. We also measured total bile acids not only in women suspected of having ICP but also in 60 women in the control group for comparative purposes.

LS and controlled attenuation parameter

LS and controlled attenuation parameter (CAP) were measured using TE (FibroScan, Echosens, Paris, France). M or XL probes were used according to the manufacturer's specifications and placed in the right lobe of the liver at the intercostal position, as described previously[30]. LS and CAP values were calculated as the medians of at least 10 consecutive measurements. In parallel to liver elastography in the control group, routine abdominal ultrasound was performed to exclude liver pathologies, such as liver cirrhosis, liver congestion, or liver tumors. In addition, the degree of liver steatosis was graded (0–3) and the spleen size was determined. Cutoff values from a recent meta-analysis were used[31]. Valid LS measurements were obtained for all the women.

Statistical methods

Statistical analyses were performed using the SPSS Statistics [version 23.0 (IBM, New York, United States), Excel 2016 (Microsoft, Redmond, United States), and GraphPad Prism 6 (GraphPad Software, San Diego, United States)]. For group comparisons, means and standard deviations were calculated, and an independent samples t-test was used. The Spearman rank-order correlation coefficient was calculated to conduct correlation analysis. Univariate and multivariate binary logistic regression analyses were used to identify the independent predictors of pregnancy complications, and receiver operating characteristic (ROC) analysis was performed.

RESULTS

Patient characteristics

For a better comparison, Table 1 presents only the characteristics of women (control and ICP) in the third trimester and after delivery. Almost all women with ICP (98 of 100, 98%) were in their third trimester, which is consistent with the literature[32]. In the control cohort, 228 of 450 patients (50.7%) were in the third trimester. A smaller number of women were followed up 1 d after delivery for both controls and ICP (n = 38 and n = 17). Supplementary Table 1 shows patient characteristics of the entire cohort in all trimesters. Accordingly, differences between the controls and patients with ICP remain. Women with ICP were significantly younger, and by definition, bile acids were significantly increased by a factor of approximately 6 (P < 0.0001). Women with ICP also had significantly elevated transaminase [predominantly alanine aminotransferase (ALT)] and bilirubin levels, but not elevated levels of alkaline phosphatase (AP) and gamma-glutamyl transferase (GGT). The levels of caspase 3-cleaved CK18, liver apoptosis marker M30, and uncleaved CK18 (M65), repres-

Table 1 Patient characteristics for the third trimester and after delivery

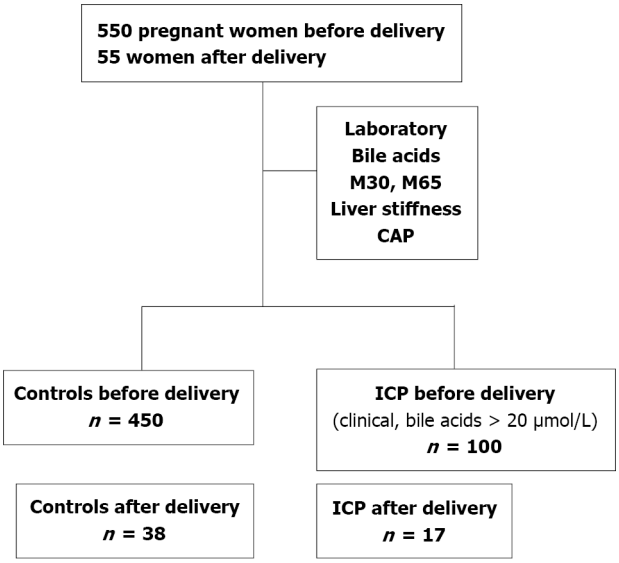
Parameters	Normal range[1]	ICP		Control	
		3. trimester, *n* = 98	Postpartal, *n* = 17	3. trimester, *n* = 228	Postpartal, *n* = 38
Age (years)		31.0 ± 4.2[a]	28.3 ± 3.5[c]	32.2 ± 5.2	32.9 ± 4.3
Bile acids (μmol/L)	2-5	39.0 ± 29.4[d]	23.7 ± 11.6[a]	6.8 ± 6.9	2.3 ± 1.2
AST (U/L)	< 35	116 ± 106[d]	88.2 ± 80[a]	26 ± 27	28 ± 6
ALT (U/L)	< 35	211 ± 202[d]	127 ± 106[b]	18 ± 26	15 ± 8
GGT (U/L)	< 40	23 ± 22	48 ± 40[a]	30 ± 55	18 ± 18
AP (U/L)	35-105	178 ± 68	182 ± 73	150 ± 95	283 ± 344
Bilirubin total (mg/dL)	< 1.3	0.65 ± 0.27[d]	0.52 ± 0.56	0.47 ± 0.26	0.65 ± 0.35
M30 (U/L)	< 200	432 ± 220[a]	293 ± 75[a]	339 ± 207	296 ± 50
M65 (U/L)	< 400	1180 ± 571[d]	777 ± 426[d]	680 ± 343	486 ± 89
Creatinine (mg/dL)	< 1.3	0.69 ± 0.38[a]	0.88 ± 0.39[a]	0.57 ± 0.12	0.61 ± 0.12
Urea (mg/dL)	< 50	19.8 ± 6.8[a]	25.5 ± 6.4[b]	16.9 ± 4.5	19.4 ± 5.3
Uric acid (mg/dL)	3.5-7.2	5.6 ± 2.4[d]	6.3 ± 2.8	3.9 ± 0.9	4.7 ± 1.0
Total protein (g/L)	66-83	67.6 ± 4.2[b]	65.2 ± 6.8	64.7 ± 4.4	66.3 ± 9.5
Albumin (g/L)	38-59	35.6 ± 2.7	36.3 ± 12.7	35.7 ± 2.7	
Leukocytes (1/nL)	3.7-10.0	8.8 ± 2.4[d]	12.0 ± 3.4[a]	10.8 ± 3.0	15.5 ± 4.8
Erythrocytes (1/pL)	4.1-5.1	3.9 ± 0.4	3.6 ± 0.6	4.0 ± 0.4	3.7 ± 0.5
Hemoglobin (g/dL)	12-16	11.4 ± 1.1	10.3 ± 1.5	11.7 ± 1.3	10.8 ± 1.5
Hematocrit (%)	36-43	33 ± 3	30 ± 6		
MCV (/pL)	80-96	86.1 ± 4.4	88.0 ± 3.5		
Platelets (1/nL)	150-360	228 ± 72	241 ± 61	227 ± 68	237 ± 61
Haptoglobin (g/L)	0.3-2.0	0.8 ± 0.4	1.0 ± 0.8		
Quick (%)	70-120	123.3 ± 5.1	119.4 ± 21.1	123.0 ± 13.0	124.5 ± 4.4
INR	< 1.1	0.91 ± 0.04	0.90 ± 0.03	0.90 ± 0.03	0.90 ± 0.03
Grade of steatosis in United States (0-3)	0	0.42 ± 0.50[a]	0.14 ± 0.38	0.18 ± 0.42	0.00 ± 0.00
Spleen size (cm)	< 11	11.4 ± 1.9	11.3 ± 1.1	11.1 ± 1.5	11.1 ± 1.7
Liver stiffness (kPa)	< 6	7.3 ± 3.0[c]	5.8 ± 1.7[d]	6.2 ± 2.3	4.2 ± 0.9
CAP (dB/m)	< 290 (S3)	206 ± 49[c]	213 ± 43	228 ± 39	224 ± 46

[1]Normal range for women.
[a]$P < 0.05$ *vs* controls in the corresponding group (3. trimester or postpartal).
[b]$P < 0.01$ *vs* controls in the corresponding group (3. trimester or postpartal).
[c]$P < 0.001$ *vs* controls in the corresponding group (3. trimester or postpartal).
[d]$P < 0.0001$ *vs* controls in the corresponding group (3. trimester or postpartal).
ICP: Intrahepatic cholestasis of pregnancy; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; AP: Alkaline phosphatase; MCV: Mean corpuscular volume; INR: International normalized ratio; CAP: Controlled attenuation parameter; M65: Soluble cytokeratin 18; M30: Caspase-cleaved cytokeratin 18 fragment.

entative of liver necrosis, were significantly elevated. However, M65 showed a twofold increase in women with ICP, whereas M30 was only slightly higher in this group. Interestingly, although all liver-related parameters decreased after delivery, GGT was the only marker with postpartum elevation compared with controls.

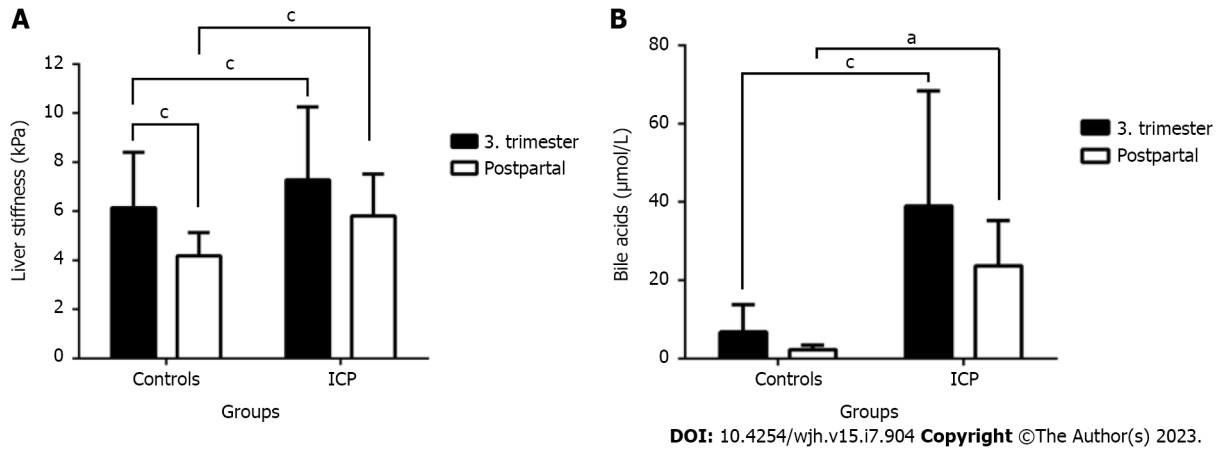
LS is significantly increased in women with ICP

As shown in Figure 2A and Table 1, LS was significantly higher in women with ICP, both before and after delivery. Moreover, in both groups, we observed an increase in LS as pregnancy progressed. In the ICP group, mean LS was significantly increased at 7.3 ± 3.0 kPa compared with the control group at 6.2 ± 2.3 kPa ($P < 0.0001$). An increase in LS to > 6 kPa was observed in 24.9% of healthy pregnant women and 44.2% of the ICP group, whereas an elevated LS of > 8



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Figure 1 Study design. ICP: Intrahepatic cholestasis of pregnancy.



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Figure 2 The 3. trimester and postpartal in the intrahepatic cholestasis of pregnancy and control group ([a]*P* < 0.05, [c]*P* < 0.001). A: Liver stiffness (mean and standard deviation); B: Bile acids (mean and standard deviation). ICP: Intrahepatic cholestasis of pregnancy.

kPa was observed in 7.1% of the control and 15.6% of the ICP group. LS of > 12.5 kPa considered above the cutoff value for cirrhosis was measured in 3.0% of the control and ICP groups. In confirmation to our initial study[26], however, postpartum LS decreased significantly in both groups to 5.8 ± 1.7 kPa in the ICP group and 4.2 ± 0.9 kPa in the control group, respectively. Finally, hepatic steatosis, as measured by the CAP, was normal in most women. It slightly but significantly increased in the ICP group during delivery, from 206 to 213 dB/m, whereas no significant changes were observed in the controls (Table 1). In summary, although LS generally increases during pregnancy, the liver is significantly stiffer in women with ICP before and after delivery than in controls without hepatic complications.

Parameters associated with LS elevation

Next, we performed Spearman's rho correlation analysis with clinical and laboratory parameters to identify potential confounders associated with elevated LS. Table 2 shows the results of the ICP, control, and total cohorts. In the ICP cohort, only a few parameters were significantly correlated with LS, namely, serum levels of bile acids and the liver apoptosis marker M30. Bilirubin levels hardly met the level of significance, whereas leukocyte count and Quick's test results were negatively correlated. No association between LS and bile acid levels was observed in the control group, and M30 Levels were weakly but significantly correlated with LS. In contrast, as described recently[26], LS significantly correlated with the duration of pregnancy, onset of gestational diabetes, body weight, mean arterial diastolic pressure, and AP and M65 levels in the total cohort. Interestingly, AST levels, which are usually highly associated with LS in liver diseases[33], were significantly correlated in the total cohort but not in the ICP or control groups alone. No correlation was observed between GGT and ALT levels in the ICP cohort. Finally, no association was observed between markers of hemolysis or anemia. In conclusion, in women with diagnosed ICP, bile acids are tightly associated with elevated LS and markers of liver apoptosis, but not with conventional liver function tests, except for bilirubin.

Table 2 Spearman Rho correlation with liver stiffness for women with intrahepatic cholestasis of pregnancy, controls and all

Parameter	Spearman rho correlation with liver stiffness		
	ICP, *n* = 100, r	Control, *n* = 450, r	All, *n* = 550, r
Bile acids total (μmol/L)	0.368[c]	0.085	0.438[d]
M30 (U/L)	0.881[b]	0.313[b]	0.385[c]
Quick (%)	-0.276[b]	0.210[a]	-0.010
Leukocytes (1/nL)	-0.223[a]	0.012	-0.203[b]
Bilirubin total (mg/dL)	0.213[a]	-0.091	0.124
MAD (mmHg)	-0.379	0.137[b]	0.154[c]
Gestational diabetes (1 or 0)	0.376	0.269[d]	0.301[d]
Pruritus (1 or 0)	0.353	-0.015	0.246[d]
Spleen size (cm)	0.340	-0.032	0.014
Uric acid (mg/dL)	0.308	0.261[a]	0.411[d]
AST (U/L)	0.146	0.103	0.327[d]
Creatinine (mg/dL)	0.281	0.057	0.163
Body weight (kg)	0.241	0.301[d]	0.317[d]
Platelets (1/nL)	-0.119	0.000	-0.074
AP (U/L)	0.237	0.329[c]	0.378[d]
Urea (mg/dL)	0.234	-0.064	0.041
Hemoglobin (g/dL)	-0.112	-0.022	-0.104
CAP (dB/m)	0.097	0.083	-0.006
M65 (U/L)	0.357	0.389[c]	0.452[d]
ALT (U/L)	0.048	0.047	0.273[d]
GGT (U/L)	-0.010	0.114	0.150

[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
[d]$P < 0.0001$.
Note that parameters are sorted first by levels of significance in the intrahepatic cholestasis of pregnancy group (p) and then the absolute correlation coefficient in descending order. Most relevant parameters are on top. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; AP: Alkaline phosphatase; ICP: Intrahepatic cholestasis of pregnancy; CAP: Controlled attenuation parameter, MAD: Mean arterial diastolic pressure; M65: Soluble cytokeratin 18; M30: Caspase-cleaved cytokeratin 18 fragment.

LS is independently associated with elevated bile acids in ICP

Figure 2B shows the levels of bile acids in both controls and women with ICP before and after delivery. Bile acid levels, which are a major criterion for the diagnosis of ICP were about six times elevated in the ICP cohort and promptly decreased after delivery. A slight but significant decrease was observed in the control group, suggesting that pregnancy causes bile acid elevation. Notably, bile acid levels were markedly elevated in the ICP cohort after delivery. Supplementary Table 2 lists the parameters associated with elevated bile acid levels. Bile acids are significantly associated with clinical features of pruritus and gestational diabetes. Among the laboratory parameters, bile acids were mostly correlated with the liver apoptosis marker M30 and the levels of ALT, AST, and bilirubin. Haptoglobin levels, leukocyte counts, and coagulation test results (Quick) were negatively associated with them. No association was observed between liver steatosis (CAP), M65 levels, AP, or splenic size. In the total cohort, bile acids were best associated with ALT, AST, and LS, in descending order. In multivariate analysis, LS remained the only parameter independently associated with elevated bile acid levels (Supplementary Table 3). In conclusion, in patients with ICP, bile acids are closely associated with liver damage in the form of apoptosis, and LS is independently associated with bile acid levels. In the third trimester, a LS of 6.5 kPa significantly discriminates between ICP and controls ($P = 0.033$) although with a modest AUROC of 0.65 (0.58–0.72, $P = 0.033$).

DISCUSSION

In this study, we noninvasively measured LS through TE and steatosis using CAP in a large cohort of pregnant women with diagnosed ICP, primarily through elevated bile acids. Our data clearly show that LS is higher in women with ICP than those in controls. Although LS decreased rapidly after delivery, as described recently[26,27], it remained significantly higher in women with ICP despite identical follow-up observation times. Women with ICP predominantly had elevated ALT levels, followed by those with elevated AST and bilirubin levels. In addition to AP levels, no differences were observed in GGT levels. Although all parameters, including LS, improved after delivery, GGT levels increased significantly in the ICP cohort. In addition, hepatic fat content, as measured using CAP, although within the normal range, was lower in patients with ICP than in controls. Finally, the liver apoptosis marker M30 showed the highest association with bile acid levels in univariate regression analysis, whereas LS remained the strongest independent predictor of bile acid levels > 20 mol/L in multivariate regression analysis.

In confirmation of earlier reports[26-28], the present study demonstrates that noninvasive assessment of LS through elastography is feasible and well accepted in pregnant women. In contrast to reports from internal medicine departments [34], elastography can be performed in all women. Second, we showed that LS is significantly elevated in women with ICP and higher than that in controls, which is remarkable, as we and others showed that LS is generally and reversibly elevated in the third trimester[26,27]. Consequently, and comparable to the previously reported LS elevation in women with preeclampsia, LS can be considered a feasible and noninvasive tool for screening, identifying, and following women with pregnancy-related liver complications.

What are the confounding factors for LS elevation in women with ICP? In contrast to initial beliefs, LS can be elevated because of many confounding factors, including inflammation, arterial and venous pressure elevation, and physiological conditions such as meal intake[22,24]. Mechanic cholestasis has been demonstrated to reversibly increase LS[35]. Of note, however, continued elevation of LS owing to these confounders has a negative impact on the liver, and the first long-term mortality data demonstrated that LS is one of the best long-term predictors of liver-related and all-cause mortality[22]. LS measurement can also be used to identify and monitor pregnant women with preexisting liver cirrhosis and predict hepatic decompensation[28]. The first study in women with uncomplicated pregnancies showed that elevated LS was significantly correlated with AP, leukocytes, gestational age, and an increase in body weight[26]. In the present study, in women with ICP, the confounders were completely different, and LS was tightly associated with elevated serum levels of bile acids and serum markers of liver apoptosis (M30). This is particularly interesting with regard to the fact that in liver diseases, such as alcoholic liver disease or viral hepatitis, LS elevation is typically associated with transaminase levels, namely, AST but not ALT[33]. Although we show here that LS is an independent predictor of elevated bile acids, the performance of LS in predicting ICP was only moderate and lower than that in the previous smaller ICP cohort[26].

To the best of our knowledge, this is the first study to show an exceptionally strong association between the established serum liver apoptosis marker (M30) with levels and bile acid levels in women with ICP and its association with elevated LS. In the multivariate analysis, LS remained the only parameter independently associated with elevated bile acid levels. In patients with liver disease, the association between liver apoptosis and LS elevation has already been shown both at the histological level[36] and using serum markers, such as M30[29]. The tight association of bile acids with LS and M30 levels in pregnancy is new. Bile acids are synthesized in hepatocytes as essential components for bile formation[9,17]. Owing to their detergent nature, however, they are highly cytotoxic and can disrupt cellular membranes if not protected, *e.g.*, by phospholipids[9,17,19]. Specifically, serum cholic acid becomes the primary bile acid in women with ICP, in contrast to normal pregnant and non-pregnant women, whose proportion is similar to that of chenodeoxycholic acid[37]. Typical examples are cholestatic liver diseases, such as primary biliary cirrhosis, which ultimately cause chronic bile duct inflammation, and later cirrhosis and cancer. Even simple mechanical cholestasis through obstruction of the major bile ducts by biliary stones can cause severe tissue damage.

In the last three decades, many gene mutations have been discovered that can cause cholestasis through the impairment of hepatocyte or cholangiocyte transport proteins relevant to bile formation[8-10,18-20]. These findings have resulted in a group of diseases known as progressive familial intrahepatic cholestasis. The normal GGT levels in our ICP cohort are a strong argument for a genetic cause. Hormonal changes/normalization after delivery with subsequent normalization of bile acid export through the hepatocellular apical membrane are considered important for the role of sex hormones in ICP[9]. In line with this, we observed a postpartum increase in GGT in our ICP cohort, suggesting a reinduction of GGT with the onset of bile flow.

Surprisingly, hepatic steatosis, as measured by CAP, which is now widely explored in patients with fatty liver[38], did not show any conclusive data in women with normal pregnancy or with ICP. The reason for this remains unclear because we expected that at least some women would present with steatosis, which can be a severe complication of pregnancy. We also briefly discuss some of the limitations of our study, which are mostly due to the challenging setting of performing clinical studies during late pregnancy, particularly in women with suspected complications. Serum was not available for all women to allow for the subsequent measurement of markers such as M30 and M65. In addition, we managed to measure LS sequentially before and after delivery in only a few cases in the same person. Another limitation with regard to postpartum follow-up measurements was that we only included women 24 h after delivery. This short time may explain why some parameters did not reach statistical significance.

CONCLUSION

In conclusion, we showed in a large cohort that women with ICP show significantly elevated LS compared with women

with uncomplicated pregnancies. In contrast to the large body of evidence in the liver literature, elevated LS in ICP is primarily correlated with the accumulation of bile acids known to be highly toxic to hepatocytes, and liver apoptosis, as measured through M30 levels, but not with transaminases, bilirubin, or GGT. We also showed for the first time that typically low GGT levels in ICP increase after delivery. Consequently, we believe that screening for LS in pregnancy is not "another diagnostic tool" to further complicate the already intensive surveillance during pregnancy, but could provide a novel non-invasive strategy to early identify women at risk for complications.

ARTICLE HIGHLIGHTS

Research background

Intrahepatic cholestasis of pregnancy (ICP) is a rare but severe hepatic complication for both mother and unborn child. Diagnosis is normally based on elevated serum levels of bile acids. Unfortunately, these blood tests usually take several hours, even at maximum care facilities. So far, there are no screening test for liver disease in pregnancy besides serological testing for viral hepatitis in the third trimester.

Research motivation

Measurement of liver stiffness (LS) through elastographic techniques has become the novel gold standard for the non-invasive diagnosis of liver cirrhosis often avoiding invasive liver biopsies. LS is not only highly correlated to the hepatic fibrosis stage but can also be elevated due to other important confounding factors such as inflammation, congestion or cholestasis. For these reasons, liver elastography could be an ideal diagnostic tool to address hepatic complications during pregnancy.

Research objectives

The aim of the present study was to specifically explore LS in a large cohort of women with ICP before and after delivery compared to a control group with uncomplicated pregnancy.

Research methods

LS and the hepatic steatosis marker controlled attenuation parameter (CAP) were measured in 100 pregnant women with ICP using transient elastography (Fibroscan, Echosens, Paris, France). In 17 cases, LS could be measured after delivery. A large cohort of women with uncomplicated pregnancy served as control group. Routine laboratory, levels of bile acids and the apoptosis marker M30 were also measured.

Research results

In the third trimester, women with ICP show a significantly increased LS at 7.3 ± 3.0 kPa compared to controls (6.2 ± 2.3 kPa, $P < 0.0001$). LS decreases significantly 24 h after deliver and remains higher in ICP (5.8 ± 1.7 kPa *vs* 4.2 ± 0.9 kPa, $P < 0.0001$). In ICP, LS is mainly correlated with levels of bile acids and the apoptosis marker M30. No correlation was seen with GGT and GGT even increased after delivery in women with ICP.

Research conclusions

In conclusion, LS is significantly elevated in ICP which is most likely due to toxic bile acid accumulation and hepatocyte apoptosis. In association with conventional laboratory markers, LS provides additional non-invasive information to rapidly identify women at risk for ICP.

Research perspectives

In the future, elastography should be further validated in order to early identify women at risk for complications. Moreover, elastography studies should be combined with genetic risk assessment, as several mutations of bile transport proteins are involved in the development of ICP.

FOOTNOTES

Author contributions: Nees J, Fluhr H, and Mueller S designed and coordinated the study; Nees J and Ammon FJ performed the experiments; Nees J, Mueller S, and Mueller J analyzed and interpreted the data; Nees J and Mueller S wrote the manuscript.

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ORIGINAL ARTICLE

Observational Study

Evaluation of the nutritional status of patients with liver cirrhosis

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Abstract

BACKGROUND

Progressive malnutrition coexists with liver diseases, particularly in patients with cirrhosis. Early diagnosis of malnutrition in patients with advanced stages of chronic liver disease and the implementation of appropriate nutritional treatment for malnourished patients should be an integral part of the therapeutic process.

AIM

To evaluate the nutritional status of patients with various severities of advanced liver fibrosis, using various nutritional status parameters.

METHODS

This study involved 118 patients with liver cirrhosis who were classified into three groups according to their Child-Pugh score. The nutritional status of the patients in each group was assessed using different methods. The average values obtained from the measurements were calculated for each research group. The influence of disease stage on the examined parameters of nutritional status was determined using one-way analysis of variance. To investigate the relationship between the parameters determining nutritional status and the stage of disease advancement, a correlation analysis was performed.

RESULTS

The Child-Pugh A group had the highest mean body weight (76.42 kg), highest mean body mass index (BMI) (26.72 kg/m^2), and largest mean arm circumference (27.64 cm). In the Child-Pugh B group, the mean scores of all examined variables were lower than those of the Child-Pugh A group, whereas the mean body weight and BMI of the Child-Pugh C group were higher than those of the Child-Pugh B group. There was a very strong correlation between the Child-Pugh classification

and subjective global assessment score; a very strong correlation between the Child-Pugh classification and arm circumference; a strong correlation between the Child-Pugh classification and body weight, albumin concentration, fat-free mass index, muscle mass index, phase angle, and BMI; and an average correlation between Child-Pugh classification and fat mass index. Notably, these indicators deteriorated with disease progression.

CONCLUSION

Advanced liver fibrosis leads to the deterioration of many nutritional status parameters. The extent of malnutrition increases with the progression of liver fibrosis. The Child-Pugh score reflects the nutritional status.

Key Words: Liver cirrhosis; Fibrosis; Nutritional status; Malnourishment; Sarcopenia

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Core Tip: Early diagnosis of malnutrition in patients with advanced stages of chronic liver disease and the implementation of appropriate nutritional treatments for malnourishment should be an integral part of the therapeutic process. It is important to properly assess the nutritional status of these patients as this can be the basis for therapeutic plans. It is advisable to determine which method of assessing nutritional status is appropriate for patients with cirrhosis since they usually develop specific complications of progressive organ failure.

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INTRODUCTION

Liver fibrosis develops in response to damaging factors with various etiologies (Table 1)[1]. It is a consequence of chronic liver disease[1,2]. Collagen deposition, scarring, and excessive accumulation of extracellular matrix elements have been observed[1]. The formation of scar tissue leads to the loss of contact between hepatocytes, blood vessels and bile ducts, resulting in the impairment of organ function[3]. Cirrhosis is the final stage of advanced fibrosis of the liver parenchyma [4]. The Global Burden of Disease estimates that cirrhosis affects 50 million adults worldwide and causes one million deaths annually[5]. The clinical advancement of the disease and risk of mortality can be assessed using the Child-Pugh scale. The scale considers ascites, hepatic encephalopathy, prothrombin time, serum bilirubin, and albumin and divides patients into three groups: (1) With good prognosis; (2) with moderate prognosis; and (3) with poor prognosis[6,7].

Chronic liver disease with advanced fibrosis and cirrhosis may be accompanied by progressive malnutrition[8]. Malnutrition is caused when the amount of food consumed is less than the required amount (hypoalimentation), often due to an incorrect understanding of the essence of the so-called "liver diet", digestive and absorption disorders that are caused by the slowing down of digestive processes (including secretion of bile acids), metabolic disorders consisting of limited protein synthesis and increased protein catabolism, and acceleration of basic metabolic processes[9]. The complications of liver cirrhosis include hepatic encephalopathy, ascites and decreased concentrations of albumin, coagulation factors, and transport proteins[9,10]. Early diagnosis of malnutrition in patients with advanced stages of chronic liver disease and implementation of appropriate nutritional treatment for malnourished patients should be an integral part of the therapeutic process[11,12] to provide support to the patient, prevent complications, and improve treatment and prognosis[13,14].

Malnutrition is usually diagnosed by assessing the patient's nutritional status. For this purpose, anthropometric measurements and a subjective global assessment (SGA) scale (SGA of nutritional status) are commonly used[15,16]. Laboratory tests, hand grip strength measurements that enable sarcopenia identification and body composition analysis using electrical bioimpedance are carried out for a thorough analysis of the nutritional status[17-21]. Among the numerous methods of assessing nutritional status, it is important to determine which one is the most appropriate for patients with liver fibrosis given the specific complications of progressive organ failure.

This study aimed to assess the nutritional status of patients diagnosed with advanced liver fibrosis at the cirrhosis stage using various methods of nutritional status assessment. In addition, we assessed whether the Child-Pugh score correlates with patients' nutritional status and which methods of assessing nutritional status are appropriate for patients with advanced liver fibrosis.

Table 1 Causes of liver fibrosis leading to cirrhosis

Etiological factor	Diseases causing liver fibrosis
Viral infections	Chronic hepatitis B
	Chronic hepatitis D
	Chronic hepatitis C
	Chronic hepatitis E
Autoimmune	Autoimmune hepatitis
	Primary biliary cirrhosis
	Primary sclerosing cholangitis
	Autoimmune cholangitis
Metabolic	Nonalcoholic fatty liver disease
	Hemochromatosis
	Wilson's disease
	a_1-antitrypsin deficiency
Toxic	Alcoholic liver disease
	Drugs, industrial toxins

MATERIALS AND METHODS

Description of the study group and criteria for patient inclusion

This study was conducted between May and November 2020 and included 118 Caucasian adult patients of Polish nationality who attended the ID Clinic in Mysłowice, Poland. First, informed consent was obtained to ensure voluntary participation. The following inclusion criteria were used: Liver cirrhosis based on clinical symptoms, imaging studies, and transient elastography[22] and no extrahepatic, acute, or chronic disease affecting the nutritional status. Liver cirrhosis was diagnosed by non-invasive transient elastography method using the Fibroscan® 502 Touch device, while in patients with ascites, which is a contraindication to elastography, the diagnosis was established based on clinical data, imaging, and laboratory results. The causes of cirrhosis in the study group were: Alcoholic hepatitis in 49 patients (42%), hepatitis C virus infection in 42 (36%), hepatitis B virus infection in 17 (14%), non-alcoholic steatohepatitis in 10 patients (8%). The study included 55 women and 63 men aged 37-81 years, who were classified into three groups according to the Child-Pugh scale. The Child-Pugh A group included 52 patients (Child-Pugh B: 34 patients; C: 32 patients). The mean age in each group was 58.84 years (Child-Pugh A), 56.94 years (Child-Pugh B), and 58.78 years (Child-Pugh C). The study was reviewed and approved by the Hospital Review Board. The study's planning, conduct, and reporting were in line with the tenets outlined in the Declaration of Helsinki.

Assessment of nutritional status

The nutritional status of patients in each group was assessed using the following methods: (1) Anthropometric measurements: Body weight (kg), height (cm), and body mass index (BMI) (kg/m^2), calculated according to the World Health Organization classification[23]; (2) Measurement and calculation of the circumference of the shoulder muscle [mid-arm muscle circumference (MAMC)]. MAMC = circumference of the arm in the middle of its length (mm) - 3.14 the thickness of the skin fold over the triceps muscle (mm)[12]; and (3) Body composition analysis by the bioelectrical impedance method, using the In Body 770 device. This device has received international certifications from the International Organization for Standardization (ISO), namely ISO 9001: 2015 and ISO 13485: 2016, and medical certification from the International Electrotechnical Commission (IEC), namely EN60601-1 and IEC 60601-1-2.

The following parameters were specified: (1) Fat-free mass index (FFMI) (kg/m^2): The cut-off points for FFMI were based on the criteria for diagnosing malnutrition developed by the European Society for Clinical Nutrition and Metabolism consensus statement: < 15 for women and < 17 kg/m^2 for men[24]; (2) Muscle mass index (MMI): The cutoff points for determining the MMI were established based on the standards of the measuring device[25]; (3) Fat mass index (FMI): The cutoff points for determining FMI were based on the standards of the measuring device[25]; (4) Extracellular water content (ECW) (kL): The cutoff points for determining the ECW were based on the standards of the measuring device[25]; (5) Phase angle (PA) (Xc/R)[26]: The cutoff points for determining the PA were based on the standards provided by the measuring device[22,25]; (6) Grip strength (kg) was measured using a DHD-3 SAEHAN hand dynamometer. The cutoff points for the assessment of weak muscle strength were based on European studies[27]. Grip strength values of 26-32 kg in men and 16-20 kg in women were classified as moderately strong, while those < 26 kg and < 16 kg were classified as weak grip strength[27]; (7) Laboratory albumin test results: Value of 3.5-5.0 g/dL were considered normal albumin concentrations[28]; and (8) A questionnaire survey was conducted using the SGA scale for the subjective assessment of nutritional status[29].

All measurements were taken on each patient on the same day.

Calculations and statistical analyses

Average values obtained from the analyses and measurements were calculated for each group. One-way analysis of variance was used to determine the influence of disease stage on the examined nutritional status parameters. The hypotheses were verified using an F-test for analysis of variance. The significance level of = 0.05 was assumed. Fisher's Least Significant Difference test was used to identify homogeneous groups. Correlation analysis was performed to examine the relationship between the parameters that determine nutritional status and the degree of disease advancement. Statistical analysis was performed using the R statistical package. To obtain transparent conclusions regarding the most appropriate methods of assessing the nutritional status of patients with liver fibrosis, the results of our research were compared with those of other recent studies.

RESULTS

Tables 2 and 3 present the results of the anthropometric measurements of body weight, BMI, and arm circumference, assigned to groups according to the Child-Pugh classification. The results were not significantly different depending on the etiology of cirrhosis.

The Child-Pugh A group had the highest mean body weight (76.42 kg), highest mean BMI (26.72 kg/m^2), and largest mean arm circumference (27.64 cm). In the Child-Pugh B group, the mean scores of all examined variables were lower than those of the Child-Pugh A group, whereas the mean body weight and BMI of the Child-Pugh C group were higher than those of the Child-Pugh B group. The mean circumferences of the arm muscle were as follows: 27.64 cm (Child-Pugh A); 25.95 cm (Child-Pugh B); and 25.20 cm (Child-Pugh C).

Underweight was the most common condition in the Child-Pugh B group; this group had the highest proportion of patients with normal body weight. Overweight and obesity were predominant in the Child-Pugh A group.

Tables 4 and 5 show the results of the body composition analysis: FFMI (kg/m^2), MMI (kg/m^2), FMI (kg/m^2), ECW (L), and PA (Xc/R) (reactance/resistance) of patients assigned to groups according to the Child-Pugh classification.

The highest mean values of FFMI, MMI, FMI, and PA were achieved by patients in the Child-Pugh A group. The mean ECW increased for each subsequent group; however, no significant differences were observed among the groups.

In all, 13.4% of the Child-Pugh A group and 64.7% of the B group had FFMI scores below the cutoff point. In the C group, almost half of the participants had scores below the cutoff. Only one person (1.9%) in the A group had MMIs below the cutoff point. In the B group, 29.4%, and in the C group, 56.2% had MMIs below the cutoff point.

Further, 42.3% of the Child-Pugh A group, 52.9% of the B group, and 59.7% of the C group had FMIs below the cutoff point; 26.9% in Child-Pugh A group, 11.7% in the B group, and 12.5% in the C had FMIs that were exceedingly higher than the cutoff point.

The prevalence of high ECW (above the standard) increased with disease progression. In the Child-A group, half of the patients had above-standard ECW, whereas 70% and over 90% of the Child-B and Child-C groups had above-standard ECW.

Further, 73% of the Child-Pugh A group, 74.6% of the B group, and 100% of the C group had suboptimal PA.

The hand grip strengths of patients in the groups according to the Child-Pugh classification are presented in Figure 1.

In the Child-Pugh A group, patients aged over 70 years had weak or medium grip strength, as expected for this age group. In the B group, men aged 60-69 years had weak grip strength. In the C group, men aged 50-59 years and women aged over 70 years had weak grip strength. Overall, hand grip strength was lower in the B group than in the A group, while the C group had the lowest grip strength.

Honestly Significant Difference Tukey's Test shows the statistically significant differences between the three groups. Significant effects of the Child-Pugh group, gender, and age on handgrip strength have been proven.

Tables 6 and 7 show the albumin concentrations of the patients according to the Child-Pugh classification. The mean serum albumin concentration in the A group was within the reference range. The mean albumin concentration was below the reference range in the B group and significantly lower than the accepted norm in the C group.

Albumin concentrations were below the reference range in 7.6% of the A group. In the B group, 79.4% of the patients had reduced albumin levels. All the participants in the C group had concentrations below the reference range.

Table 8 presents the results of the SGA scale according to the Child-Pugh classification. In the A group, the majority of patients (78.8%) were properly nourished. In the B group, 41.1% of the respondents were properly nourished, 32.3% were at risk of malnutrition, and 26.4% were malnourished. In the C group, 18.7% were properly nourished, but the majority were at risk of malnutrition (37.5%) and malnourishment (43.7%).

The percentages of patients classified as malnourished in each Child-Pugh group, based on BMI, FFMI, MMI, FMI, ECW, albumin level, and SGA scale are presented in Figure 2.

We found that as the percentage of patients classified as malnourished increased with the advancement of the disease according to the Child-Pugh classification, the indicators deteriorated, except BMI and FFMI.

Correlations between the indicators of nutritional status are presented in Figure 3. There was a strong correlation between the Child-Pugh classification and SGA score; a very strong correlation between the Child-Pugh classification and arm circumference; a strong correlation between the Child-Pugh classification and body weight, albumin concentration, FFMI, MMI, PA, and BMI; and an average correlation between the Child-Pugh classification and FMI. The indicators decreased with disease progression.

Table 2 Mean values of anthropometric measurements in groups according to Child–Pugh classification (*n* = 118)

Parameter	A	B	C
	n = 52	*n* = 34	*n* = 32
Body weight (kg)	76.4[1]	64.1[1]	68.9[1]
BMI (kg/m²)	26.7[1]	21.9[1]	23.9[1]
Myocardial circumference (cm)	27.6[1]	25.9[1]	25.2[1]

[1]Mean values are significantly different at $P < 0.05$.
BMI: Body mass index.

Table 3 Nutritional status expressed by body mass index in relation to standards (*n* = 118)

BMI	A		B		C	
	n = 52	%	*n* = 34	%	*n* = 32	%
Underweight	2	3.8	8	24	3	9.3
Correct body weight	18	35	20	59	17	53
Overweight	21	40	6	18	11	35
Obesity	11	21	0	0	1	3.1

BMI: Body mass index.

Table 4 Mean measurement values of body composition components (*n* = 118) in groups according to Child–Pugh classification

Component	A	B	C
	n = 52	*n* = 34	*n* = 32
FFMI (kg/m²)	18.5[1]	16.6[1]	16.6[1]
MMI (kg/m²)	10.1[1]	8.8[1]	8.55[1]
FMI (kg/m²)	7.9[1]	5.25[1]	6.7[1]
ECW (l)	0.388[1]	0.396[1]	0.406[1]
PA (Xc/R)	5[1]	4.4[1]	3.9[1]

[1]Mean values are significantly different at $P < 0.05$.
FFMI: Fat free mass index; MMI: Muscle mass index; FMI: Fat mass index; ECW: Extracellular water; SGA: Subjective global assessment; PA: Phase angle.

DISCUSSION

The effect of liver fibrosis on the patients is reflected by nutritional status measurements. The percentage of underweight individuals, defined by BMI, which was interpreted in our study as possible malnutrition, was higher in the Child-Pugh C group (23.5%) than in the B group (9.3%). Similarly, among 56 patients with liver cirrhosis examined by Łapiński and Łapińska[30], malnutrition determined by BMI was found in 7% of the cohort. BMI assessment is important due to its proven relationship with mortality in patients with chronic liver failure[31]. However, this indicator may not be an appropriate tool for assessing the nutritional status of patients with advanced liver disease given the fact that the body composition is not taken into account, including possible edema and ascites, which are complications of the disease[32, 33]. Fluid retention affects the BMI, resulting in malnourished patients being classified as overweight.

In our cohort, the average circumference of the arm muscle, which reflects the amount of muscle tissue and indicates the risk of sarcopenia, decreased with disease progression. This indicator (in contrast to BMI) does not change with the occurrence of edema[34]. Similar results were obtained by Crisan *et al*[34], who examined the impact of dietary behavior and nutritional status on the outcomes of 101 hospitalized patients with cirrhosis. According to them, malnutrition examined by measuring the circumference of the arm muscle was predominant among the patients with organ decompensation as opposed to those without decompensation[34]. Moreover, in a study conducted by Gnanadeepam *et al* [35], who evaluated the level of weakness in the course of cirrhosis in 81 patients with organ decompensation, arm circumference was correlated with the level of weakness coexisting with the disease.

Table 5 Nutritional status expressed by body composition results in relation to standards (*n* = 118)

Component		A		B		C	
		n = 52	%	*n* = 34	%	*n* = 32	%
FFMI (kg/m²)	Below standard	7	13.4	22	64.7	15	46.8
	Standard	45	86.5	12	35.2	17	53.1
MMI (kg/m²)	Below standard	1	1.9	10	29.4	18	56.2
	Standard	51	98	24	70.5	14	43.7
FMI (kg/m²)	Below standard	22	42.3	18	52.9	19	59.7
	Standard	16	30.7	12	35.2	9	28.1
	Above standard	14	26.9	4	11.7	4	12.5
ECW (L)	Normal	26	50	10	29.4	3	9.3
	Above standard	20	38.4	14	41.1	7	21.8
	Much above standard	6	11.5	10	29.4	22	68.7
PA (Xc/R)	Below standard	38	73	27	74.6	32	100
	Standard	14	26.9	7	20.5	0	0

FFMI: Fat free mass index; MMI: Muscle mass index; FMI: Fat mass index; ECW: Extracellular water; SGA: Subjective global assessment; PA: Phase angle.

Table 6 Mean serum albumin values according to Child–Pugh classification (*n* = 118)

Factor	A	B	C
Albumin concentration (g/dL)	4.181	3.281	2.71[1]

[1]Mean values are significantly different at $P < 0.05$.

Table 7 Nutritional status expressed by the obtained results of albumin concentration in relation to the standards (*n* = 118)

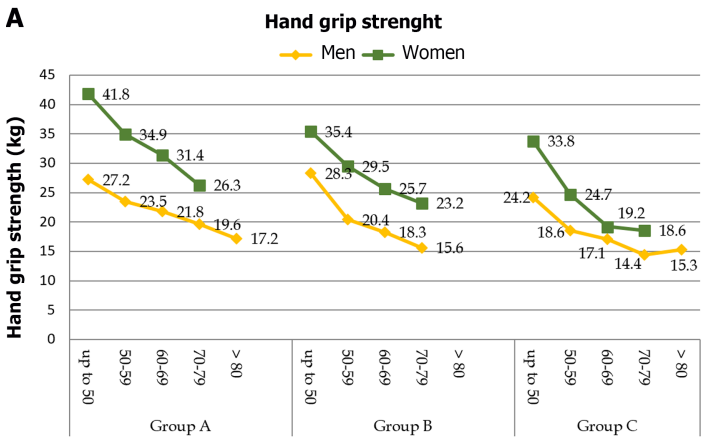
Albumin concentration (g/dL)	A		B		C	
	n	%	*n*	%	*n*	%
Below standard	4	7.6	27	79	32	100
Standard	48	92.3	7	21	0	0

Table 8 Nutritional status expressed by the results of the subjective global assessment scale (*n* = 118)

SGA	A		B		C	
	n = 52	%	*n* = 34	%	*n* = 32	%
Proper nutritional status	41	78.8	14	41.1	6	18.7
Risk of malnutrition	8	15.3	11	32.3	12	37.5
Malnutrition	3	5.7	9	26.4	14	43.7

SGA: Subjective global assessment.

Body composition data indicated a decrease in the average FFMI, skeletal MMI, and PA, with an increase in disease severity and a simultaneous increase in the average ECW. Considering the coexistence of liver diseases with disturbances in the balance between the amount of intracellular and extracellular water and the tendency of water to accumulate outside the cells, the ECW index, without considering water retention in the body, can lead to incorrect estimations of the nutritional status of the patients[36]. In patients with liver diseases, when interpreting the results of body composition analysis, it is advantageous to use the PA, which indicates the ratio of the water level inside the cells to that in the



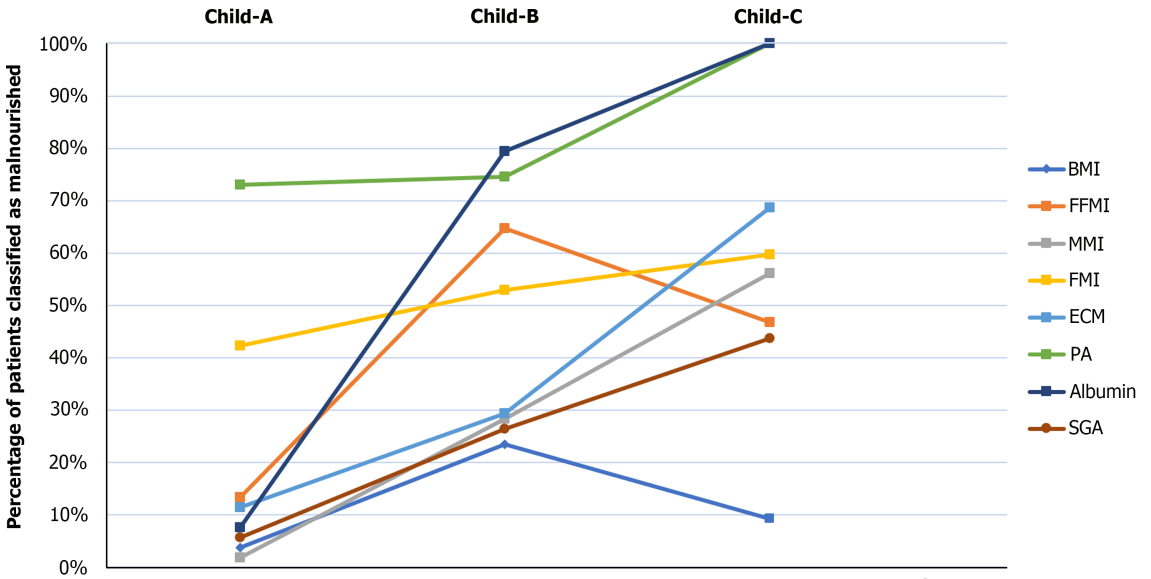
B

HSD Tukey's test of differences

HSD Tukey's teat		
Group	Hand grip strength mean	*P* value
C	21.44	$P < 0.05$
B	24.55	$P < 0.05$
A	28.01	$P < 0.05$

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Figure 1 Arithmetic mean of the measurement of hand grip strength expressed in kilograms, considering the Child-Pugh classification, age, and sex of the patients. Honestly Significant Difference Tukey's Test of differences (n = 118). A: Arithmetic mean of the measurement of hand grip strength; B: Tukey's Test of differences. HSD: Honestly Significant Difference.



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Figure 2 Percentage of patients classified as malnourished in Child-Pugh groups, based on body mass index, fat free mass index, muscle mass index, fat mass index, extracellular water, albumin concentration, and subjective global assessment score. BMI: Body mass index; FFMI: Fat free mass index; MMI: Muscle mass index; FMI: Fat mass index; ECW: Extracellular water; PA: Phase angle; SGA: Subjective global assessment.

extracellular spaces[37]. Luengpradidgun *et al*[38] also noted that among 30 patients in their study group with cirrhosis and sarcopenia, measurements based on electrical bioimpedance revealed that only six patients had co-occurring malnutrition. These data indicate the need for caution when assessing the nutritional status of this patient group.

The average hand grip strength in the study groups decreased with disease progression. This result was expected because progressive sarcopenia accompanies chronic liver inflammation that eventually leads to fibrosis[38]. Similar results were obtained by Nishikawa *et al*[39], who examined 241 patients with chronic liver disease and classified them according to Child-Pugh scores into groups A and B. Lower grip strength was observed in group B than in group A.

The blood test results indicated a decrease in the mean concentration of serum albumin levels with disease progression, as expected. The deterioration of the organs results in decreased albumin production. Previous studies have indicated the need for including albumin therapy in the treatment plan of patients with advanced liver fibrosis and cirrhosis[40,41].

We used the standardized SGA scale for assessing the nutritional status of the patients; the SGA scale is commonly used in the assessment of patients with liver diseases at risk of malnutrition[12,42]. Aldana Ledesma *et al*[42] compared various tools for assessing malnutrition and sarcopenia in patients with cirrhosis and observed the highest percentage of properly nourished patients in the Child-Pugh A and B groups. This finding is similar to the results of the present study.

When assessing the nutritional status of patients with advanced liver fibrosis, it is necessary to consider their overall condition. The correct assessment method should consider factors such as low protein synthesis, extracellular water accumulation, edema formation, and loss of muscle mass. Furthermore, the extent of hepatic fibrosis, as a result of

	Child	Body mass	SGA	Albumin	FFMI	MMI	FMI	ECW	PA	BMI	Arm muscle circumferene
Child		-0.68	1.00	-0.61	-0.68	-0.62	-0.46	n.s	-0.67	-0.65	-0.87
Body mass	-0.68		-0.71	0.55	0.77	0.66	0.85	n.s	0.58	0.99	0.79
SGA	1.00	-0.71		-0.57	-0.66	-0.59	-0.45	n.s	-0.63	-0.67	-0.85
Albumin	-0.61	0.55	-0.57		0.95	0.99	0.80	0.63	0.96	0.64	0.88
FFMI	-0.68	0.77	-0.66	0.95		0.99	0.92	0.55	0.93	0.84	0.93
MMI	-0.62	0.66	-0.59	0,99	0.99		0.88	0.61	0.96	0.75	0.91
FMI	-0.46	0.85	-0.45	0.80	0.92	0.88		0.52	0.76	0.91	0.78
ECW	n.s	n.s	n.s	0.63	0.55	0.61	0.52		0.57	0.27	0.38
PA	-0.67	0.58	-0.63	0.96	0.93	0.96	0.76	0.57		0.67	0.93
BMI	-0.65	0.99	-0.67	0.64	0.84	0.75	0.91	0.27	0.67		0.82
Arm muscle circumference	-0.87	0.79	-0.85	0,88	0.93	0.91	0.78	0.38	0.93	0.82	

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Figure 3 Correlations between the analyzed indicators of nutritional status. No correlation coefficient means no significant relationship between the tested parameters ($P < 0.05$). SGA: Subjective global assessment; FFMI: Fat free mass index; MMI: Muscle mass index; FMI: Fat mass index; ECW: Extracellular water; PA: Phase angle; BMI: Body mass index.

chronic inflammation, is reflected by the nutritional status of the patients, where the greater the level of organ deterioration, the greater the decline in the nutritional status.

The strengths of our study are the extensive study methods of malnutrition measurement and statistical analysis, which allows choosing the most appropriate measurement tool in the future. Our study also has certain limitations. This study had a relatively small sample size from one medical facility; therefore, further studies with larger sample sizes are recommended to validate our findings.

CONCLUSION

Advanced liver fibrosis leads to a reduction in various nutritional status parameters. Malnutrition among patients worsens with the progression of liver fibrosis, and the level of deterioration of this organ is indicated by the Child-Pugh scores.

Owing to its multifactorial etiology and numerous related complications, the identification of malnutrition in the course of chronic liver diseases is difficult. Therefore, a comprehensive assessment method involving a combination of available clinical, anthropometric, and biochemical methods is required for this patient population. We found that serum albumin concentration, arm circumference, lean body mass, skeletal muscle mass, phase angle, hand grip strength, and SGA score were useful parameters for assessing the nutri-tional status of patients with liver cirrhosis.

ARTICLE HIGHLIGHTS

Research background

Chronic liver disease with advanced fibrosis and cirrhosis may be accompanied by progressive malnutrition. Early diagnosis of malnutrition in patients with advanced stages of chronic liver disease and implementation of appropriate nutritional treatment for malnourished patients should be an integral part of the therapeutic process.

Research motivation

Among the numerous methods of assessing nutritional status, it is important to determine which one is the most appropriate for patients with liver fibrosis given the specific complications of progressive organ failure.

Research objectives

The aim was to assess the nutritional status of patients diagnosed with advanced liver fibrosis at the cirrhosis stage using various methods of nutritional status assessment. We tried to find out which methods of assessing nutritional status are the most appropriate for patients with advanced liver fibrosis.

Research methods

The study group contained 88 patients with advanced liver fibrosis. Patients were classified into three groups according to the Child-Pugh scale. The nutritional status was assessed using many methods: Electrical bioimpedance method, albumin concentration, mid-armmuscle circumference, body mass index (BMI), subjective global assessment (SGA) of nutritional status scale, and hand grip strength. To draw conclusions, proper statistical analyzes were performed.

Research results

There was a strong correlation between the Child-Pugh classification and SGA score; a very strong correlation between the Child-Pugh classification and arm circumference; a strong correlation between the Child-Pugh classification and body weight, albumin concentration, fat-free mass index, muscle mass index, phase angle, and BMI; and an average correlation between the Child-Pugh classification and fat mass index. The indicators decreased with disease progression.

Research conclusions

Malnutrition among patients worsens with the progression of liver fibrosis, and the level of deterioration of this organ is indicated by the Child-Pugh scores. We found that serum albumin concentration, arm circumference, lean body mass, skeletal muscle mass, phase angle, hand grip strength, and SGA score were useful parameters for assessing the nutritional status of patients with liver cirrhosis.

Research perspectives

Another important step in the study of the nutritional status of patients with advanced liver fibrosis seems to be the analysis of patients' diets to prepare individualized recommendations, adequate to progressive malnutrition.

FOOTNOTES

Author contributions: Janota B designed research and wrote the paper; Krupowicz A collected data and wrote the paper; Noras K performed statistical analysis; Janczewska E designed research and supervised the paper.

Institutional review board statement: The study was reviewed and approved by the Hospital Review Board. Planning, conduct, and reporting of the study were in line with the tenets outlined in the Declaration of Helsinki.

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

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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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SYSTEMATIC REVIEWS

Associations between irritable bowel syndrome and non-alcoholic fatty liver disease: A systematic review

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Abstract

BACKGROUND

Irritable bowel syndrome (IBS) is associated with obesity and metabolic syndrome. IBS and non-alcoholic fatty liver disease (NAFLD) are highly prevalent entities worldwide and may share similar mechanisms including gut dysbiosis, impaired intestinal mucosal barrier and immune system activation.

AIM

To systematically review their association according to the Preferred Reporting Items for Systemic Review and Meta-analyses guidelines.

METHODS

PubMed, EMBASE and Cochrane Database of Systematic Reviews were searched for relevant papers. Manual searches were also performed.

RESULTS

Six studies were included. Both IBS and NAFLD subjects had significantly more metabolic risk factors like hypertension, obesity, dyslipidaemia and diabetes. Our review showed that 23.2% to 29.4% of NAFLD patients had IBS. IBS was significantly higher in NAFLD patients compared with patients without NAFLD (23.2% *vs* 12.5%, $P < 0.01$). A higher proportion of IBS patients had NAFLD (65.8% to 74.0%). IBS patients were three times more likely to have NAFLD compared with non-IBS patients ($P < 0.001$). Two studies showed a significant correlation bet-

ween the severity of IBS and NAFLD. The proportion of NAFLD subjects with IBS increased with NAFLD severity.

CONCLUSION
Further prospective studies are warranted to evaluate the relationship and shared pathways between IBS and NAFLD, potentially leading to the development of future therapeutics.

Key Words: Irritable bowel syndrome; Functional gastrointestinal disorder; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Gut dysbiosis; Metabolic syndrome

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Core Tip: The relationship between irritable bowel syndrome (IBS) and non-alcoholic fatty liver disease (NAFLD) is increasingly recognised but their shared mechanisms remain poorly elucidated. We evaluate the association between IBS and NAFLD and discuss the risk factors and possible common mechanistic pathways including the brain-gut-liver axis, gut dysbiosis and translocation, altered hypothalamic-pituitary-adrenal axis and sleep quality.

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INTRODUCTION

The global surge in obesity has heralded the increasing prevalence of non-alcoholic fatty liver disease (NAFLD), which is the hepatic manifestation of the metabolic syndrome. NAFLD has emerged as the most common chronic liver disease and is increasingly recognised as a leading cause of morbidity and mortality[1]. It affects up to 25%-30% of the general population but is highly prevalent (up to 50%-90%) in patients with obesity and features of the metabolic syndrome[1,2]. Irritable bowel syndrome (IBS) is a disorder of gut-brain interaction, characterised by chronic abdominal pain and altered bowel movements without an organic cause[3]. Given the degree of heterogeneity in criteria used for the diagnosis of IBS, the true global prevalence of IBS remains elusive but is estimated to affect 1 in 10 people globally[4,5]. IBS is a complex condition that is caused by a myriad of factors with interplay of genetics, epigenetics, immune activation, gut dysbiosis, abnormal gut-brain interactions, visceral hypersensitivity, altered gut motility, eating behaviours, psychological stressors, and environmental factors[6,7]. To date, studies have found an interesting relationship between obesity and IBS. As NAFLD is closely linked with obesity, insulin resistance and the metabolic risk factors, emerging evidence has shown a possible correlation between NAFLD and IBS due to purported shared underlying pathophysiological links[8].

IBS is significantly associated with a higher prevalence of the metabolic syndrome and a multitude of studies have determined a link and common pathogenic mechanisms between these two conditions. Obesity and metabolic syndrome are found more frequently in IBS patients compared with controls[9]. A cross-sectional study from Japan showed a positive association between IBS and increased prevalence of metabolic syndrome and triglyceride levels. The odds ratio (OR) (95%CI) in IBS subjects were 2.01 (1.13-3.55) and 1.50 (1.03-2.18) respectively as compared with non-IBS subjects[10]. There is a higher occurrence of pre-diabetes and higher low-density lipoprotein (LDL) levels in patients with IBS[11,12]. In a large population-based cohort study, increased bowel movement frequency was associated with elevated risks of cardiovascular disease, diabetes, heart failure and chronic kidney disease[13]. In IBS subjects, an elevated body mass index (BMI) is associated with significantly faster colonic and rectosigmoid transit and higher bowel frequency[14].

Obesity and the metabolic syndrome are linked with insulin resistance, oxidative stress, chronic low-grade inflammation, abnormal lipid metabolism and gut microbiota alterations, all of which play key roles in the pathogenesis of NAFLD and the more progressive non-alcoholic steatohepatitis (NASH)[15,16]. The gut-liver axis is implicated in the development of NAFLD and similar mechanisms have also been shown to be pivotal to the pathogenesis of IBS[17,18]. Several studies have highlighted the correlation between NAFLD and IBS. Lee *et al*[19] demonstrated an increased prevalence of elevated alanine aminotransferase and gamma-glutamyl transferase levels and metabolic syndrome in IBS patients[19].

In this systematic review, we aim to evaluate the association between IBS and NAFLD including the common mechanistic pathways and overlapping risk factors.

MATERIALS AND METHODS

Search strategy and screening

The Preferred Reporting Items for Systemic Review and Meta-analyses (PRISMA) guidelines were used for the purposes

of this systematic review. A two-step approach was adopted to identify studies: (1) A systematic search of electronic databases; and (2) A manual search of direct citations from potentially relevant papers and other peer-reviewed journals.

The following electronic libraries-PubMed, Embase and the Cochrane Database of Systemic Reviews were searched from inception to March 2023 to identify papers studying the associations between IBS and NAFLD. The uses of search strategies were described in Table 1.

Manual searches of direct citations from potentially relevant papers and other peer-reviewed journals were also performed to identify additional studies not included in the systemic search. No filters were used to refine search results. No linguistic or geographical restrictions were imposed.

The titles of all papers retrieved by the literature search were screened for relevance and any studies of obvious irrelevance were excluded. All full manuscripts of relevant papers were then retrieved and subsequently screened in its entirety. The entire screening process was done by two independent researchers to identify studies that met the study selection criteria. Consensus was reached regarding any discrepancies that arose without the need for a third independent reviewer.

Eligibility criteria

Studies that met the following inclusion criteria were included: (1) Observational studies that investigated the relevance between IBS and NAFLD that included cross-sectional, case-control or cohort studies; (2) Population studies that showed the relevance between IBS and NAFLD; and (3) Human studies. Due to predictions of limited number of studies regarding the topic of interest, a planned option of expansion of criteria was put in place to include studies published in the Chinese language, of which all of the authors are also proficient in.

Data extraction

The data extraction process was done independently as per the PRISMA checklist. Publication details were first extracted, which included: (1) Author's name; (2) Year and Country of study; and (3) Methodology (sample characteristics, study design, and modalities used to diagnose IBS and NAFLD). Further data extraction included any risk factors that predisposed patients to the incidence of IBS and NAFLD if present that included risk estimate, including OR with corresponding 95%CIs about the association between IBS and NAFLD.

Risk of bias assessment

All eligible studies were assessed for risk of bias using Cochrane Risk of Bias Assessment (Figure 1).

Search results

The search from PubMed, Embase and Cochrane identified 509 studies along with 2 studies *via* manual search. 441 studies were screened for after the removal of 70 duplicates. 428 studies were excluded and 13 studies were retrieved for and screened in full. Among these 13 studies, 7 studies were excluded for reasons that included (1) Review articles (2) No full papers available; and (3) Non-English or non-Chinese language (Figure 2). The final 6 studies were included in this systematic review (Table 2).

RESULTS

The studies that were included demonstrated associations between NAFLD and IBS. The criteria used to identify IBS patients included ROME III criteria (Ke *et al*[20], Hasanian *et al*[21], Zheng *et al*[22]) and ROME IV criteria (Franco *et al*[23]), whilst Wu *et al*[24] selected IBS patients according to international classification of diseases (ICD)-10 coding[20-24]. Singh *et al*[25], Hasanian *et al*[21], Ke *et al*[20], and Zheng *et al*[22] used ultrasonography for the diagnosis of NAFLD whereas the fatty liver index (FLI) was used by Wu *et al*[24] to assess for NAFLD[20-22,24,25]. Franco *et al*[23] recruited patients with NAFLD based on ICD-10 codes[23]. The mode of diagnosis of IBS was not stated by Singh *et al*[25].

All the included studies demonstrate overlap between IBS and NAFLD, either *via* a substantial proportion of sample population having both IBS and NAFLD, or *via* meta-analysis that showed increased risk of also having IBS or NAFLD should either condition be present. Two studies reported that patients with IBS had increased risk of metabolic syndrome, including higher BMI, dyslipidemia and diabetes mellitus[20,21]. One study demonstrated the same for the IBS and NAFLD overlap group compared to the group that had either IBS or NAFLD[22].

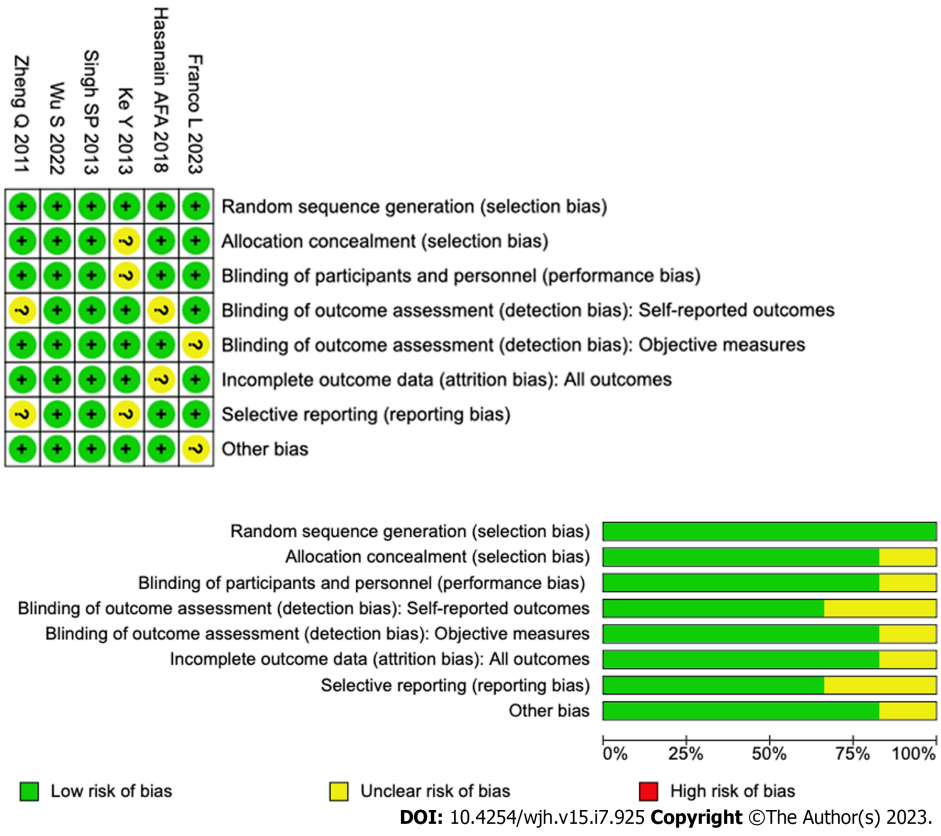
NAFLD in IBS patients

The 100 IBS patients were included in the study by Hasanian *et al*[21] according to the ROME III diagnostic criteria[21]. BMI and waist circumference were obtained. Laboratory investigations included fasting serum glucose levels, lipid profile, liver chemistry profile, international normalized ratio and a complete blood count. NAFLD was diagnosed in 74% of the study population using abdominal ultrasonography. Furthermore, it was noted that a higher prevalence of moderate/severe NAFLD were found among patients with moderate/severe IBS compared to mild IBS (22.4% *vs* 4.8%). This could signify a potential association between higher grades of NAFLD with more severe IBS. Multi-variate analysis affirmed the association of moderate/severe NAFLD with moderate/severe IBS which was independent of other risk factors of IBS (OR: 2.4, 95%CI: 1.3-62.7, P = 0.026). Metabolic syndrome was also found to be independently associated with moderate/severe IBS (OR: 3.1, 95%CI: 1.8-54.6, P = 0.011). The study observed that the most frequent metabolic parameter in IBS patients was high BMI (89%)[21].

Table 1 Search terms used

Database	
PubMed	(Irritable bowel syndrome[Mesh] OR "Irritable bowel syndrome"[tiab] OR "irritable colon*"[tiab] OR IBS[tiab]) AND (Fatty Liver[Mesh] OR (fatty[tiab] AND (liver*[tiab] OR hepat*[tiab]) OR steatohepat*[tiab] OR NAFL*[tiab] OR NASH*[tiab]))
EMBASE	('irritable colon'/exp OR 'irritable bowel syndrome':ab,ti OR 'irritable colon':ab,ti OR 'IBS':ab,ti) AND ('Fatty Liver'/exp OR (fatty:ab,ti AND (liver*:ab,ti OR hepat*:ab,ti) OR steatohepat*:ab,ti OR NAFL*:ab,ti OR NASH*:ab,ti))
CENTRAL	#1 MeSH descriptor: [Irritable Bowel Syndrome] explode all trees
	#2 ('irritable bowel syndrome' OR 'irritable colon' OR 'IBS'):ti,ab,kw
	#3 MeSH descriptor: [Fatty Liver] explode all trees
	#4 fatty:ab,ti AND (liver*:ab,ti OR hepat*:ab,ti) OR steatohepat*:ab,ti OR NAFL*:ab,ti OR NASH*:ab,ti
	#5 (#1 or #2) and (#3 or #4)

CENTRAL: Cochrane Central Register of Controlled Trials.



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Figure 1 Cochrane risk of bias assessment.

IBS in NAFLD patients

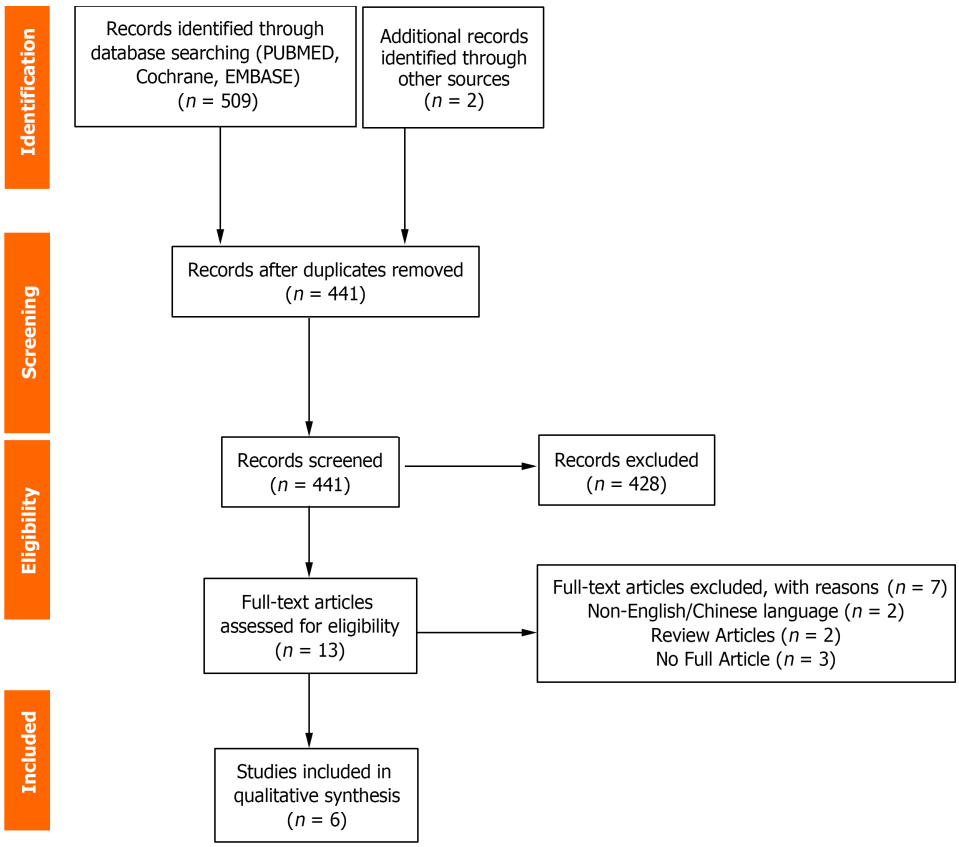
Ke *et al*[20] aimed to detect the prevalence of IBS in NAFLD patients as well as normal patients. Patients who underwent health examination in Urumqi, China were randomised into 2 groups: NAFLD and normal controls. IBS was diagnosed using the ROME III criteria. NAFLD was diagnosed using ultrasound based on the 2006 Revised Diagnostic criteria by Fatty Liver and Alcoholic Liver Disease Group of Chinese Medical association Hepatology Branch. 65.8% of adults with IBS-like symptoms had NAFLD while the detection rate of IBS was higher in NAFLD patients compared to normal (23.2% *vs* 12.5%, $P < 0.01$), suggesting an association between NAFLD and IBS-like symptoms. NAFLD patients were subsequently subdivided based on severity into: (1) Mild: 212; (2) moderate: 188; and (3) severe: 48. The prevalence of IBS in NAFLD patients increased with the severity of NAFLD as noted by the IBS detection rate for the groups being 11.3%, 27.7% and 58.3% respectively. Further analysis showed that more IBS symptoms were experienced with increasing severity of NAFLD. Multivariate logistic regression analysis demonstrated that IBS-like symptoms were closely related to ethnicity (OR: 0.316, 95%CI: 0.134-0.745, $P = 0.008$), fatty liver (OR: 0.525; 95%CI: 0.278-0.991, $P = 0.047$), BMI (OR: 0.918; 95%CI: 0.844-1.000, $P = 0.049$) and triglyceride levels (OR: 0.855; 95%CI: 0.739-0.988; $P = 0.034$)[20].

Table 2 Summary of study findings

Ref.	Location	Sample characteristics	Study design	IBS diagnosis	NAFLD diagnosis	IBS and NAFLD overlap	Associated risk factors
Ke *et al* [20], 2013	China	No. of patients: 945. NAFLD population: 470 (226 males, 222 females). Total without NAFLD: 475 (198 males, 234 females). Note: DM was in exclusion criteria	Cross-sectional study	ROME III	Ultrasound; Chinese Society of Hepatology, Chinese Medical Association diagnosis criteria for NAFLD	IBS incidence: 104 (23.2%) of NAFLD patients *vs* 54 (12.5%) of patients without NAFLD. NAFLD incidence in patients with IBS symptoms: 65.8% of patients with IBS-like symptoms had NAFLD. Higher detection rate of IBS-like symptoms with more severe NAFLD (P < 0.05). Mild NAFLD (Group A): 24 out of 212 (11.3%). Moderate NAFLD (Group B): 52 out of 188 (27.7%). Severe NAFLD (Group C): 28 out of 48 (58.3%)	Risk factors for IBS-like symptoms: BMI (P = 0.049). Triglycerides (P = 0.034). Fatty liver (P = 0.047). Ethnicity-Han, Uygur (P = 0.008)
Hasanian *et al*[21], 2018	Egypt	100 consecutive patients diagnosed with IBS (49 males, 51 females): 45% IBS-C 23% IBS-D 32% IBS-M. Further divided into: Mild IBS (n = 42); Moderate IBS (n = 43); Severe IBS (n = 15)	Cross-sectional study	ROME III	Ultrasound	74% of IBS patients had NAFLD. Moderate/severe NAFLD significantly associated with moderate/severe IBS. 22.4% moderate/severe IBS patients (95%CI: 15.8%-31.5%) *vs* 4.8% mild IBS patients: (95%CI: 2.7%-7.9%) (P = 0.001)	Predictors of moderate/severe IBS: NAFLD (P = 0.026); Metabolic Syndrome (P = 0.011)
Zheng *et al*[22], 2011	China	No. of patients: 200 (89 males, 111 females) (1) Both NAFLD and IBS: 25; (2) Either NAFLD or IBS: 36; and (3) Neither IBS nor NAFLD: 139	Cross-sectional study	ROME III	Ultrasound; NAFLD diagnosis based on Chinese Society of Hepatology, Chinese Medical Association	25 subjects (12.5%) had both NAFLD and IBS	NAFLD and IBS overlap group compared to group without NAFLD and IBS Overlap had higher: BMI (P = 0.045); TG (P = 0.035); TC (P = 0.038); HDL-C (P = 0.045); LDL-C (P = 0.031); FBG (P = 0.023). NAFLD and IBS overlap group compared to the either NAFLD or IBS group had higher: Hypertension (P = 0.041); Obesity (P = 0.034); Dyslipidemia (P = 0.020); Diabetes (P = 0.037); Digestive system diseases (P = 0.037). GIT sensory threshold: NAFLD and IBS overlap group had lower threshold of the following compared to either NAFLD or IBS group: FSV (P = 0.034); DSV (P = 0.032); MTV (P = 0.035); PSV (P = 0.027)
Franco *et al*[23], 2022	United States	No. of patients: 130 patients with NAFLD (49 males, 81 females)	Cross-sectional study	ROME IV	ICD-10 Code	38 (29.2%) patients with NAFLD fulfilled Rome IV IBS criteria	Increased prevalence of depression (18.4% *vs* 5.4%, P = 0.01) and anxiety (31.6% *vs* 9.8%, P = 0.002) in NAFLD patients with IBS compared to those without IBS. Independent predictors of IBS in NAFLD: Female gender (OR: 5.69, P = 0.001); Depression (OR: 1.23, P < 0.001); BMI (OR: 0.90, P = 0.02)
Wu *et al* [24], 2022	United Kingdom	No. of patients: 396838 (189759 males, 207079 females). NAFLD population: 153203	Prospective cohort study; United Kingdom Biobank	ICD-10 Code	Fatty Liver Index	7129 cases of incident IBS in NAFLD patients (cumulative incidence rate of 1.49 (95%CI: 1.46–1.53) per 1000 person-years). NAFLD patients showed a 13% higher risk of developing IBS (HR = 1.13, 1.05-1.17). Increased risk of IBS in higher FLI quartile	Subgroup analysis showed increased IBS risk for: Age (P < 0.003); Gender (Female) (P < 0.001)

Singh *et al*[25], 2013	India	No. of patients: 632 patients with incidentally detected NAFLD (484 males, 148 females)	Retrospective analysis	Not documented	Ultrasound diagnosis and grading of liver steatosis by two radiologists in a blinded study	186 (29.4%) out of 632 NAFLD patients had IBS	-

IBS: Irritable bowel syndrome; NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; FBG: Fasting blood glucose; FSV: First sensation volume; DSV: Defaecating sensation volume; MTV: Maximum tolerable volume; PSV: Painful sensation volume; ICD-10: International classification of diseases-10.



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Figure 2 Preferred Reporting Items for Systemic Review and Meta-analyses flowchart.

A cross-sectional study by Franco *et al*[23] included 130 NAFLD patients of which up to 29.2% of patients had co-existing IBS according to ROME IV criteria[23]. A higher prevalence of depression (18.4%) and anxiety (31.6%) was detected using the Hospital Anxiety Depression Scale in NAFLD patients with IBS compared to those without IBS (5.4% and 9.8%) respectively. Female gender (OR: 5.69, 95%CI: 2.01-16.12, $P = 0.001$) and depression (OR: 1.23, 95%CI: 1.10-1.38, $P < 0.001$) were independent risk factors for IBS[23].

A recent prospective cohort study followed 153203 patients diagnosed with NAFLD using FLI over 12.4 years[24]. IBS patients were determined *via* ICD-10 codes and diagnosis was based on self-report, primary care and hospital admission data. 7129 cases of incident IBS were detected with a cumulative incidence rate of 1.49 (95%CI: 1.46-1.53) per 1000 person-years. NAFLD patients showed a 13% higher risk of developing IBS (HR = 1.13, 1.05-1.17) compared with non-NAFLD patients. The highest FLI quartile was associated with a significant increase in risk of IBS compared to the lowest FLI quartile (HR q4 *vs* q1 = 1.21, 1.13-1.30, $P < 0.001$). This positive association between NAFLD and IBS was also observed by per SD change of FLI (adjusted HR = 1.08, 1.05-1.10) and predominantly in females[24].

Singh *et al*[25] showed that a proportion of NAFLD patients initially presented with IBS, though this was not elaborated upon. Out of 16225 patients with various gastrointestinal complaints, 632 patients with NAFLD were included. These patients attended the clinic not because of NAFLD but rather for a variety of gastrointestinal symptoms and the initial reason for evaluation for 29.4% (186) of these individuals with NAFLD was IBS[25].

Patients with both IBS and NAFLD

Zheng *et al*[22] grouped patients into those with IBS and NAFLD overlap and those without[22]. 200 subjects were recruited and split into 3 groups: (1) Both IBS and NAFLD; (2) either IBS or NAFLD; and (3) neither NAFLD nor IBS. IBS was diagnosed according to the ROME III criteria while NAFLD was diagnosed based on the Chinese Society of Liver Diseases on NAFLD. Out of 200 subjects, 25 (12.5%) had both IBS and NAFLD while 36 (18%) had either NAFLD or IBS. The rest of the 139 (69.5%) subjects had neither IBS nor NAFLD. Results suggested that the combined effect of IBS and NAFLD can implicate the metabolic parameters of patients. The IBS and NAFLD overlap group had a significant higherly incidence of hypertension (8% *vs* 5.56% $P = 0.041$), obesity (12% *vs* 5.56%, $P = 0.034$), dyslipidemia (12% *vs* 2.78%, $P = 0.020$), diabetes (4.0% *vs* 2.78%, $P = 0.037$) and digestive illnesses (24.0% *vs* 11.11%, $P = 0.037$) compared to the group with either IBS or NAFLD. The extent of dyslipidemia was greater in the IBS and NAFLD overlap group compared to the group with either IBS or NAFLD as higher triglycerides (2.34 ± 1.22 *vs* 1.71 ± 0.98, $P = 0.035$), total cholesterol (5.78 ± 1.57 *vs* 4.99 ± 1.06, $P = 0.038$), LDL cholesterol (LDL-C) (3.34 ± 1.12 *vs* 2.90 ± 0.99, $P = 0.023$) were noted. The IBS and NAFLD overlap group had a higher BMI (26.30 ± 3.03 *vs* 25.12 ± 2.59, $P = 0.045$) and higher fasting blood glucose levels (6.02 ± 1.01 *vs* 5.11 ± 0.97, $P = 0.023$)[22].

Anorectal manometry assessment was also performed which suggested that when both IBS and NAFLD were present the multiple risk factors from both conditions had a synergistic effect in irritation of the gastrointestinal tract. There was decreased gastrointestinal volume sensory threshold for patients in the IBS and NAFLD overlap group in comparison with the group that had either IBS or NAFLD as indicated by the decreased first sensation volume (22.56 ± 6.04 *vs* 30.27 ± 5.38, $P = 0.034$), defaecating sensation volume (63.22 ± 5.29 *vs* 78.34 ± 6.41, $P = 0.032$), maximum tolerable volume (82.39 ± 7.45 *vs* 131.78 ± 23.22, $P = 0.035$) and painful sensation volume (132.56 ± 19.29 *vs* 228.32 ± 17.36, $P = 0.027$) in the IBS and NAFLD overlap group[22].

DISCUSSION

The results overall support the possible association between IBS and NAFLD. NAFLD was more prevalent in patients with IBS compared to those without IBS. Individuals with both IBS and NAFLD overlap had more metabolic risk factors including high BMI, hypertension, dyslipidemia, and diabetes. The proportion of NAFLD patients with IBS increased along with the severity. IBS patients were three times more likely to have NAFLD compared with non-IBS patients ($P < 0.001$) with a significant correlation between the severity of IBS and NAFLD.

IBS and NAFLD have been postulated to share similar characteristics which are further discussed (Figure 3).

The brain-gut-liver axis and microbiome

The bidirectional relationship between the gut and the liver is well-established. The gut-liver axis involving gut dysbiosis, intestinal barrier dysfunction, intestinal dysmotility plays a vital role in the pathogenesis of NAFLD[18,26]. Changes in the intestinal microbiota is associated with severity of hepatic fat deposition through several mechanisms: Increasing low-grade mucosal inflammation, immune system activation, altering intestinal permeability, bile acid metabolism, dietary choline metabolism, and generating endogenous ethanol[27,28]. The brain-gut axis likewise shares a bidirectional relationship and similar changes in the brain-gut axis are implicated in the pathogenesis of IBS. The hypothalamic-pituitary-adrenal (HPA) axis and serotonin (5-HT) signalling are some of the pathways affected by dysregulation in the brain-gut axis[28,29]. These alterations lead to abnormal gut motility and visceral hypersensitivity[30]. Emerging evidence suggests a link between NAFLD and IBS symptoms such as diarrhoea. Population-based data from the National Health and Nutrition Examination Surveys revealed that NAFLD and diabetes were independently associated with diarrhoea as opposed to constipation or normal bowel patterns even after adjusting for BMI[31]. This is consistent with our results showing a correlation between IBS and NAFLD with the common mechanism of gut dysbiosis.

The gut microbiota has a significant role in the regulation of the various mechanisms and dysregulation of the gut microbiome contributes to the development of both NAFLD and IBS[26,32]. NAFLD patients have been shown to have increased *Firmicutes, Proteobacteria, Actinobacteria, Escherichia*, *Clostridium*, and *Bacteroides*, and decreased *Bifdobacterium*, *Prevotella* and *Faecalibacterium*[27,33-35]. Somewhat similarly, IBS patients have shown increased abundance of *Ruminococcus*, *Streptococci, Firmicutes*, and decreased *Bifidobacterium*, *Faecalibacterium*, and *Lactobacillus*[18,36-38]. However, no specific microbial signature exists for IBS and NAFLD patients to distinguish them from healthy controls, in part due to the variability of sequencing techniques and population groups, as well as various confounding factors such as dietary habits[35,39].

Chronic inflammation and immune system activation

Activation of the innate and adaptive immune pathways has been implicated in the pathogenesis of IBS, both in the intestinal mucosa and neuroinflammation *via* the brain-gut axis. This involves an overall state of inflammatory overdrive, a dysregulated HPA axis and serotonergic signaling[40]. IBS patients display persistent signs of low-grade mucosal inflammation with increased counts of $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocytes, mast cells[41]. Enhanced expression of pro-inflammatory cytokines including interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-6, IL-8 are observed in IBS patients[18,41,42]. Higher baseline TNF-α, lipopolysaccharide-induced TNF-α, IL-6 and IL-8 levels were significantly correlated with increased bowel frequency and severity of IBS symptoms[42,43].

NAFLD shares similar points of contact with IBS with a low-grade chronic inflammation as a main driver of disease progression in NAFLD[18,44]. Obesity triggers activation of innate and adaptive immune pathways and adipose tissue inflammation exacerbates NASH[18]. Liver metabolism is affected directly *via* circulating free fatty acids (FFA) from food,

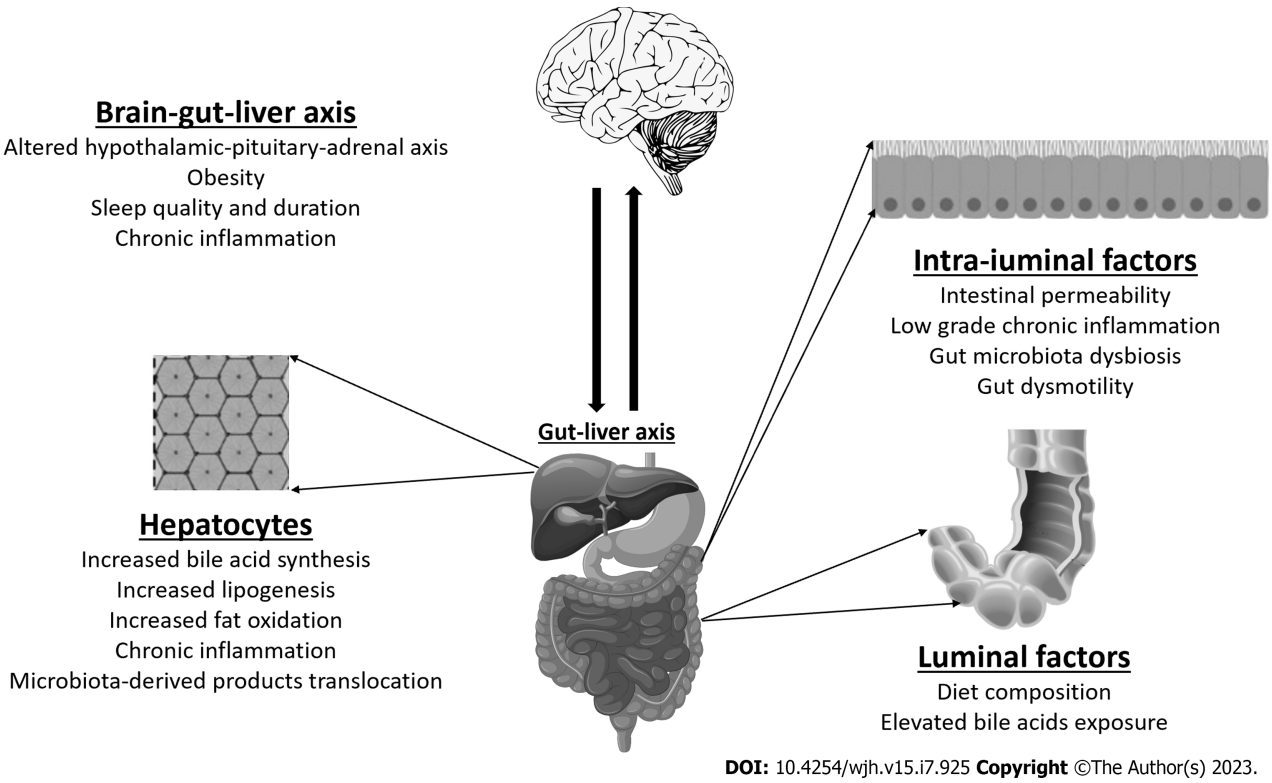


Figure 3 Postulated pathophysiology.

adipose tissue, intestinal bacteria, and indirectly *via* pro-inflammatory cytokines[18,45]. Higher levels of IL-6, C-reactive protein, TNF-α are detected in NAFLD subjects[18,46]. FFA binds toll-like receptors (TLR) on immune cells and in the liver, contributing to activity of the immune system. FFA are also involved in production of reactive oxygen species, mitochondrial dysfunction and endoplasmic reticulum stress in the liver[18,46]. Increased natural killer (NK) cell and NKT cell activity is linked with hepatic expression of inflammatory cytokines and activation of Kupffer cells[47]. All these mediators drive the inflammatory cascade and consequent fibrogenesis in NAFLD.

Bile acids

Bile acid-mediated mechanisms are involved in the pathophysiology of both NAFLD and IBS. There is bile acid signaling dysregulation resulting in increased bile acid production and bile acid malabsorption[48]. Altered bile acid metabolism with defective farnesoid X receptor (FXR) and fibroblast growth factor 19 (FGF19) contributes to abnormal hepatic lipid metabolism in NAFLD[48,49]. Patients with IBS have higher colonic bile acid exposure compared with healthy controls which affects bowel habits in IBS patients, predominantly in the IBS-D subgroup[50,51]. This stimulates colonic motility with acceleration of colonic transit, activation of visceral fluid sensation and fluid secretion[52].

In NAFLD, patients were found to have higher total faecal bile acid levels, increased rates of bile acid synthesis in the liver and a predominance of primary bile acids in the stool[53]. Primary unconjugated faecal bile acids correlated with the degree of hepatic steatosis, the presence of ballooning and severity of fibrosis in NASH subjects[53]. A retrospective study described an increased prevalence of NAFLD in individuals with bile acid diarrhoea[54]. These findings were further confirmed in a prospective study of 127 NAFLD patients that showed a correlation between increased hepatic bile acid production and diarrhoea with increased NAFLD fibrosis scores[49].

Small intestinal bacterial overgrowth

A higher prevalence of small intestinal bacterial overgrowth (SIBO) has been reported in patients with IBS[55]. Studies have demonstrated that IBS patients were more likely to develop SIBO compared with healthy controls, predominantly of the diarrhoea subtype[56,57]. SIBO is also found to be more prevalent in NAFLD patients attributed to proposed mechanisms including endotoxaemia and induction of TLR and pro-inflammatory cytokines[58,59]. A study by Sabaté *et al*[60] showed an association between SIBO and severity of hepatic steatosis in obese individuals[60].

Intestinal permeability

Impaired intestinal permeability is a key factor in the development of IBS[61]. Alterations in gut barrier function were observed in IBS patients which correlated with severity of symptoms[62,63]. A subgroup of IBS-D patients with increased intestinal permeability experienced more severe IBS symptoms and visceral hypersensitivity[62]. This is in part due to bacterial translocation and inflammatory agents through disruption of the epithelial tight junctions[7,18].

NAFLD is similarly associated with increased gut permeability which has an important role in the pathogenesis of NASH[64]. Several studies have described increased intestinal permeability in correlation with the degree of hepatic steatosis[65,66]. Impaired intestinal permeability allows for translocation of bacterial-derived products into the portal circulation and increasing hepatic exposure to harmful substances resulting in inflammation and fibrosis[66]. Conversely, a study by Luther *et al*[67] suggests that initial hepatic injury may contribute to impaired intestinal permeability although the mechanism is undetermined[67].

Obesity and metabolic syndrome

A multitude of studies denotes the association between IBS and obesity including the metabolic syndrome. Talley *et al*[68] demonstrated that older age, less early satiety, increased stool frequency and heartburn were all independently associated with increasing BMI[68]. Visceral abdominal obesity is correlated with an increased risk of developing IBS, primarily diarrhoea-predominant IBS. This is attributed to alteration in visceral fat metabolism which triggers production of adipokines and immunologic factors[69]. IBS patients have an augmented visceral perception of luminal stimuli, dysmotility and abdominal pain related to increased visceral adiposity[70]. Conversely, recent studies have shown that IBS subjects with morbid obesity achieved significant improvement in bowel symptoms after undergoing weight loss intervention[71].

The interplay between NAFLD, obesity and the metabolic syndrome with insulin resistance as a key pathogenic driver has been well-established. A meta-analysis by Li *et al*[72] showed that obese individuals had a 3.5-fold increased risk of developing NAFLD which has a dose-dependent relationship with BMI[72]. A high pooled prevalence of NAFLD was found in type 2 diabetes patients, with 60% of diabetes patients being diagnosed with NAFLD[73].

Sleep

Poor sleep quality and circadian misalignment have been implicated in the pathogenesis of IBS[74]. Exacerbation of symptoms have been observed after a night of poor sleep in IBS patients[74]. Likewise, impaired sleep quality, short sleep duration and daytime sleepiness are associated with NAFLD risk with correlation with insulin resistance[75,76]. The hypothalamus-pituitary-adrenal axis can also be affected with impaired cortisol metabolism leading to hepatic steatosis [75]. In addition, obstructive sleep apnoea could have deleterious effects on liver metabolism in the disease progression of NAFLD[76].

Clinical impact

In view of the postulated shared mechanisms underlying IBS and NAFLD, therapeutic strategies for IBS may also be beneficial for patients with NAFLD and vice versa. Lifestyle modification with diet and exercise leading to weight loss remains the cornerstone of NAFLD management[77]. Weight loss in IBS subjects showed marked improvement in bowel symptoms along with subjective well-being[71].

Rifaximin has been established as an effective drug in improving global IBS symptoms and bloating[78]. The administration of Rifaximin in NAFLD demonstrated effects including the reduction of serum endotoxin, pro-inflammatory cytokines, NAFLD-liver fat score and improvement in insulin resistance[79,80]. The postulated mechanism could be due to Rifaximin's effect on gram-negative bacteria leading to the inhibition of endotoxin proinflammatory cytokine production in NAFLD patients[79]. Several studies have shown that probiotics can lead to decrease in serum cytokine levels, oxidative stress markers, liver fat and biochemistry in NAFLD[81-83]. Similarly, studies have identified that probiotics can aid in alleviating IBS symptoms[84,85].

Obeticholic acid (OCA), a semi-synthetic FXR agonist, has been shown to stimulate FGF19 and decrease bile acid synthesis, improve stool form and diarrhoea in patients with IBS-D symptoms[86]. The promising impact of OCA on NASH and its associated metabolic features has been described with significant improvement in fibrosis and NASH histology[87]. Lubiprostone has been demonstrated to be effective in treating global IBS-C symptoms[88]. It has also been shown to decrease hepatic enzyme levels in NAFLD patients with constipation. Greater reduction in hepatic steatosis levels and levels of endotoxin were seen in those with improved intestinal permeability[89]. This may provide a basis for the role of Lubiprostone in a subset of NAFLD patients.

There are several limitations. The number of studies included was small given the lack of data on this topic to date. Different ultrasonographic diagnostic criteria was used for NAFLD. One study used codes from the International Disease Classification of diseases for identification of IBS while the other did not disclose how IBS patients were diagnosed, resulting in heterogeneity among the studies. Statistical analysis was unable to be carried out as not all studies assessed OR and 95%CI.

CONCLUSION

In conclusion, the evidence supports the association between IBS and NAFLD. IBS and NAFLD may co-exist and patients with IBS should be assessed for NAFLD and vice versa. Given the common postulated pathophysiology of both conditions, this may form the basis for further studies to assess suitability and benefits of utilising known therapeutics for IBS to treat NAFLD. This may guide future therapeutic strategies, especially in patients who suffer from both conditions. However, further prospective studies are required to confirm this association.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease with the rise of obesity and metabolic syndrome. Functional gastrointestinal disorders like irritable bowel syndrome (IBS) are increasing in prevalence.

Research motivation

At present, there is limited understanding regarding the links between the two conditions despite there being suggestions of possible overlap between IBS and NAFLD. We hope to explore literature to assess this overlap and also possible common pathophysiological links.

Research objectives

To review the current literature regarding the overlap of NAFLD and IBS and potentially identify common pathophysiological links which may show potential for utilizing common therapeutics to treat both conditions.

Research methods

A systematic search was done to assess current literature showing overlap between NAFLD and IBS in human subjects from PubMed, EMBASE and Cochrane.

Research results

We identified studies showing overlap between NAFLD and IBS. Both IBS and NAFLD patients demonstrated more metabolic risk factors like obesity, hypertension, dyslipidaemia and diabetes. IBS was seen to be more common in NAFLD patients and vice versa. Common pathophysiological links included the brain-gut-liver axis, intestinal permeability, gut microbiota dysbiosis, bile acid signalling dysregulation, obesity and metabolic syndrome.

Research conclusions

Our systematic review summarizes the current literature regarding IBS and NAFLD and demonstrates overlap between the two conditions. Common pathophysiological links were identified between both conditions.

Research perspectives

The evidence supports the association between IBS and NAFLD. With common postulated pathophysiology of both conditions discussed, further studies would be useful to further strengthen the association between both conditions and also look into possible common therapeutics.

FOOTNOTES

Author contributions: Siah KTH contributed to the design and implementation of the research and supervision; Ng JJJ and Loo WM performed the analysis of the results and writing of the manuscript.

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REVIEW

Liver transplant in primary sclerosing cholangitis: Current trends and future directions

Yash R Shah, Natalia Nombera-Aznaran, David Guevara-Lazo, Ernesto Calderon-Martinez, Angad Tiwari, SriLakshmiDevi Kanumilli, Purva Shah, Bhanu Siva Mohan Pinnam, Hassam Ali, Dushyant Singh Dahiya

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Abstract

Primary sclerosing cholangitis (PSC) is a chronic and progressive immune-mediated cholangiopathy causing biliary tree inflammation and scarring, leading to liver cirrhosis and end-stage liver disease. Diagnosis of PSC is challenging due to its nonspecific symptoms and overlap with other liver diseases. Despite the rising incidence of PSC, there is no proven medical therapy that can alter the natural history of the disease. While liver transplantation (LT) is the most effective approach for managing advanced liver disease caused by PSC, post-transplanta-

tion recurrence of PSC remains a challenge. Therefore, ongoing research aims to develop better therapies for PSC, and continued efforts are necessary to improve outcomes for patients with PSC. This article provides an overview of PSC's pathogenesis, clinical presentation, and management options, including LT trends and future aspects. It also highlights the need for improved therapeutic options and ethical considerations in providing equitable access to LT for patients with PSC. Additionally, the impact of liver transplant on the quality of life and psychological outcomes of patients with PSC is discussed. Ongoing research into PSC's pathogenesis and post-transplant recurrence is crucial for improved understanding of the disease and more effective treatment options.

Key Words: Primary sclerosing cholangitis; Liver transplantation; Management; Psychosocial outcomes; Pathogenesis

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Core Tip: Despite recent advancements in the field of hepatology, therapeutic options for the medical management of primary sclerosing cholangitis (PSC) are limited. Liver transplantation (LT) remains the primary treatment for patients with end stage liver disease (ESLD) secondary to PSC. Both deceased donor liver transplant and living donor liver transplant have demonstrated successful outcomes in patients with ESLD. Psychosocial patient factors also play a significant role in the outcome LT. Addressing ethical issues is crucial to ensure healthcare equity. Recent developments in digital technology and stem cell therapy suggest a promising future for LT in PSC.

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic immune-mediated cholangiopathy characterized by inflammation and scarring of the biliary tree, affecting both intrahepatic and extrahepatic bile ducts. Its etiology remains idiopathic and it presents a wide spectrum of symptoms and complications. PSC is a rare condition with a global incidence rate ranging from 0 to 1.58 cases per 100000 per year, and a prevalence bracket of 0 to 31.7 cases per 100000 persons[1]. Recent research northern Europe has shown an increasing frequency of both new cases and overall instances of PSC[1]. As compared to the adult population, the incidence and prevalence of PSC is lower in the pediatric population at 0.2 and 1.5 per 100000 children[2].

Currently, there is no proven no medical therapy to treat effectively or alter the natural history of PSC[3]. As a result, the prognosis of this condition is poor, and it is strongly associated with an elevated risk of developing liver cirrhosis and end-stage liver disease, often necessitating liver transplantation (LT)[4]. The development and progression of PSC involve a combination of genetic susceptibility and environmental factors, although the contribution of genetic factors remains limited[5]. On the other hand, environmental factors, particularly the gut microbiota, have gained increasing attention in PSC development[6]. Additionally, approximately 70% of PSC patients have concomitant inflammatory bowel disease (IBD), which serves as a strong risk factor for colon, bile duct, and gallbladder cancers[7]. The co-occurrence of IBD and PSC is evident, with 2%-7.5% of IBD patients developing PSC[4].

Advancements in noninvasive imaging techniques, such as magnetic resonance cholangiopancreatography (MRCP), have improved the understanding and diagnosis of PSC, including its relationship to LT. PSC accounts for approximately 10% of all liver transplants performed annually[4-8]. However, post-transplant recurrence of PSC has been reported, highlighting the need for a better understanding of its underlying pathogenesis and the development of more comprehensive therapeutic strategies[9]. Despite these challenges, long-term outcomes following transplantation are encouraging, with a 5-year survival rate of 89% and favorable graft survival rates[10].

PSC is a poorly understood domain and has been presented with a huge void in terms of concrete solutions that are yet to be fulfilled. In this review, we discuss the current understanding of PSC's pathogenesis, clinical presentation, management options, the scope of LT, and associated challenges.

DISCUSSION

Pathogenesis of PSC

Inflammation and fibrosis of the bile ducts are two primary processes in the pathogenesis of PSC. However, the mechanism of inflammation and fibrosis in PSC are not fully understood. It is believed that various factors such as Ischemic, traumatic, infectious, autoimmune, or toxic injuries cause damage to cells, leading to the release of "danger-

associated molecular patterns (DAMPs)". These DAMPs activate the innate immune system through "pattern recognition receptors"[11]. Chronic inflammatory response mediated by DAMPs and the recruitment and activation of innate or adaptive immune cells play a critical role in initiating and perpetuating the activation of profibrogenic cells into myofibroblasts through the release of cytokines, chemokines, and reactive oxygen species (ROS). ROS and oxidative stress can induce hepatocyte injury, cell death, and parenchymal cell proliferation, along with altered remodeling and increased expression of tissue inhibitors of metalloproteinases[12]. Additionally, certain cytokines produced by damaged cells, such as interleukin (IL)-1a, IL-33, and others, directly or indirectly promote the development of a Th2 immune response, which is believed to promote fibrosis. Th2 immune response is recognized to have profibrotic properties through the release of IL-4, IL-5, IL-10, and IL-13[11,13]. However, the exact mechanisms and interactions between these processes are still being investigated in the context of PSC pathogenesis.

Role of Bile Ducts in PSC

Cholangiocytes can be activated by various insults such as infections, cholestasis, *etc*., leading to increased proliferation along with pro-fibrotic and pro-inflammatory secretions through pleiotropic autocrine and paracrine mechanisms[14]. Persistent biliary cell damage causes an inflammatory reaction that leads to a pathological reparative reaction with excessive deposition of scar tissue around the injured ducts. The biliary epithelium is exposed to cytokines and chemokines secreted by innate and adaptive immune cells in response to DAMPs. If biliary homeostasis is not restored, there will be a maladaptive chronic inflammatory response stimulating the deposition of connective tissue (Figure 1)[14].

Genetic and Environmental factors in pathogenesis of PSC

Genetic factors play a significant role in PSC pathogenesis. Studies have demonstrated an increased risk of PSC among first-degree relatives of patients with the disease[15]. Genome-wide association studies have identified over 20 susceptibility genes for PSC, with the human leukocyte antigen (HLA) complex on chromosome six showing the strongest association[16-20]. Patients with PSC exhibit chromosomal instability and immunosenescence, as evidenced by higher rates of short telomere length and telomere aggregates compared to patients with IBD[21,22]. It is important to note that genetic findings explain less than 10% of the disease liability, while environmental factors account for over 50% of it[22].

The microbiome has also been implicated in PSC pathogenesis, with bacteria potentially triggering an aberrant immune response and perpetuating inflammation[23]. Studies have shown an enrichment of *Barnesiallaceae* and *Blautia* families and *Barnesiellacaeae* genus in PSC patients. Microbiome shifts associated with PSC are observed in *Clostridiales* and *Bacteroidales* orders, with more than 80% of shifts occurring within the former order. However, the causal relationship between these shifts remain unclear due to limited sample size[24]. Some environmental triggers have been investigated, indicating a higher prevalence of PSC in rural areas and a possible connection to agricultural activities, pesticides, or fertilizers[25]. Close contact with dogs or cats has also been identified as a potential trigger, suggesting the pathogenic role of an unidentified agent such as a toxin or microbiome[26]. Additionally, coffee consumption and smoking have been suggested as protective factors against PSC[27].

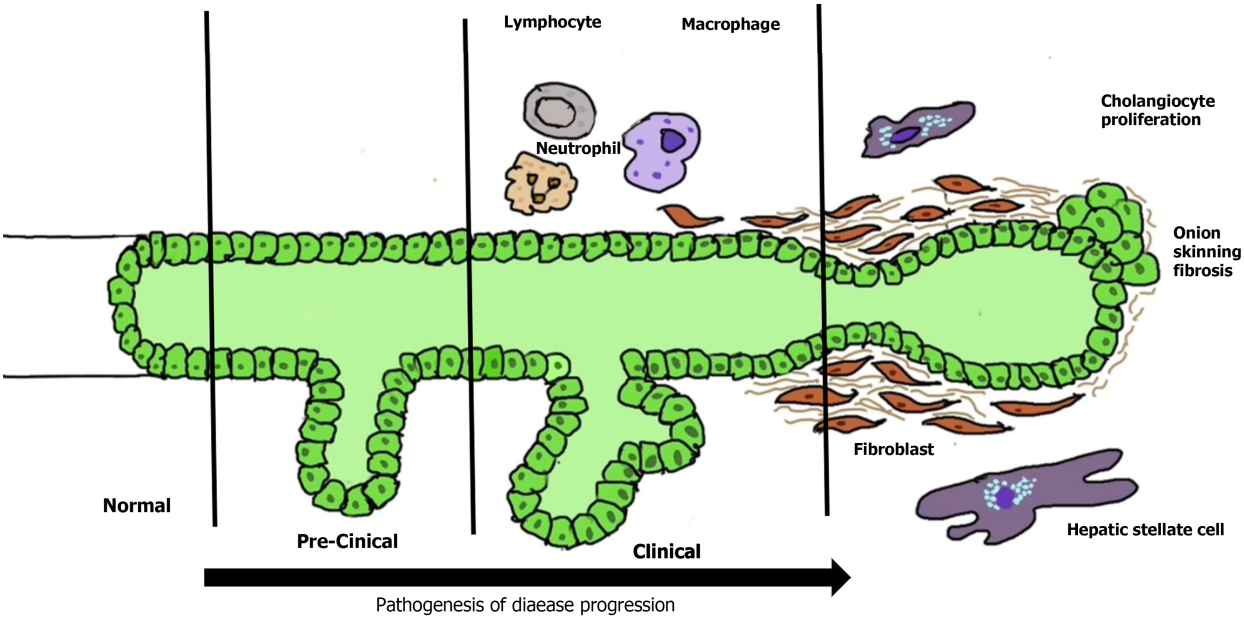
Clinical Presentation and Diagnosis of PSC

PSC is characterized by bile duct injury and fibrosis, leading to a variety of symptoms and signs. It is commonly associated with IBD[12]. It typically affects individuals between the ages of 30 and 40, with a higher prevalence in men [28]. PSC patients are identified during general health examinations or the investigation for another disease, and about 50% patients are asymptomatic[29]. When symptoms occur, the most common are pruritus, fatigue, and right upper quadrant pain; less frequent symptoms are weight loss and fever[29,30]. Physical exam often reveals jaundice, hepatomegaly, splenomegaly, and excoriation marks[29,31]. PSC is a progressive cholestatic liver disease associated with complications such as bacterial cholangitis, dominant strictures, gallbladder polyps, adenocarcinoma, and cholangiocarcinoma (CCA)[29,30,32]. Disease progression may differ in children due to absence of other risk factors like alcohol abuse or polypharmacy that can lead to faster progression of the liver disease[33].

Diagnosis of PSC relies on the presence of cholestasis markers [alkaline phosphatase (ALP) and gamma-glutamyl (GGT) transferase], characteristic bile duct changes on imaging and the exclusion of secondary causes[29,20]. The elevation of serum ALP is the commonest marker[29,32]. ALP is not reliable in children as it can be elevated due to high bone turnover. So, GGT transferase is more commonly used as a diagnostic marker in the pediatric population[34]. However, the transient blockage of the strictured bile ducts can create fluctuations in ALP and bilirubin levels. The total serum bilirubin level is usually normal, but an increase or fluctuations in bilirubin levels indicate the presence of dominant strictures or advanced liver disease[32,35]. The dominant strictures are present in around 45% of adult patients at the diagnosis of PSC as compared to < 5% in the pediatric population[34]. Additionally, serum aminotransferases are elevated 2-3 times the upper limit of normal[29]. In cases of a high level of serum aminotransferases, autoimmune hepatitis should be ruled out[36].

PSC is commonly associated with an underlying IBD, with ulcerative colitis (UC) being the most prevalent. Both PSC and UC have an autoimmune component, which is reflected in the presence of autoantibodies. The most frequently reported autoantibodies in PSC and UC are perinuclear antineutrophil cytoplasmic antibodies, found in 26%-94% of PSC cases and 50%-70% of UC cases[37,38]. Additional autoantibodies reported in PSC include antinuclear antibodies (present in 8%-77% of patients) and smooth muscle antibodies (present in 0%-83% of patients)[32,38]. However, it's important to highlight that these autoantibodies lack specificity and are not necessary for a diagnosis of PSC.

Imaging techniques such as abdominal X-ray and ultrasound can reveal abnormal bile ducts and exclude gallstones. However, these techniques are unable to provide a clear view of intrahepatic biliary ducts. Additionally, sclerosis does not dilate the ducts enough to be seen on imaging, resulting in suboptimal assessment in suspected cases of PSC[39]. For



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Figure 1 Pathogenesis of disease progression in primary sclerosing cholangitis.

this reason, cholangiography assessment is essential for the diagnosis of PSC, as the morphological features of PSC mainly involve biliary ductal changes, while liver parenchymal changes develop later[39]. The common imaging findings in PSC seen on MRCP or endoscopic retrograde cholangiopancreatography (ERCP) include intrahepatic and extrahepatic bile duct strictures, which alternate with normal or dilated bile ducts showing a beading appearance (Figure 2)[39-41]. MRCP is preferred as an initial non-invasive imaging method, while ERCP is reserved for therapeutic interventions[30,32,42,43]. The sensitivity and specificity for ERCP in the diagnosis of PSC are 89% and 80% respectively[44]. MRCP has shown a sensitivity of 86% and specificity of 94% for diagnosis of PSC, with superior cost-effectiveness compared to ERCP[45,46].

Staging, Prognosis, and Management of PSC

PSC is staged using a four-stage system first developed by Ludwig *et al*[47] in 1978, which is shown in Table 1. Several good prognostic factors have been identified, including young age, female sex, small duct phenotype, and the presence of Crohn's disease[48]. In early disease, the Mayo PSC risk score can be useful in predicting short-term survival, but it cannot predict the need for LT[49,50]. However, a meta-analysis has shown that the United Kingdom-PSC score and the PSC risk estimate tool are better at predicting long-term risk[51-53]. The components of each prognostic score are listed in Table 2[49,52-57].

The management of PSC focuses on slowing the disease progression and managing its complications. However, there is no definitive treatment to halt the disease process. LT is a viable option for advanced cases and has shown favorable outcomes.

Ursodeoxycholic acid (UDCA), a hydrophilic bile acid, is commonly used in the treatment of cholestatic liver diseases and is extensively studied in PSC[58]. Its mechanisms of action include protecting cholangiocytes against cytotoxic hydrophobic bile acids in early stages, stimulating hepatobiliary secretion in more advanced stages, and protection of hepatocytes against bile acid-induced apoptosis in all stages[59,60]. UDCA has been shown to improve liver function tests, its impact on survival rates, prevention of CCA, or clinical symptoms is inconclusive[61-63]. However, other data has shown that meaningful reductions in ALP levels have been associated with better outcomes in PSC[64-66]. In addition, withdrawing UDCA may be associated with increase in fatigue, pruritus, liver biochemistries, and Mayo PSC risk score[58,67]. American Association for the Study of Liver Diseases (AASLD) updated their guidelines on PSC management in 2022, to suggest a dose of 13-23 mg/kg/d of UDCA, with continued use if there is a reduction or normalization of ALP levels and/or improvement of symptoms after 12 mo of treatment[68].

Immunosuppressive therapies, including glucocorticoids, cyclosporine, tacrolimus, methotrexate, and mycophenolate mofetil, have been explored for PSC treatment. However, a systematic review concluded that these agents, either as monotherapy or in combination do not reduce the risk of mortality or LT, and monotherapy may increase adverse effects [69]. Recent findings from a meta-analysis suggest that immune-modulating therapy may benefit patients with high baseline levels of ALP (> 420 U/L) and aspartate transaminase (> 80 U/L)[70]. Nevertheless, immunosuppressive agents should be reserved for patients with overlap syndromes such as autoimmune hepatitis-PSC or IgG4-associated cholangitis[68]. Ongoing clinical trials are investigating potential treatments options, such as cilofexor (a nonsteroidal farnesoid X receptor agonist), and 24-nor UDCA (a derivative of UDCA), which show promising results[71,72].

Medical management of PSC has several limitations, despite various drugs being investigated, a recent meta-analysis concluded that there is currently insufficient evidence to show differences in effectiveness measures, such as mortality,

Table 1 Staging of primary sclerosing cholangitis based on the four-stage system developed

Primary sclerosing cholangitis stages		Histological finding
Stage I	Portal stage	Presence of portal hepatitis and edema confined to the portal triads with mononuclear infiltration
Stage II	Periportal stage	The inflammation progresses to the periportal space causing periductal fibrosis with dilation of the portal triads. There is absence of bridging necrosis or septal fibrosis
Stage III	Septal stage	Characterized by the presence of fibrous septae and/or bridging fibrosis
Stage IV	Cirrhosis	Established cirrhosis with the presence of fibrous septa and nodular regeneration

Table 2 Overview of the clinical scores for predicting prognosis in primary sclerosing cholangitis and its components that include serum-based biomarkers and clinical features

Clinical scores	Components
Mayo risk score	Age, bilirubin, histological stage, hemoglobin and presence of inflammatory bowel disease
Revised Mayo risk score	Age, bilirubin, albumin, aspartate aminotransferase and variceal bleeding
Amsterdam–Oxford model	Primary sclerosis cholangitis (PSC) subtype, age at PSC diagnosis, albumin, alkaline phosphatase, aspartate aminotransferase, bilirubin and platelets
Short-term United Kingdom-pSC risk score	Bilirubin, albumin, hemoglobin, and platelets count at diagnosis
Long-term United Kingdom-pSC risk score	Age at diagnosis, bilirubin at the second year, alkaline phosphatase at the second year, albumin at the second year, platelets at the second year, presence of extrahepatic biliary disease at diagnosis, and variceal hemorrhage by the second year
Primary sclerosing cholangitis risk estimate tool	Bilirubin, albumin, alkaline phosphatase, platelets, aspartate transaminases, hemoglobin, sodium, PSC duration and age
Model for end stage liver disease	Dialysis at least twice in the past week, creatinine, bilirubin, international normalized ratio and sodium
Child-Pugh score	Bilirubin, albumin, international normalized ratio, ascites and encephalopathy

health-related quality of life, cirrhosis, or LT between any active pharmacological intervention and no intervention[73]. The high risk of bias in most assessed trials further underscores the need for well-designed randomized controlled trials with adequate follow-up in order to improve pharmacological management of patients with PSC. An overview of various clinical trials and meta-analysis assessing the efficacy and adverse effects of medications used in management of PSC is described in Table 3[61-63,69,70,72,74-76].

While most cases of PSC are characterized by multifocal bile duct strictures, a few have a localized high-grade stricture (dominant stricture) superimposed on diffuse disease that can cause jaundice or cholangitis[77]. Furthermore, CCA may appear as a dominant stricture[78,79]. Hence, brush cytology of the biliary tree, endobiliary biopsy, and fluorescence in situ hybridization should be performed to assess it[68]. AASLD recommends ERCP for the evaluation of relevant strictures as well as new-onset or worsening pruritus, unexplained weight loss, worsening serum liver test abnormalities, rising serum cancer antigen 19-9, recurrent bacterial cholangitis, or progressive bile duct dilation[68]. However, it is important to consider that PSC patients undergoing ERCP have an increased risk of bacterial cholangitis and pancreatitis, so antimicrobial prophylaxis should be administered before the procedure[80-84].

Liver Transplant in PSC

Indications for liver transplant in PSC: LT is performed in patients with PSC when medical therapy has reached its limits[85]. PSC is a hepatic condition with a variable clinical course. LT becomes necessary when the patient develops end-stage liver disease and complications related to portal hypertension, such as ascites, hepatic encephalopathy, variceal hemorrhage, or spontaneous bacterial peritonitis[51,85]. In 2006, United Network Of Organ Sharing reported that 6650 patients received liver transplants, while 17221 were on the waiting list[86]. To address the insufficient number of deceased donors and long wait times, living donor liver transplant (LDLT) emerged as an alternative with favorable outcomes for acute and chronic liver diseases, provided appropriate selection criteria[87,88].

In the United States, Model For End Stage Liver Disease (MELD) score is used by Organ Procurement and Transportation in Network (OPTN) to prioritize liver transplant recipients. LT is considered when MELD score is ≥ 15, indicating hepatocellular dysfunction[85]. The MELD score incorporates the patient's serum bilirubin level, international normalized ratio, and serum creatinine level[89]. MELD exceptions for LT are granted to patients with at least two admissions within a 1-year period for acute cholangitis with a documented bloodstream in infection or with evidence of sepsis requiring vasopressors for hemodynamic instability, as well as those with a diagnosis of CCA[90]. The inclusion and exclusion criteria for LT in patients with CCA are detailed in Table 4[68,91]. The LT process involves a multidisciplinary team with different roles, as outlined in Table 5[92].

Table 3 Overview of important clinical trials and meta-analysis assessing medications used in management of primary sclerosing cholangitis

Ref.	Year	Type	Objective	Results							
				Death	Symptoms (fatigue, pruritus)	Liver transplantation	Histological improvement	Marker values (bilirubin, GGT, ALP, ALT or AST)	Cholangiographic changes	Cholangiocarcinoma	Adverse events
Ursodeoxycholic acid											
Shi *et al* [74]	2009	Meta-analysis of RCT (8 RCT, 465 patients)	Evaluate the effect and safety of UDCA in PSC	No significant effect	No significant effect	No significant effect	Significant difference	No significant effect	No significant effect on improvement	No significant difference on incidence	No significant difference on incidence
Othman *et al*[61]	2012	Meta-analysis of RCT (7 RCT, 553 patients)	Investigate the efficacy of UDCA in PSC	No significant effect	No significant effect	No significant effect	No significant effect	Significantly decrease ALP, GGT, bilirubin, ALT or AST	No significant effect on improvement	No significant difference on incidence	No significant difference on incidence
Poropat *et al*[62]	2011	Meta-analysis of RCT (8 RCT, 592 patients)	Assess the beneficial and harmful effects of BA for patients with PSC	No significant effect	No significant effect	No significant effect	No significant effect	Significantly decrease ALP, GGT, bilirubin or AST. Not significant effect on albumin	No significant effect on improvement	No significant difference on incidence	No significant difference on incidence
Triantos *et al*[63]	2011	Meta-analysis of RCT (8 RCT, 567 patients)	Evaluate if UDCA is useful for PSC	No significant effect	No significant effect	No significant effect	No significant effect	Not reported	Not reported	No significant difference on incidence	Not reported
Immunosuppressive therapies: glucocorticoids, cyclosporine, tacrolimus, methotrexate and mycophenolate mofetil											
Peng *et al* [69]	2017	Meta-analysis of RCT (7 RCT, 266 patients)	Evaluate the safety and efficiency of IA for the treatment of PSC	No significant effect	Not reported	No significant effect	Not reported	No significant improvement on liver biochemistry except AST	Not reported	Not reported	Significant increase on incidence
Liu *et al* [70]	2022	Meta-analysis (7 RCT and 14 observational, 737 patients)	Assess the efficacy and adverse effects of immunomodulators in adult patients with PSC	Not reported	Not reported	Not reported	Not reported	Significantly decrease ALP. Not significant effect on bilirubin and AST	Not reported	Not reported	16.1% of patients had severe AEs[1]
Antibiotics											
Shah *et al* [75]	2019	Meta-analysis of clinical trials (3 RCT and 2 open labeled trials, 124 patients)	Assess the effect of antibiotic therapy (vancomycin, metronidazole, rifaximin and minocycline) in PSC with or without inflammatory bowel disease	Not reported	Not reported	Not reported	Not reported	Significant reduction in ALP and bilirubin	Not reported	Not reported	8.9 % of patients had severe AEs[1]
Probiotics											

Vleggaar *et al*[76]	2008	RCT that included 14 patients	Assess potential beneficial effects of probiotics in PSC	Not reported	No significant effect	Not reported	Not reported	No significant effect on bilirubin, ALP, GGT, AST, ALT, prothrombin, albumin or bile salts	Not reported	Not reported	Not reported
Newer drugs											
Fickert *et al*[72]	2017	RCT that included 161 patients	Evaluate the safety and efficacy of three doses of oral nor UDCA compared with placebo in patients with PSC	Not reported	No significant effect	Not reported	Not reported	Significantly decrease ALP, GGT, ALT or AST	Not reported	Not reported	14 patients had severe AE[1]

[1]Results of this outcome were not included in the meta-analysis. It was derived from the assessment of individual studies. GGT: Gamma-glutamyl; ALP: Alkaline phosphatase; AE: Adverse effects; RCT: Randomized controlled trial; AST: Aspartate Aminotransferase; ALT: Alanine transaminase; PSC: Primary sclerosing cholangitis; UDCA: Ursodeoxycholic acid; BA: Bile acids.

Psychosocial evaluation in liver transplant candidates: Patients with PSC may experience anxiety, depression, substance use disorder, or other psychological symptoms due to the chronic and potentially progressive nature of the disease[93]. 35%-65% of transplant candidates meet criteria for an internalizing disorder as a result of waiting and anticipating surgery[94]. Chronic illness can impact the quality of life, especially in case of conditions like PSC where patients may have unpredictable flares of symptoms. The psychological evaluation can also help in identifying coping strategies that patients can use to manage their symptoms and improve their overall well-being[93]. Various psychological instruments used for psychosocial evaluation in transplant candidates are listed in Table 6[93].

Overall, the medical and psychological evaluation in PSC plays a crucial role in assessing the severity of the disease and identifying any associated conditions, as well as addressing psychological factors that may affect the patient's quality of life[93].

Ethical considerations in liver transplant candidates: Organ transplantation raises significant ethical considerations, making it one of the most controversial disciplines in medicine[95]. Key ethical concerns related to organ retrieval include accurately diagnosing brain death, respecting the patient's known wishes regarding organ donation, and upholding the principle of altruism in living organ donation[96]. When it comes to living organ donors, ensuring their understanding of the surgery's risks, benefits and potential complications is crucial, especially during the informed consent process. Comprehensive discussions on short-term and long-term outcomes should take place at this stage[97]. There is notable regional variability in the application and acceptance of MELD exceptions for LT. A study revealed that, despite OPTN's clinical criteria, nearly 80% of exception applications for PSC and cholangitis were approved by regional review boards regardless of the indication[98]. This highlights the need for a standardized national review board to ensure equitable access to LT for patients with PSC and bacterial cholangitis[98].

Sex-based disparities in organ transplantation are also a concern. The MELD score, which relies on creatinine levels, underestimates true renal function in females due to lower muscle mass. Additionally, men face an increased risk of hepatocellular carcinoma, which can lead to MELD exceptions[95]. A analysis of waitlisted candidates from the scientific registry for transplant recipients showed that Hispanics with MELD scores < 20 had an 8% lower deceased donor liver transplant (DDLT) rate compared to Whites[99]. Asian patients with MELD score < 15, on the other hand, had a 24% higher DDLT rate compared to Whites, but this rate dropped by 46% for Asian patients with MELD scores between 30-40 compared to Whites[99]. As the field of LT continues to evolve, addressing ethical concerns requires filling knowledge

Table 4 Inclusion and exclusion criteria for patients with cholangiocarcinoma being considered for Model For End Stage Liver Disease exception points

Inclusion criteria	Exclusion criteria
Evidence of positive tumor cells or cells strongly suspicious for CCA on biopsy	Evidence of extra hepatic disease or lymph node enlargement
Malignant appearing stricture on radiograph and 1 of the following criteria (a or b or c)	Previous malignancy excluding skin or cervical cancer within 5 yr before diagnosis of cholangiocarcinoma
a. Ca 19-19 > 100 U/mL in the absence of acute bacterial cholangitis	History of abdominal radiotherapy
b. Polysomy on fluorescence in-situ hybridization	Uncontrolled infection before treatment
c. Hilar mass < 3 cm in radial diameter on cross-sectional imaging	Prior attempt of surgical tumors reaction and subsequent violation of tumor plane
-	Any medical condition precluding transplantation
-	Any transperitoneal biopsy including percutaneous and/or endoscopic ultrasonographic-guided fine needle aspiration

CCA: Cholangiocarcinoma.

Table 5 Role of multidisciplinary team involved in the process of liver transplantation

Role	Description
Transplant hepatologist	A medical doctor who specializes in liver disease
Liver transplant surgeon	Evaluates the patient and determines whether a liver transplant is the best option by considering surgical contraindications
Transplant nurse coordinator	Serves as the primary contact for the patient throughout the transplant process, ensures that testing is up-to-date, and provides education on the transplant process
Transplant social worker	Focuses on the psychological and social aspects of end-stage liver disease and provides mental health support as needed
Transplant nutritionist	Assesses the patient's nutritional status, including weight patterns and dietary intake, and makes recommendations for an optimal diet
Financial coordinator	Reviews the patient's medical insurance coverage and assists with obtaining adequate coverage for the transplant
Transplant pharmacist	Reviews the patient's medication list for any contraindications before the transplant and provides education on new medications after the transplant

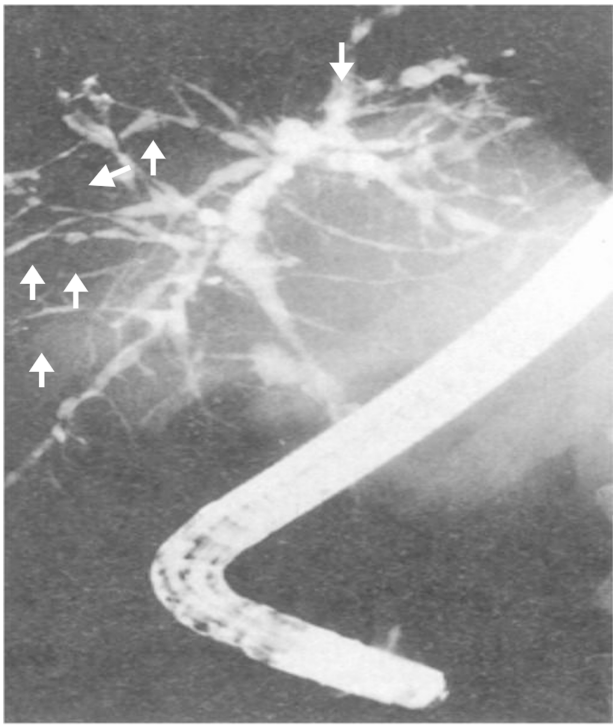
gaps with robust and carefully gathered data that go beyond the informed consent of donors[95].

Outcomes of liver transplant in patients with PSC: The one-year and five-year survival rates were better in patients with LT for impaired quality of life (97.4% and 94.9%) as compared to patients with LT for end-stage liver disease (91.4% and 88.6% respectively) based on a retrospective study on 74 patients with LT[100]. The one-year and five-year survival rates for patients with suspicion of neoplasia prior to the LT were 95.8% and 74.1% respectively[100]. A larger study of 6071 patients had similar outcomes with patient survival rate of 89.7%, 79.8%, 70.7%, 58.3%, 43.8% and 20.4% respectively at 1, 5, 10, 15, 20, and 30 years respectively[101]. Based on a study of 6911 LT patients from the OPTN database, the unadjusted survival rate was significantly higher among the LDLT group as compared to the DDLT group[102]. The most common factors associated with death after LT were infections, malignancies, cardiovascular diseases, graft failure (GF) due to rejection, and hepatic artery thrombosis (HAT)[101,103,104].

Patients with LT for PSC have an acute cellular rejection (ACR) rate of 20-40%, requiring additional immunosuppression[105]. ACR does not affect long-term graft or survival outcomes in patients with LT, as opposed to patients with renal transplant[105]. A retrospective study of patients with a diagnosis of PSC (24 patients) and PSC-autoimmune hepatitis overlap (2 patients) without evidence of CCA at the time of LDLT showed allograft rejection successfully managed by immunosuppression in 11.5% patients, postoperative bile leak in 7.6% patients managed conservatively, and biliary stricture in 11.5% patients with successful ERCP and biliary stent placement[103]. Biliary strictures and bile leaks are other common complications after LT with an incidence of 5%-15% in patients who received DDLT and 28%-32% in recipients of right lobe LDLT[106]. The mean time interval for presentation of biliary strictures after LT is 5-8 mo[106]. Biliary strictures are classified into anastomotic variant and non-anastomotic (NAS) variant. NAS variants may be caused due to HAT and non-HAT etiologies like chronic ductopenic rejection, ABO incompatibility, PSC causing recurrent or ischemic strictures, age of the donors, duration of use of vasopressors, prolonged cold and warm ischemia times, preservative injury, and donation after cardiac deaths[106]. NAS variants related to ischemia usually present within one year and those related to immunological factors present after one year of LT[107].

Table 6 Psychological instruments for psychosocial evaluation of transplant patients

Instrument name	Description	Scoring/rating	Reliability	Validity
Beck depression inventory	Self-report measure of depressive symptoms	21-item scale, higher scores indicate more severe depressive symptoms	High test-retest reliability, internal consistency, and concurrent validity	Established validity in measuring depressive symptoms in various populations
Hamilton depression rating scale	Clinician-rated scale to assess severity of depressive symptoms	17-item scale, higher scores indicate more severe depressive symptoms	High inter-rater reliability, internal consistency, and concurrent validity	Established validity in measuring depressive symptoms in various populations
General health questionnaire	Self-report measure of general mental health	12-item or 28-item scale, higher scores indicate poorer mental health	High internal consistency, test-retest reliability, and concurrent validity	Widely used in assessing mental health in general populations
Primary care evaluation of mental disorders-patient health questionnaire	Self-report measure of common mental disorders	9-item scale, higher scores indicate greater severity of mental disorder symptoms	High sensitivity and specificity, test-retest reliability, and convergent validity	Widely used in primary care settings to screen for mental disorders
Transplant evaluation rating scale	Clinician-rated scale to assess psychosocial functioning in transplant recipients	10 aspects of psychosocial functioning rated on a 5-point scale, higher scores indicate better adjustment	Good inter-rater reliability and validity in liver transplant recipients	Specific to evaluating psychosocial functioning in transplant recipients
Psychosocial assessment of candidates for transplantation	Clinician-rated scale to assess psychosocial acceptability of transplant candidates	8 subscales rated on a 5-point scale, with initial and final overall ratings	Established reliability and validity in evaluating psychosocial acceptability of transplant candidates	Widely used in evaluating transplant candidate suitability
Stanford integrated psychosocial assessment for transplantation	Clinician-rated scale to assess psychosocial functioning in transplant candidates	Comprehensive assessment covering multiple domains of psychosocial functioning	Limited data on reliability and validity, but shows promise in transplant candidate evaluation	Developed specifically for evaluating psychosocial functioning in transplant candidates



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Figure 2 A cholangiography showing common findings in primary sclerosing cholangitis *i.e* the presence of multiple short narrowings (shown by arrows) and dilatations in the intra and extrahepatic bile ducts, creating a distinctive "beaded pattern".

A study based on the review of 22 publications with a total of 1399 patients who underwent LT for PSC showed that the recurrence rate of PSC was around 18.5%, ranging from 5.7%-59.1%[108]. Another study with a patient population of 230 had a recurrence rate of 23.5% with a median of 4.6 years after LT[108]. Some of the most common factors related to an increased risk of recurrence of PSC are presence of HLA-DRB1*08 in the donor or recipient, absence of donor HLA

DR52, older and younger recipients, male recipients, development of UC after LT, requirement of a longer duration of maintenance therapy with steroids (> 3 mo), steroid resistant ACR, and the presence of CCA or concurrent infection with cytomegalovirus in the donor[108]. Due to a high recurrence rate of PSC in patients and a 4-fold increase in the risk of GF or mortality within 5 years of LT; liver re-transplant (ReLT) is considered to extend survival[109].

Quality of life and psychological outcomes after liver transplant: The health-related quality of life (HRQOL) and employment after LT depends on the etiology of the ESLD. In a cross-sectional study of 356 patients post LT, the return to employment rates within six months were highest amongst patients with PSC (2.4 times) and alcoholic cirrhosis (2.5 times) as compared to patients with primary biliary cirrhosis. However, post LT HRQOL was comparable among different ESLD etiologies[110]. Early retirement was also significantly higher, reaching 83% in patients with PSC[110]. Most commonly reported symptoms of physical distress after LT were fatigue, muscle weakness, increased appetite, headache, backache, and bruising which were higher in females over one year as compared to men[111]. The most commonly reported symptoms of psychological distress at one year were sleeplessness and mood swings, followed by nervousness, depression, and difficulty concentrating[111]. Recipients of LT rated their overall health as 7.17 ± 2.22 out of a possible score of 10 based on a questionnaire adapted from Karnofsky functional performance scale, medical outcomes study short form (SF-36), and psychosocial adjustment to illness scale, with 10 being the best outcome[112]. The greatest benefit reported post LT was "being alive". The worst factor reported about being a LT recipient was dependence on medications and the cost of insurance and medications[112].

Future directions in management of PSC: Recent advancement in digital technology have opened up new possibilities of enhancing the understanding of liver anatomy and vascular structures through the creation of three-dimensional (3D) liver models using data from computed tomography and magnetic resonance imaging scans[113]. PSC often involves the development of strictures and narrowing of the bile ducts, making LT surgery more challenging[114]. The emerging technique of 3D liver transplant offers surgeons assistance in planning surgical procedures, including precise identification of blood vessels and bile ducts, improving the accuracy and efficiency of transplant surgeries[115,116].

Stem cell therapy and gene therapy represent two emerging treatments with potential for managing PSC. Stem cell therapy involves using stem cells to repair and regenerate damaged liver tissue[117]. Various types of stem cells , such as mesenchymal stem cells, induced pluripotent stem cells, and embryonic stem cells, have been investigated for their potential in treating liver diseases, including PSC. Studies indicate that stem cell therapy may reduce inflammation and promote tissue regeneration in PSC cases[117,118]. Gene therapy, on the other hand, utilizes genes to modify the expression of specific proteins involved in the development and progression of the disease. One potential target for gene therapy in PSC is the nuclear factor kappa B (NF-κB) pathway, which plays a role in liver inflammation regulation. Inhibiting the NF-κB pathway has shown promise in reducing inflammation and fibrosis in PSC[119]. Despite the potential benefits of stem cell and gene therapies in PSC, further research is needed to determine their safety and efficacy. Ongoing clinical trials are evaluating the use of these therapies in PSC treatment, and their outcomes will determine their future role in managing the disease[117].

CONCLUSION

LT plays a very crucial role in the management of patients with PSC due to limited options and studies on outcomes with medical management. This review offers a summary of clinical features, diagnosis, medical management and a detailed discussion on the indications, clinical and psychosocial outcomes, ethical dilemmas, and future aspects in the field of liver transplant for management of PSC. The review also highlights the important aspect of pReLT psychosocial evaluation, as well as psychosocial outcomes post-transplant, which plays a pivotal role in preventing mental health crises in the patients. Significant efforts need to be directed towards addressing the ethical issues in liver transplant for equity of the care. Patients with PSC will also greatly benefit from more advances in the medical management of PSC.

FOOTNOTES

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MINIREVIEWS

Prognostic and diagnostic scoring models in acute alcohol-associated hepatitis: A review comparing the performance of different scoring systems

Jad Mitri, Mohammad Almeqdadi, Raffi Karagozian

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Abstract

Alcohol-associated hepatitis (AAH) is a severe form of liver disease caused by alcohol consumption. In the absence of confounding factors, clinical features and laboratory markers are sufficient to diagnose AAH, rule out alternative causes of liver injury and assess disease severity. Due to the elevated mortality of AAH, assessing the prognosis is a radical step in management. The Maddrey discriminant function (MDF) is the first established clinical prognostic score for AAH and was commonly used in the earliest AAH clinical trials. A MDF > 32 indicates a poor prognosis and a potential benefit of initiating corticosteroids. The model for end stage liver disease (MELD) score has been studied for AAH prognostication and new evidence suggests MELD may predict mortality more accurately than MDF. The Lille score is usually combined to MDF or MELD score after corticosteroid initiation and offers the advantage of assessing response to treatment a 4-7 d into the course. Other commonly used scores include the Glasgow Alcoholic Hepatitis Score and the Age Bilirubin international normalized ratio Creatinine model. Clinical AAH correlate adequately with histologic severity scores and leave little indication for liver biopsy in assessing AAH prognosis. AAH presenting as acute on chronic liver failure (ACLF) is so far prognosticated with ACLF-specific scoring systems. New artificial intelligence-generated prognostic models have emerged and are being studied for use in AAH. Acute kidney injury (AKI) is one possible complication of AAH and is significantly associated with increased AAH mortality. Predicting AKI and alcohol relapse are important steps in the management of AAH. The aim of this review is to discuss

the performance and limitations of different scoring models for AAH mortality, emphasize the most useful tools in prognostication and review predictors of recurrence.

Key Words: Alcohol-associated hepatitis; Prognostic scores; Mortality; Maddrey discriminant function; Model for end stage liver disease; Acute kidney injury

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Core Tip: Clinical prognostic scores for alcohol-associated hepatitis (AAH) are reliable and commonly minimize the need for histological assessment. Model for end stage liver disease (MELD) score is recently showing superiority compared to the commonly used Maddrey Discriminant function for AAH prognostication. Combining MELD at diagnosis with day 4 (or day 7) Lille score when managing severe AAH would be interesting to validate as a superior mean of assessing AAH prognosis. Acute kidney injury is a complication of AAH with significant impact on mortality. It is therefore important to account for when managing AAH.

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INTRODUCTION

Alcohol consumption could result in numerous liver diseases, the most severe one being alcohol-associated hepatitis (AAH). AAH, otherwise known as alcoholic hepatitis, is clinically characterized by rapidly progressing jaundice, malaise, tender hepatomegaly, and discreet systemic inflammatory response syndrome (SIRS) features[1]. While the burden of this disease is well known, little improvement in survival has been noted over the years[2]. Therefore, research and development for AAH are desperately needed to improve patients' outcomes and reduce its morbidity and mortality. In fact, since the coronavirus disease 2019 pandemic, the incidence of AAH has increased by over 50%, with a subsequent increase in referrals to liver transplant centers for patients with AAH[3,4]. Additionally, liver transplant waiting list additions increased by 105.6% and liver transplant recipients increased by 411.8% in patients with AAH[5]. The mortality of AAH may be as high as 30% at 28 d and surpass 50% at 1 year[1]. Several prognostic scores have been created and studied throughout the years in an attempt to predict the mortality of AAH. For instance, the Maddrey discriminant function (MDF), conceived in 1978, has been the first[6] and the most discussed score for the assessment of disease severity and guidance of treatment initiation. However, emerging data has supported other prognostic scores such as the model for end stage liver disease (MELD) score. Among others scores, the dynamic Lille score is renowned for its ability to assess the response of AAH to therapy as the disease progresses. There is no consensus regarding the superiority of one score compared to the other. This review aims to discuss the most recent evidence regarding the clinical relevance and performance of the available AAH prognostic scores.

DIAGNOSIS AND SEVERITY OF ALCOHOL-ASSOCIATED HEPATITIS: CLINICAL, BIOLOGICAL AND HISTOLOGICAL FEATURES

AAH is a potentially fatal complication of chronic alcohol abuse that commonly occurs after a sudden increase in alcohol consumption. Although AAH may present abruptly, it most often progresses insidiously over days or weeks with patients complaining of fatigue and malaise followed by anorexia, nausea and vomiting before developing ascites or jaundice[7]. While the latter are the most important symptoms for diagnosis in clinical practice settings, other signs and symptoms may be seen including tender hepatomegaly, low-grade fever, and abdominal pain[8]. The diagnosis is mainly clinical, however abdominal imaging should also be performed to rule out obstructive biliary disease. Additional workup should rule out acute viral hepatitis, severe autoimmune liver disease and Wilson disease[9,10]. The gold-standard diagnostic test remains liver biopsy. The decision of performing biopsy should be guided by the pre-test probability of AAH and should consider the risk of complications such as bleeding. As per the AAH consortia in 2016 outlining the consensus criteria for the diagnosis of AAH[9], the clinical diagnosis of AAH is based on the presence of typical clinical features as well as laboratory tests that help rule out other causes of liver injury and guide treatment decisions. The most important clinical feature is the onset of jaundice within 8 wk of heavy alcohol consumption overlying a daily consumption that is superior to 40 g/d in women and 60 g/d in men for at least 6 mo. Serum bilirubin level is typically above 3 mg/dL. Other important features include elevated transaminases (aspartate aminotransferase AST > 50, AST to alanine aminotransferase ratio ALT ratio > 1.5) that do not surpass 400 IU/L[9].

The diagnosis of definite, probable, or possible AAH depends on the presence of those typical clinical features as well as laboratory tests that help rule out other causes of liver injury and guide treatment decisions (Table 1)[9,11]. While histological confirmation remains the gold standard, this approach not always necessary when other clinical and laboratory features are clearly suggestive of AAH. Liver biopsy may also help guide treatment decisions in some cases, for example, when there is uncertainty about the severity of liver injury or if drug-induced liver injury is suspected[10]. Histological features of AAH include microvesicular steatosis, periportal Mallory-Denk bodies, and neutrophilic infiltration in the portal areas. Altamirano *et al*[12] proposed an AAH histologic scoring system that included degree of fibrosis, neutrophil infiltration, type of bilirubinostasis, and presence mega-mitochondria. The authors were able to demonstrate that this scoring system was correlated with severity of liver dysfunction as well as mortality. However, this scoring system has not yet been validated in large cohorts and thus not yet routinely used clinically.

While histologic scoring systems may help to assess the severity of AAH, practically, clinical features remain the most important practical determinant of prognosis[8]. Patients with more severe disease are more likely to require hospitalization and have a higher mortality rate. Multiple clinical scoring systems that assess the severity of liver disease of any cause exist, including MELD, Child-Turcotte-Pugh and Chronic Liver Failure Consortium-C (CLIF-C) ACLF (acute-on-chronic liver failure) scores[13]. Some scores were particularly aimed at predicting outcomes in AAH and are widely used including MDF and Age Bilirubin international normalized ratio (INR) Creatinine model (ABIC). Scoring criteria, clinical application and interpretation of relevant AAH prognostic scores are detailed in Table 2. While all these scoring systems have some value, they are far from perfect and need to be interpreted with caution. Head-to-head comparisons of these scores are lacking and it is unclear which, if any, is superior.

Patients with severe AAH have increased short-term mortality rates, causes include portal hypertension complications, multiorgan failure (liver, kidney) and infections[14]. Assessment of the severity of AAH remains a complex task that requires careful clinical evaluation as well as consideration of multiple laboratory and imaging tests. The relevance of clinical prognostic scores to AAH pathophysiology is explained and illustrated in Figure 1.

PROGNOSTIC SCORING SYSTEMS

Maddrey discriminant function

MDF was the first prognostic score found for AAH. It was found through the discriminant analysis of biologic parameters associated with mortality in AAH. This was how, in an early clinical trial, Maddrey *et al*[6] found an independent association with death from AAH between the increase in prothrombin time (PT) and total bilirubin levels at the start of their study.

Discriminant Function = 4.6 × (Pt's PT-control PT) + TBili.

Data on MDF sets the cutoff for severe AAH at 32, where patients with a score lower than 32 have a proven survival rate of 90% at 30 d without steroid therapy, which defines AAH as mild to moderate when MDF < 32. On the other hand, patients with MDF 32 or higher showed mortality exceeding 20%-30% at 30 d (severe AAH = MDF ≥ 32), and can be used as a threshold for initiation steroid therapy if no contraindications exist[15].

MDF was largely used in randomized controlled trials evaluating benefit of steroid therapy in AAH, which reported heterogenous results. In a meta-analysis of 418 patients with AAH, decreased 1-mo mortality after corticosteroid therapy *vs* placebo was proven only in severe AAH (defined as MDF > 32) or in patients with hepatic encephalopathy (relative risk reduction of 36%)[16].

In a post-hoc multivariate analysis of the STOPAH trial (a large study that studied the effect of prednisolone *vs* pentoxifylline on 28-d mortality), treatment with prednisolone displayed significantly improved survival at 28 d, which was limited to short term mortality when MDF > 32. No significant difference in mortality at 90 d or 1 year was found. Of note, the original STOPAH trial did not show any benefit with prednisolone *vs* placebo on 28-d mortality[17]. Because the trial was stopped prematurely (difficult to follow patients out long-term), 33 individuals were not included in 90 d or 1 year follow up and another 159 could not be followed for a full year. Even though the investigators met their goal enrollment of 1026 patients, the lower mortality than expected and use of MDF without liver biopsy probably led to many misclassifications. Furthermore, no taper was used in prednisolone treatment which may have caused harm to patients when they stopped taking the medication.

The above evidence elicits the role of MDF in AAH severity assessment and treatment decisions since it was commonly applied in the concerned trials. However, it has some drawbacks which give grounds for studies on evaluating potential superiority of other scores. MDF is calculated using PT and bilirubin. Despite its wide use in mortality prediction, the MDF lacks some important components which would strongly predict prognosis, such as serum creatinine[18]. Moreover, PT is dependent on the control subject measurement, which creates variability among laboratories. These drawbacks make it mandatory to review research on other scores that might display better performance in AAH mortality prediction than MDF. There have been increasing reports that the MELD can exhibit superiority in AAH mortality prediction compared to MDF[18,19].

MELD score

The MELD is based on INR, bilirubin and creatinine. The new MELD-Na score also encompasses sodium levels. It is a widely used tool in prognostic and severity assessment of AAH. MELD score demonstrates comparable performance to MDF in mortality prediction at 1 mo (Se = 86%, Sp = 86%)[20,21] and at 90 d (Se = 75%, Sp = 75%). For example, a MELD score above 20 predicts 20% mortality at 90 d[22]. Concerning the initiation of corticosteroid therapy in patients with AAH, benefit was proven for patients with MELD > 20, with evidence being the strongest in the range 25 to 39[23]. MELD

Table 1 Alcohol-associated hepatitis diagnosis probability in clinically suspected Alcohol-associated hepatitis[11]

Category	Potential confounding factors[1]	Biopsy indication
Definite AAH	N/A	AAH clinically diagnosed and biopsy proven. Biopsy may inform of the mechanism of injury
Probable AAH	No confounding factors	AAH clinically diagnosed, biopsy not indicated
Possible AAH	Potential confounding factor present	AAH clinically diagnosed but biopsy is indicated for confirmation

[1]Potential confounding factors: (1) Possible ischemic hepatitis (hypotension, severe upper gastrointestinal bleed, cocaine use within 7 d); (2) Possible metabolic liver disease (Wilson disease, alpha 1 antitrypsin deficiency); (3) Hepatotoxic medication within 30 d of onset of jaundice (drug-induced liver injury); (4) Uncertain alcohol intake (if the patient denies excessive alcohol use); (5) Atypical liver function test pattern (aspartate aminotransferase < 50 or > 400 IU/L, aspartate aminotransferase/alanine aminotransferase < 1.5); and (6) positive antinuclear antibody ANA > 1:160 or smooth muscle antibody SMA > 1:80; AAH: Alcohol-associated hepatitis.

Table 2 Alcohol-associated hepatitis prognostic scores: Components, purpose, clinical application and interpretation

Clinical score	Components	Purpose	Clinical application	Interpretation
MELD	INR, bilirubin (total), Creatinine, Sodium	Assess severity of liver disease and predict short-term mortality	Calculate on initial presentation	MELD ≥ 20 = severe AAH
Maddrey Discriminant function	PT (measured and control), bilirubin (total)	Assess severity and prognosis of alcoholic hepatitis	Calculate on initial presentation	MDF ≥ 32 = severe AAH
GAHS	Age, WBC, BUN, Bilirubin, PT (measured and control)	Assess severity and prognosis of alcoholic hepatitis	Calculate on initial presentation	GAHS ≥ 9 = severe AAH
ABIC	Age, bilirubin, INR, PT (measured and control)	Assess prognosis of alcoholic hepatitis in patients on steroid therapy	Use in patients on steroid therapy	< 6.71 low mortality risk; 6.71-8.99 intermediate mortality risk; ≥ 9.00 high mortality risk
Lille score	Age, bilirubin (initial, and day 4 OR day 7), albumin, creatinine, PT	Assess response to corticosteroid therapy in patients with alcoholic hepatitis	Use in patients on steroid therapy, at day 4 and/or day 7 to assess response and indication to continue steroids	< 0.45 at day 4-7 = favorable response to steroid therapy; > 0.45 at day 4-7 = little/no response to steroid therapy
Alcoholic hepatitis histological score	Histologic features of liver injury	Assess severity and prognosis of alcoholic hepatitis	Calculate on biopsy based on: Fibrosis stage, bilirubinostasis, polymorpho-nuclear infiltration, and megamito-chondria	0-3: Mild AAH; 4-5: Moderate AAH; 6-9: Severe AAH

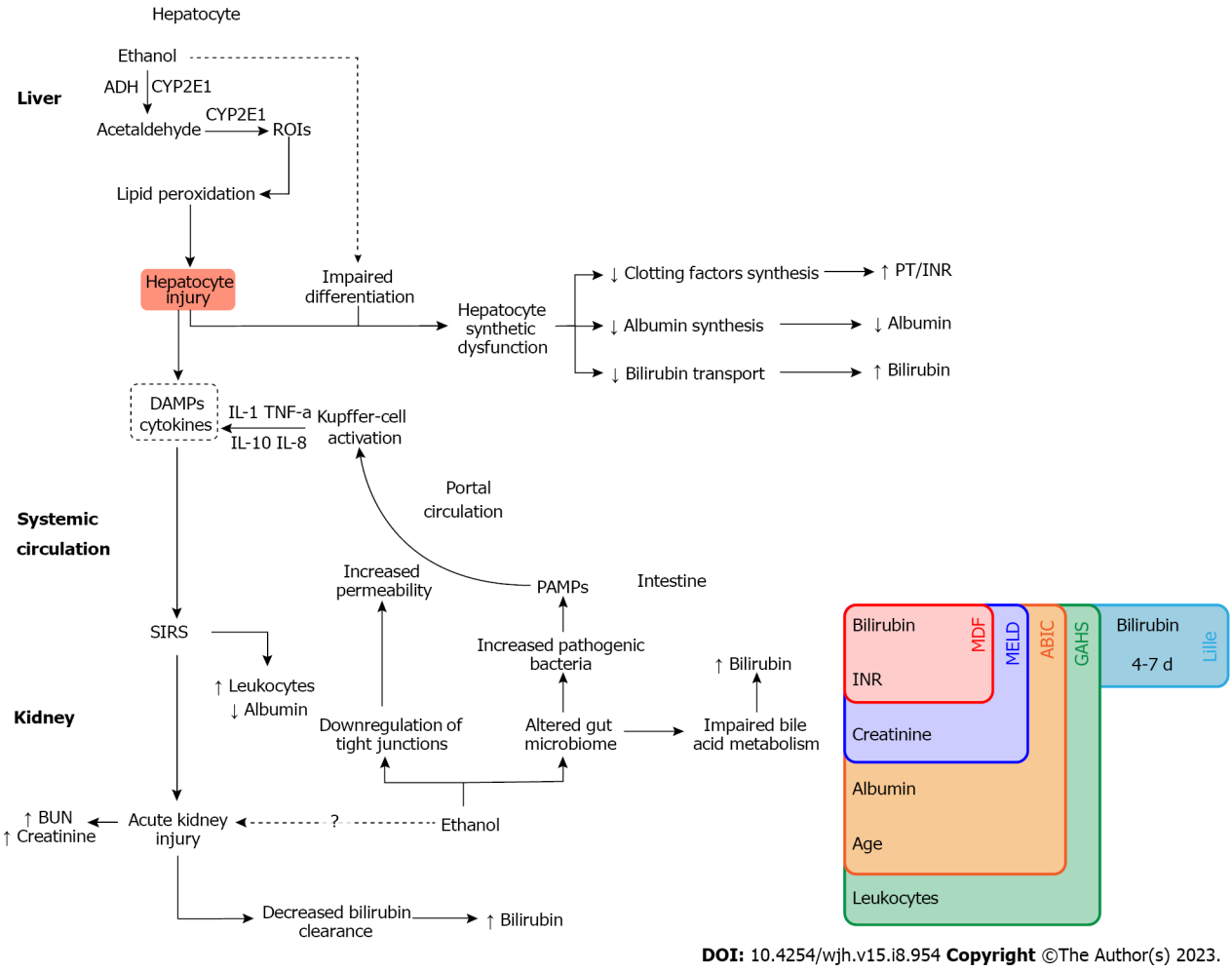
MELD: Model for end-stage liver disease; INR: International normalized ratio; BUN: Blood urea nitrogen; WBC: White blood cells; MDF: Maddrey discriminant function; AAH: Alcohol-associated hepatitis; PT: Prothrombin time; GAHS: Glasgow alcoholic hepatitis score; ABIC: Age-bilirubin-INR-creatinine score.

score is widely used in prioritizing transplant receipt in patients with cirrhosis, that includes patients with severe AAH who are considered to have acute-on-chronic liver failure.

Limitations of MELD score are related to elevated creatinine levels accentuating predicted mortality even when liver function is recovering. Acute kidney injury (AKI) is only one potential complication of AAH, the others (such as portal hypertension, infections, multiorgan failure) are not accounted for in the MELD score. Also, creatinine levels have interpersonal variability with factors such as sex, nutrition, and age, which could also create heterogeneity in MELD score profiles among individuals with similar degrees of hepatic injury, which might not correlate adequately with mortality levels. Sodium levels are also prone to fluctuation related to diuretic/free water administration rather than liver disease. Moreover, it is documented that MELD score for the same individual could differ depending on laboratory measurement of its variables (INR+++ > creatinine > bilirubin)[24-27].

MELD vs MDF: Which score is superior?

Multiple studies have compared MELD and MDF scores in predicting outcomes in AAH. A recent multinational retrospective analysis by Morales-Arráez *et al*[18](n = 2581), proved MELD superiority to MDF with a significant difference in the area under the curve in predicting mortality at 1 mo and 3 mo. The studied population was diversified by recruiting patients throughout 85 tertiary centers in 11 different countries from 3 different continents. The diversity of the population in the study of Morales-Arráez *et al*[18] reinforces findings by a previous analysis of the STOPAH trial by Forrest *et al*[28] in terms of MELD score superiority to MDF in AAH mortality/severity prediction, a finding reflected by



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Figure 1 Pathophysiology of alcohol-associated hepatitis and correlation with prognostic scores. As hepatocytes metabolize ethanol, reactive oxygen species are generated and mediate hepatocyte injury through lipid peroxidation. Injured hepatocytes become unable to adequately perform their functions, this includes albumin and clotting factor synthesis as well as bilirubin transport. Damaged hepatocytes release inflammatory molecules such as danger-associated molecular patterns known as "DAMPs" which favor a systemic inflammatory response system (SIRS). With systemic inflammation, white blood cell count rises, and albumin concentration decreases. SIRS also precipitates acute kidney injury, resulting in a rise in serum creatinine and body urea nitrogen concentrations and causes decreased bilirubin clearance. Large amounts of ethanol alter the gut microbiome and increase intestinal permeability through the downregulation of tight junctions which impairs bile acid metabolism. Furthermore, there is favored growth of pathogenic bacteria that pathogen-associated molecular patterns (PAMPs). PAMPs reach the portal circulation through increased intestinal permeability and activate Kupffer cells in the liver. Which in turn amplifies systemic inflammation and the resulting consequences through cytokine secretion[8]. PAMP: Pathogen-associated molecular patterns; SIRS: Systemic inflammatory response system; BUN: Blood urea nitrogen; MDF: Maddrey discriminant function; INR: International normalized ratio; MELD: Model for end-stage liver disease; GAHS: Glasgow alcoholic hepatitis score; PT: Prothrombin time.

multiple other database analyses of more homogeneous populations around the world[29-31]. More details on the advantages and disadvantages of both scores as well as differences in scoring criteria are listed in Table 3. To analyze the evidence mentioned above: Despite the disadvantages of including creatinine levels in the MELD score and the abundance of MDF use and validation in early AAH trials, accounting for AKI in AAH prognosis is a large advantage of the MELD score over MDF as it addresses an important determinant of AAH mortality. Furthermore, using INR minimizes laboratory-dependent differences in PT values which provides a notable advantage to using MELD over MDF.

A glimpse of other prognostic scores

Glasgow alcoholic hepatitis score: It is based on total bilirubin, age, blood urea nitrogen (BUN), PT and leukocyte count (white blood cell count; WBC). WBC and BUN are variables unique to GAH. GAH has demonstrated superior specificity and accuracy in predicting mortality in comparison to MDF or MELD, however GAH sensitivity to 1- and 3-mo mortality is inferior to MDF or MELD[32]. While the concern for short-term mortality of AAH is substantial, a test with high sensitivity is preferred. GAH adds benefit in clinical decision making by complementing MDF: If MDF > 32, a GAHS 9 or greater is more accurate in predicting mortality, therefore in filtering steroid treatment indications. GAHS was only studied in a relatively homogenous population from one country, population, thus making it solely validated in the United Kingdom[33].

The ABIC model: Age, bilirubin, INR and serum creatinine level classifies patients into categories according to their survival risk. Risk groups are low, medium, and high, with respective survival rates of 100%-70%-25%. ABIC model is

Table 3 Alcohol-associated hepatitis prognostic scores advantages and limitations

Clinical score	Components	Advantages	Limitations
MELD	INR, bilirubin (total), creatinine, sodium	MELD or MELD-Na ≥ 20 predicts high mortality at 30 d, consider corticosteroid therapy	(1) Mortality overestimation with elevated creatinine levels; (2) interpersonal variability of creatinine levels; (3) extrahepatic causes of sodium fluctuations; and (4) does not account for markers of AAH complications other than kidney and liver failure
Maddrey discriminant function	PT (measured and control), bilirubin (total)	MDF ≥ 32 predicts high mortality at 30 d, consider corticosteroid therapy. Oldest, most commonly used score	(1) AKI and other AAH complications not reflected in MDF; (2) PT use instead of INR; and (3) low specificity
GAHS	Age, WBC, BUN, bilirubin, PT (measured and control)	GAHS ≥ 9 is in favor of high mortality, helpful for selecting candidates for steroid treatment	(1) Only studied on the British population; and (2) lower sensitivity for short-term mortality compared to MELD/MDF
ABIC	Age, Bilirubin, INR, PT (measured and control)	Score < 6.71 has high negative predictive value to detect patients with low risk	(1) Not used for deciding on steroid initiation; and (2) low accuracy for predicting mortality in severe group
Lille score	Age, bilirubin (initial, and day 4 OR day 7), albumin, creatinine, and PT	Lille score ≤ 0.45 at day 7 (or 4) implies good response to corticosteroids	(1) Complex to calculate; (2) uses PT instead of INR; and (3) bias secondary to elevated creatinine levels and interpersonal variability of creatinine
Alcoholic hepatitis histological score	Histologic features of liver injury	Can be combined with clinical prognostic scores for more accurate mortality risk stratification	(1) Requires liver biopsy (invasive); and (2) static

MELD: Model for end-stage liver disease; INR: International normalized ratio; BUN: Blood urea nitrogen; AKI: Acute kidney injury; WBC: White blood cells; MDF: Maddrey discriminant function; AAH: Alcohol-associated hepatitis; PT: Prothrombin time; GAHS: Glasgow alcoholic hepatitis score; ABIC: Age-bilirubin-INR-creatinine score.

helpful in prognosticating patients with AAH who were initiated on steroid treatment. However, ABIC model is not commonly used in assessing the indications for treatment initiation[34].

Static vs dynamic scores

As stativity brings bias into prognostic scoring for multiple reasons, some of which are mentioned above, dynamic scoring has been proposed and studied. Lille score adds dynamicity through the incorporation of bilirubin levels at 2 points in time: baseline levels and levels at day 7 of steroid therapy. Lille score is based on the concept that a decrease in bilirubin levels at the first week of treatment is a sign of good prognosis meaning that a score lower than 0.45 is suggestive of steroid treatment benefits outweighing the risks[35]. On the other hand, a Lille score higher than 0.45 reflects a lack of response to steroids and therefore a low likelihood of benefiting from additional days of treatment[36]. New studies are in favor of calculating Lille score at day 4 with comparable performance to day 7, this reduces the limitation of having to wait for 7 d[37].

Despite recent studies favoring MELD over MDF, combining MELD score (static) with Lille score (dynamic) would be interesting to evaluate on large populations in future studies on this matter given fluctuating course of disease, need for treatment response assessment and superior performance of combinations compared to single scores.

One limitation of clinical prognostic scores is performance in the long term. Few studies evaluate long term performance. In one of the studies, mortality of AAH patients at 1 year was found to be significantly lower when MELD < 20, 10.4% *vs* 31.4% MELD > 20 (P < 0.001)[38]. In another retrospective study, patients with MDF < 32 had a 50% mortality at 5 years, but the study did not feature any comparison to patients with MDF > 32[39]. In a comparison of the most commonly used scores in 44 patients with biopsy proven AAH: GAHS, MDF, MELD, and ABIC scores all performed poorly in survival prediction after the 6-mo mark[40].

Prognostication of AAH presenting as acute on chronic liver failure

Alcohol is an important trigger for decompensation of chronic liver disease, including ACLF. The AAH scores mentioned above fail to encompass multiple organ failure beyond acute kidney injury. ACLF prognostic scores are applied to patients with severe AAH complicated by organ failure as mortality rates are similar in ACLF whether infection or AAH are incriminated. Notable prognostic scores for ACLF are: CLIF-C (European) ACLF, Asian Pacific Association for the Study of the Liver acute-on-chronic liver failure Research Consortium (AARC), North American Consortium for the Study of End-Stage Liver Disease (NACSELD)[41].

Clinical score correlation with histologic severity

As previously discussed, clinical data is the cornerstone of AAH prognostication. Histologic severity has been studied, with AHHS (alcoholic hepatitis histological score) being proposed by Altamirano *et al*[12] to predict 90-d mortality through the combination of histological parameters that were most strongly associated to death. Overall, no statistical difference was found among MELD, ABIC and AHHS in 90-d mortality prediction. However, there are cases with added

benefit to combining clinical and histological scores. In patients with MELD < 21, 90-d survival was higher when AHHS was < 5 compared to 5 or higher (94% *vs* 72%; *P* = 0.001). Similarly, patients with ABIC B (medium risk) and AHHS < 5 have shown a potentially lower risk of death at 90 d, *vs* a moderate risk of death at 90 d (95% *vs* 70% survival, *P* = 0.003) for ABIC B patients with AHHS 5 or higher[12,42].

Role of artificial intelligence in AAH prognostication

As artificial intelligence (AI) has been more commonly incorporated in health care, there have been attempts of optimizing AAH prognostication through AI. Of note, a multicenter retrospective cohort by Kezer *et al*[43] validated a new 30-d mortality scoring system based on age, BUN, albumin, bilirubin and INR. The score was derived through AI: The Mortality Index for Alcohol-Associated Hepatitis. Performance showed comparable accuracy to clinical scores, however superiority was only demonstrated compared to MDF but not to MELD[43].

In a recent abstract by Dunn *et al*[44], a new AI-generated score was created with the aim of predicting 90-d survival in AAH and validated in a multicenter international retrospective cohort. The score incorporates age, INR, bilirubin, creatinine, albumin, blood urea nitrogen and neutrophil to lymphocyte ratio. The abstract reports statistical superiority to MDF, MELD, MELD-Na, MELD 3.0. ABIC, GAHS. Steroid use showed decreased mortality at 30 d in those with ALCHAIN score 0.30-0.70.

ROLE OF SCORING SYSTEMS IN PREDICTING KIDNEY INJURY

As previously discussed, AAH elevated short-term mortality correlates with numerous complications, developing AKI is one of them. AKI is an important prognostic determinant in AAH, which makes predicting AKI risk in a patient with AAH an important step in management. It has been demonstrated that patients with liver failure and/or fulfillment of the SIRS criteria in addition to the nephrotoxic effects of alcohol are linked to the occurrence of AKI[45-47].

In a multicentric prospective cohort conducted by Sujan *et al*[48], AAH with AKI were more likely to have hepatic encephalopathy, SIRS criteria upon admission, higher MELD, baseline bilirubin, creatinine and INR. In a second phase, the study developed a risk score for AKI. AUROC was 0.74 (95%CI: 0.69-0.80; *P* < 0.001). AKI risk score incorporates SIRS, hepatic encephalopathy presence and MELD score on admission. The score stratifies AKI risk to three categories: Low (< 3), moderate (3-4), and high (> 4). Patients with AKI risk score classified as high had significantly higher short-term mortality compared to those with moderate and low AKI risk scores (90-d survival respectively 47% *vs* 68% *vs* 88%, *P* value < 0.001)[48].

PREDICTING ALCOHOL RECIDIVISM

Treating AAH includes minimizing the risk for recurrence. Alcohol recidivism prediction is routinely done when evaluating patients for liver transplant. In the United States, 6 mo of alcohol abstinence are usually required for liver transplant (LT) consideration in most centers. This period is useful in terms of observing patients with AAH for clinical improvement/adherence to the treatment plan (abstinence), and even possibly the dissipation of the need for LT. However, the pitfall of the 6-month abstinence condition involves depriving patients with overall poor prognosis and high mortality rates from receiving a curative intervention[49].

Parallel to AAH severity scoring tools, Sustained alcohol use post-liver transplantation score, Stanford Integrated Psychosocial Assessment for Transplantation, Alcohol Relapse Risk Assessment, and High-Risk Alcoholism Relapse are all scores developed to be used when considering patients with AAH for LT. So far, these scores have not formally been used alone for selecting LT candidates given the high stakes. More studies are required to optimize our understanding of their reliability[50].

CONCLUSION

The mortality of patients with severe AAH emphasizes the need for accurate prognostication when managing cases of AAH. Many clinical scores have been studied and used, the most common notable being MELD, MDF and Lille score. While MDF is the oldest and the most popularly used score (MDF > 32) to determine the indication for corticosteroid initiation in AAH, MELD score has been increasingly showing superiority in assessing AAH severity. Dynamic prognostication is superior to static. Therefore, initiating steroids for a MELD of 20 or above and continuing them for a day 7 Lille score < 0.45 (favorable response to steroids) is the logical approach towards managing severe AAH. However, more research on AAH is necessary to improve our understanding of the major driving factors that will lead the way to improving our prediction models.

FOOTNOTES

Author contributions: Mitri J and Almeqdadi M reviewed the literature, planned the outline, and created the tables and figure; Mitri J

wrote the manuscript with contributions and supervision from Almeqdadi M and Karagozian R; Almeqdadi M and Karagozian R proofread the manuscript; Almeqdadi M designed figure 1; Mitri J, Almeqdadi M, and Karagozian R revised the manuscript and wrote the comments to reviewers.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Tenofovir alafenamide significantly increased serum lipid levels compared with entecavir therapy in chronic hepatitis B virus patients

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Abstract

BACKGROUND

Tenofovir alafenamide (TAF) has a serum lipid-raising effect in patients with HIV; however, its effect on serum lipids and nonalcoholic fatty liver disease (NAFLD) risk in patients with chronic hepatitis B (CHB) is unclear.

AIM

To compare the effects of TAF and entecavir (ETV) on serum lipid levels in patients with CHB.

METHODS

In this retrospective cohort study, the data including the clinical features, serum lipids, and metabolic factors of patients with CHB at baseline and approximately 1 year after TAF or ETV treatment were collected and analyzed. We used propensity score-matched models to assess the effects on high-density lipoprotein, low-density lipoprotein, triglycerides, and total cholesterol (TCHO).

RESULTS

A total of 336 patients (75.60% male) were included; 63.69% received TAF and 36.31% received ETV. Compared with the ETV group, the TAF group had significantly higher TCHO levels after treatment (4.67 ± 0.90 *vs* 4.36 ± 1.05, $P = 0.006$). In a propensity score-matched model for body mass index, age, sex, smoking, drinking, presence of comorbidities such as NAFLD, cirrhosis, diabetes mellitus, and hypertension, TAF-treated patients had significantly increased TCHO levels compared to that at baseline ($P = 0.019$). There was no difference for the ETV group. Body mass index, sex, hypertension, baseline TCHO, and creatine kinase-MB isoenzyme levels were significantly associated with elevated TCHO levels in logistic regression analysis. However, 1-year TAF treatment did not increase the incidence of NAFLD.

CONCLUSION

A greater increase in TCHO was observed in patients with CHB receiving TAF compared to those receiving ETV. However, TAF-induced dyslipidemia did not increase the incidence of NAFLD.

Key Words: Tenofovir alafenamide; Entecavir; Hepatitis B virus; Serum lipid; Metabolic factor

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Core Tip: This study compared the effects of tenofovir alafenamide (TAF) and entecavir on serum lipid levels in chronic hepatitis B patients. The results suggested that TAF-treated patients had significantly increased triglycerides, and total cholesterol levels compared to that at baseline, while there was no difference in the entecavir group. However, dyslipidemia caused by TAF therapy did not increase the incidence of nonalcoholic fatty liver disease. Our findings indicated that the potential impact of anti-viral therapy on the lipid profile may be an important consideration in the treatment choices for chronic hepatitis B patients with abnormal metabolic factors.

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INTRODUCTION

Hepatitis B virus (HBV) infects approximately 240 million people worldwide, including approximately 86 million people in China[1]. Chronic hepatitis B (CHB) may lead to decompensation of cirrhosis and hepatocellular carcinoma (HCC), which are the leading causes of mortality in patients with CHB[2]. Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome (MetS), with a cumulative prevalence of 24% worldwide[3]. In recent decades, the prevalence of NAFLD has significantly increased in China, leading to the coexistence of NAFLD and CHB. Dyslipidemia, which is characterized by high triglyceride (TG), high total cholesterol (TCHO), low high-density lipoprotein (HDL), and high low-density lipoprotein levels, is strongly associated with NAFLD and MetS[4].

Interferon and nucleoside analog therapies cannot completely eradicate HBV infection[5]. Many patients require long-term anti-HBV therapy with potent oral drugs tenofovir alafenamide (TAF) and entecavir (ETV), which are recommended as first-line treatment in HBV clinical practice guidelines[6]. TAF has also recently been incorporated into antiretroviral regimens for people with HIV, and we observed its impact on serum lipid levels in these individuals. A prospective cohort study showed that patients with HIV infection treated with a TAF-containing regimen had significantly worse blood lipid levels, especially those with higher LDL and TCHO[7]. In a recent real-world study, switching from tenofovir disoproxil fumarate (TDF) to a TAF-containing regimen in HIV-infected patients resulted in a significant increase in serum lipid profiles[8]. However, data on the effects of TAF on serum lipid levels in patients with HIV may be limited by the potentially confounding effects of concomitant antiretroviral HIV drugs.

The effect of ETV on serum lipid profiles has not yet been reported in postmarketing studies. A retrospective cohort study showed greater reductions in TCHO, LDL, and HDL levels in patients with CHB treated with TDF than in those treated with ETV[9]. TAF is considered the successor of TDF; however, no studies have compared the effects of TAF and ETV on lipid profiles in HBV-treated patients. Meanwhile, there are limited data on the effects of TAF on metabolism-related complications in real-world settings. Therefore, this retrospective cohort study aimed to characterize the effect of TAF on serum lipid levels and NAFLD risk in patients with CHB, and we compared the pretreatment and post-treatment serum lipid profile changes after initiation of either TAF or ETV anti-viral therapy.

MATERIALS AND METHODS

Eligibility/study subjects

This study included all patients with CHB older than 18 years who visited the outpatient department of the Hepatology Research Institute of the First Affiliated Hospital of Fujian Medical University between January 2020 and January 2021. Information such as patient demographics, treatment history, laboratory data, and comorbidities was extracted from the electronic medical record system. CHB was defined according to the Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2019) of China[10]. Diagnosis of decompensated cirrhosis was confirmed by ultrasonography or imaging for inclusion in the study. Finally, the study included 336 participants (Figure 1) who were taking TAF or ETV and had a pretreatment serum lipid profile and repeated serum lipid assessment after initiating anti-viral therapy for 1 year. Exclusion criteria were as follows: (1) Less than 1 year of anti-viral therapy; (2) Use of other oral anti-viral drugs during the study period; (3) Use of lipid-lowering drugs; (4) Complicated with other liver disease or pregnancy; (5) Heavy alcohol intake (amounting to ethanol consumption of ≥ 40 g/day for males and ≥ 20 g/day for females).

Measurement of parameters /data collection

The collected clinical and demographic data included age, sex, body mass index (BMI), drinking and smoking habits, date of anti-viral treatment initiation, cirrhosis, and comorbidities [diabetes mellitus (DM), hypertension, and NAFLD]. A fatty liver was identified using ultrasonography. Clinical laboratory information included HBV-DNA, hepatitis B surface antigen, aspartate aminotransferase, alanine aminotransferase, glomerular filtration rate, uric acid, creatinine, creatine kinase (CK), CK-MB isoenzyme, fasting serum lipid profiles (TCHO, TG, HDL, and LDL), and baseline fasting blood glucose levels. The parameters were measured by the clinical laboratory of the First Affiliated Hospital of Fujian Medical University, and data were collected before and 1 year after the initiation of anti-viral therapy. This retrospective study was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University, China.

Statistical analysis

Statistical analyses were performed using SPSS 23.0. Normally distributed continuous variables were presented as mean ± standard deviation, which were further evaluated by Student's *t*-test for the different treatment groups. Categorical variables were described using frequencies and proportions, and Pearson's χ^2 test was used to compare categorical variables. The paired *t*-test and McNemar's test were used to assess the differences between the levels before and after treatments in the same treatment group. We calculated the pretreatment and post-treatment differences in each lipid profile component in order to evaluate the impact of anti-viral therapy on lipid profile.

Propensity score-matched models were used to assess the effect of treatment type (TAF *vs* ETV) on lipid profile component changes. All propensity score-matched models were adjusted for BMI, age, sex, fatty liver disease, cirrhosis, DM, hypertension, smoking, and drinking. We presented the average changes in the model coefficients. Finally, logistic regression analysis was used to estimate the odds ratio of the association between baseline factors and elevated TCHO levels. Statistical *P* values less than 0.05 were considered significant.

RESULTS

Summary of baseline clinical and demographic data of TAF-treated and ETV-treated CHB patients

Overall, 336 CHB patients receiving anti-viral therapy (TAF, n = 214 *vs* ETV, n = 122) were included in the study. The mean age was 46.67 years, 75.60% were male, and 30.95% were cirrhotic at baseline. Patients were older in the ETV group (P = 0.001), but the two groups had a similar rate of NAFLD (TAF: 35.05% *vs* ETV: 26.23%, P = 0.122) and BMI (TAF: 22.97 *vs* ETV: 23.78, P = 0.152). However, hypertension and cirrhosis were more prevalent in the ETV group (17.21% *vs* 7.94%, P = 0.016 and 40.98% *vs* 25.23%, P = 0.004, respectively). TAF and ETV had similar levels of hepatitis B surface antigen (3.07 *vs* 3.04, P = 0.787) and HBV-DNA (1.94 *vs* 1.86, P = 0.560) (Table 1) as well as no statistically significant differences in serum alanine aminotransferase and aspartate aminotransferase levels.

Comparison of serum lipid profiles before and after anti-viral therapy in TAF-treated vs ETV-treated CHB patients

TCHO, TG, LDL, and HDL values in the TAF-treated and ETV-treated individuals were comparable at baseline (prior to anti-viral medication). In the TAF-treated group, post-treatment serum lipoprotein levels were considerably higher than the pretreatment levels for TCHO (4.51 ± 0.93 *vs* 4.67 ± 0.90, P = 0.001) and TG (1.25 ± 0.67 *vs* 1.37 ± 0.81, P = 0.014), whereas HDL and LDL levels did not change in the TAF group over the duration of our study. Further, the TAF group showed significantly higher post-treatment TCHO levels compared with that in the ETV group (4.67 ± 0.90 *vs* 4.36 ± 1.05, P = 0.006). However, TAF treatment did not increase the incidence of NAFLD after 1 year of follow-up (Table 2). In the ETV cohort, there was no significant difference between the pretreatment and post-treatment serum lipoprotein levels.

TAF as an independent predictor of TCHO level change

Using propensity score-matched models for BMI, age, sex, smoking, drinking, and presence of comorbidities such as NAFLD, cirrhosis, DM, and hypertension, we assessed the impact of TAF compared to ETV on achieving an increase in the levels of the serum lipid profile (Table 3). Patients treated with TAF had a statistically significant increase in TCHO levels (P = 0.019), which was 5% higher than that in the baseline lipid profile.

Table 1 Summary of demographic and clinical characteristics at baseline of chronic hepatitis B patients on tenofovir alafenamide or entecavir therapy

Characteristic	TAF, *n* = 214	ETV, *n* = 122	*P* value
Age in yr	43.38 ± 10.42	49.96 ± 11.82	0.001
Male	158 (73.83)	96 (78.69)	0.387
BMI in kg/m^2	22.97 ± 2.93	23.78 ± 3.30	0.152
Smoking	16 (7.48)	15 (12.30)	0.203
Drinking	13 (6.07)	14 (11.48)	0.123
ALT in U/L	36.60 ± 36.87	30.44 ± 20.00	0.089
AST in U/L	28.01 ± 26.41	24.50 ± 13.58	0.172
logHBsAg in ng/mL	3.07 ± 0.92	3.04 ± 0.84	0.787
logDNA in IU/mL	1.94 ± 1.23	1.86 ± 1.05	0.56
CREA in μmol/L	73.68 ± 15.87	74.82 ± 16.78	0.535
UA in μmol/L	353.52 ± 89.10	357.23 ± 84.29	0.708
GFR in mL/min	103.48 ± 15.01	97.86 ± 14.17	0.001
CK in U/L	157.23 ± 444.75	122.91 ± 71.31	0.401
FBG in mmol/L	5.18 ± 0.80	5.52 ± 1.61	0.011
Concurrent diseases			
Hypertension	17 (7.94)	21 (17.21)	0.016
DM	20 (9.35)	13 (10.66)	0.844
NAFLD	75 (35.05)	32 (26.23)	0.122
Cirrhosis	54 (25.23)	50 (40.98)	0.004

Data are presented as *n* (%). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CK: Creatine kinase; CREA: Creatinine; DM: Diabetes mellitus; ETV: Entecavir; FBG: Fasting blood-glucose; GFR: Glomerular filtration rate; HBsAg: Hepatitis B surface antigen; NAFLD: nonalcoholic fatty liver disease; TAF: Tenofovir alafenamide; UA: Uric acid.

Table 2 Comparison of serum lipid profile before and after 1 year of anti-viral therapy either tenofovir alafenamide or entecavir

Characteristic	TAF, *n* = 214, statistic	*P* value[1]	ETV, *n* = 122, statistic	*P* value[1]	*P* value[2]
Pre-Tx TCHO in mmol/L	4.51 ± 0.93	0.001	4.41 ± 1.03	0.275	0.376
Post-Tx TCHO in mmol/L	4.67 ± 0.90		4.36 ± 1.05		0.006
Pre-Tx TG in mmol/L	1.25 ± 0.67	0.014	1.33 ± 0.75	0.052	0.35
Post-Tx TG in mmol/L	1.37 ± 0.81		1.24 ± 0.61		0.126
Pre-Tx HDL in mmol/L	1.32 ± 0.40	0.794	1.26 ± 0.40	0.879	0.246
Post-Tx HDL in mmol/L	1.32 ± 0.36		1.27 ± 0.41		0.285
Pre-Tx LDL in mmol/L	3.12 ± 0.90	0.785	3.06 ± 1.01	0.078	0.543
Post-Tx LDL in mmol/L	3.14 ± 0.92		3.15 ± 1.00		0.906
Pre-Tx NAFLD	75 (35.05)	0.125	32 (26.23)	1	0.122
Post-Tx NAFLD	70 (32.71)		31 (25.41)		0.16

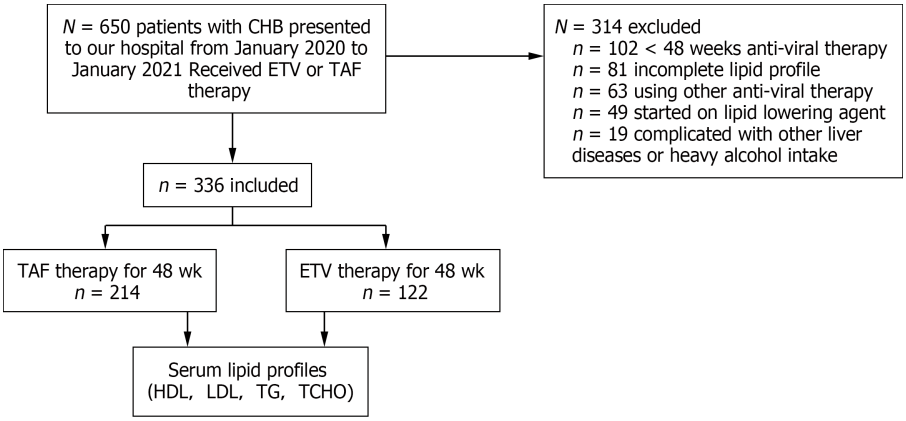
[1]Comparing the pretreatment and post-treatment variables (paired) in each therapy type.
[2]Comparing the same variable (either pretreatment or post-treatment) according to type of therapy. ETV: Entecavir; HDL: High density lipoprotein; LDL: Low-density lipoprotein; NAFLD: Nonalcoholic fatty liver disease; TAF: Tenofovir alafenamide; TCHO: Total cholesterol; TG: Triglycerides; Tx: Treatment.

Table 3 Impact of tenofovir alafenamide on achieving a higher level of total cholesterol in chronic hepatitis B patients

Characteristic	TCHO, increased change (%) by using TAF compared with ETV[1,2], OR (95%CI)	*P* value
5% higher than baseline	1.88 (1.11, 3.16)	0.019
10% higher than baseline	1.70 (0.94, 3.09)	0.081
15% higher than baseline	2.07 (0.99, 4.34)	0.055

[1]All propensity score-matched models were adjusted for body mass index, age, sex, nonalcoholic fatty liver disease, cirrhosis, diabetes mellitus, hypertension, smoking, and drinking. The increased percentage change was presented by using tenofovir alafenamide (TAF) compared with entecavir (ETV).

[2]The increased change was transformation of the treatment effect coefficients in propensity score-matched models. CI: Confidence interval; OR: Odds ratio; TCHO: Total cholesterol.



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Figure 1 Flowchart of study participants. CHB: Chronic hepatitis B; TAF: Tenofovir alafenamide; ETV: Entecavir; TCHO: Total cholesterol; HDL: High density lipoprotein; LDL: Low-density lipoprotein; TG: Triglycerides.

Risk factors were associated with elevated TCHO levels in CHB patients with 1-year TAF therapy

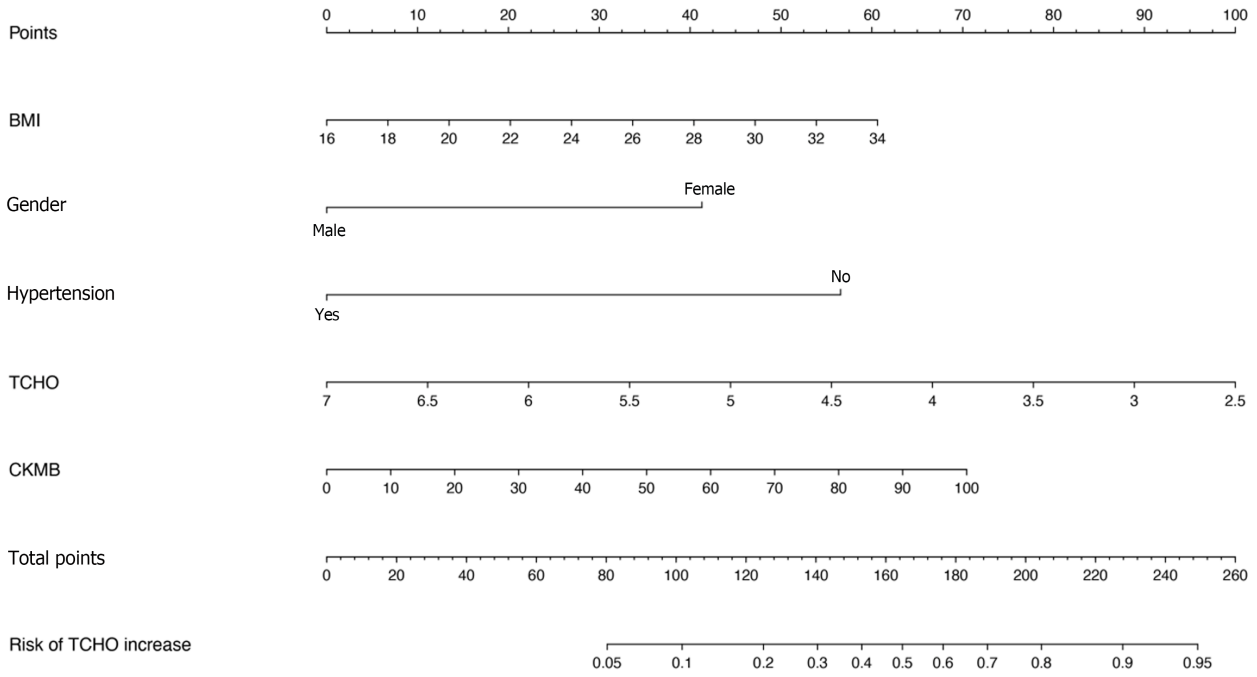
Logistic regression analysis was used to evaluate the risk factors associated with the worsening of TCHO levels (Figure 2). BMI, sex, hypertension, the baseline TCHO, and CK-MB levels were significantly associated with elevated TCHO levels. Furthermore, a nomogram incorporating statistically significant parameters in the logistic regression analysis was constructed, and the total points predicted the probability of elevated TCHO levels in individual patients.

DISCUSSION

In this real-life retrospective cohort study of 336 patients with CHB who received anti-viral therapy for 1 year, we compared fasting serum lipid profiles before and after initiation of either TAF or ETV treatment. In the TAF-treated cohort, we found a significant increase in the serum TCHO and TG levels compared with no difference in patients who received ETV. However, there were no concomitant significant changes in serum HDL or LDL levels in TAF-treated patients. Recent data have demonstrated that MetS increased the risk of progression of liver fibrosis independent of viral factors in patients with CHB[11,12]. Elevated TCHO and TG levels as MetS risk factors were related to a high risk of cirrhotic events in patients with CHB; thus, the results of this investigation may affect the decision regarding anti-viral therapy in CHB patients with cirrhotic risk factors.

TAF has been used as first-line therapy for CHB and HIV infections. Dyslipidemia and metabolic disorders have been linked to the prolonged use of TAF to treat HIV infection. Previous studies found that the serum lipid profile increased in HIV patients switching from TDF to TAF therapy, and the effect of increased serum lipids on the risk of cardiovascular events cannot be ignored[13-15]. Therefore, whether dyslipidemia due to TAF treatment in patients with CHB would lead to an increased risk of NAFLD is also a concern. Studies have reported that patients with CHB and NAFLD have better liver-related outcomes and overall mortality than those with CHB alone[16]. It is increasingly recognized that metabolic factors, which are precursors of NAFLD, can also be used to evaluate the risk of HCC in patients with CHB[17,18].

In our study, patients who received TAF therapy for 1 year did not have a significantly increased risk of developing NAFLD. However, owing to the short follow-up period of our study, the effect of TAF on increasing the incidence of NAFLD still needs to be evaluated over a longer follow-up period, and more data are required to better elucidate this.



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Figure 2 Nomogram plot predicted the probability of the elevated total cholesterol level for individual patients receiving tenofovir alafenamide therapy. BMI: Body mass index; CK-MB: Creatine kinase-MB isoenzyme; TCHO: Total cholesterol.

It was unclear how TAF increased lipid concentrations, but the mechanism was independent of the patient's HIV status because a similar effect of TAF has been reported in HIV-negative patients receiving CHB therapy. Notably, a higher proportion of TAF-treated CHB patients experienced higher levels of LDL than the TDF group at the 48-wk follow-up[19,20]; however, the use of lipid-lowering agents was not described. In our study, the increased TCHO levels with TAF treatment represented a small change in the propensity score-matched model (5% higher than baseline, $P = 0.019$), but we did not find a significant change in the LDL profile compared to the ETV treatment group. Regular serum lipid measurements might not accurately capture the intricate alterations in lipid metabolism brought on by anti-viral medication. A recent study showed that TDF modulates lipid metabolism by upregulating hepatic CD36 *via* PPAR-a activation in patients with HBV infection[21]. However, the mechanism by which TAF increases serum lipid profiles remains unclear, and further studies are required.

In addition, in multivariate logistic regression analysis, metabolic factors, including BMI, hypertension, baseline TCHO, and CK-MB levels, were significantly associated with elevated TCHO levels, demonstrating an important impact of metabolic factors in terms of the elevated lipid profile caused by TAF. Previous studies have reported the effects of MetS on the adverse outcomes of fibrosis in patients with CHB[22,23]. Furthermore, some studies have confirmed that metabolic factors positively affected the risk of HCC in patients with HBV infection[17,24]. Based on the results mentioned above, the treatment options for patients with CHB who have the aforementioned aberrant metabolic variables may need to consider the potential effects of TAF medication on blood lipid levels.

Our study had some limitations. First, not all relevant clinical data were obtained from the treatment databases, and important data elements, such as lifestyle risk factors (*i.e.* exercise and diet) and family history, were not fully documented. Second, this was not a multicenter study, and we adjusted for baseline factors through a propensity score-matched model. Therefore, the observed increase in the TCHO profile may be due to the effect of anti-viral treatment. Third, the effect of TAF on the incidence of NAFLD cannot be elucidated well because of the short follow-up time. However, this study provided real-world data from China regarding the relationship between TAF-treated patients with CHB and changes in serum lipid levels. Large-scale multicenter prospective studies should be conducted in the future to further evaluate the effects of CHB and anti-HBV therapies on the risk of dyslipidemia, NAFLD, cirrhosis, and HCC.

CONCLUSION

In this real-life retrospective cohort study in China, we found that TAF was significantly associated with higher TCHO levels, whereas ETV had no effect in patients with CHB treated for 1 year. Risk factors (BMI, sex, baseline TCHO and CK-MB levels) were significantly associated with elevated TCHO levels. In the future, we will focus on increased NAFLD risk and not just changes in serum lipid profiles. Further studies will help elucidate the effect of TAF on long-term metabolic-related complications in patients with CHB.

ARTICLE HIGHLIGHTS

Research background

In recent decades, the prevalence of nonalcoholic fatty liver disease (NAFLD) has significantly increased in China, leading to the coexistence of NAFLD and chronic hepatitis B (CHB). Many patients require long-term anti-hepatitis B virus (HBV) therapy with potent oral drugs tenofovir alafenamide (TAF) and entecavir (ETV), which are recommended as first-line treatment in HBV clinical practice guidelines. However, no studies have compared the effects of TAF and ETV on lipid profiles in HBV-treated patients. Meanwhile, there are limited data on the effects of TAF on metabolism-related complications in real-world settings.

Research motivation

Many patients require long-term anti-HBV therapy with the potent oral drugs TAF and ETV, which are recommended as first-line treatment in HBV clinical practice guidelines. TAF has a serum lipid-raising effect in patients with HIV; however, its effect on serum lipids and NAFLD risk in patients with CHB is unclear.

Research objectives

This retrospective cohort study aimed to characterize the effect of TAF on serum lipid levels and NAFLD risk in patients with CHB, and we compared the pretreatment and post-treatment serum lipid profile changes after initiation of either TAF or ETV anti-viral therapy.

Research methods

The data including the clinical features, serum lipids, and metabolic factors of patients with CHB at baseline and approximately 1 year after TAF or ETV treatment were collected and analyzed. We used propensity score-matched models to assess the effects on high-density lipoprotein, low-density lipoprotein, triglycerides, and total cholesterol (TCHO).

Research results

Compared with the ETV group, the TAF group had significantly higher TCHO levels after treatment (4.67 ± 0.90 *vs* 4.36 ± 1.05, P=0.006). In a propensity score-matched model, TAF-treated patients had significantly increased TCHO levels compared to that at baseline (P = 0.019), while there was no difference for the ETV group. Body mass index, sex, hypertension, baseline TCHO, and creatine kinase-MB isoenzyme levels were significantly associated with elevated TCHO levels in logistic regression analysis. However, 1-year TAF treatment did not increase the incidence of NAFLD.

Research conclusions

Our study found that a greater increase in TCHO was observed in patients with CHB receiving TAF than in those receiving ETV; however, TAF-induced dyslipidemia did not increase the incidence of NAFLD.

Research perspectives

This was not a multicenter study, and most of patients with CHB in this study were Asians. Large-scale multicenter prospective studies should be conducted in the future to further evaluate the effects of CHB and anti-HBV therapies on the risk of dyslipidemia, NAFLD, cirrhosis, and hepatocellular carcinoma.

FOOTNOTES

Author contributions: Lai RM and Lin S contributed equally to this work; Lai RM and Lin S conceived and designed the study; Chen TB and Zhou JH contributed to the data analysis and manuscript feedback; Li N and Wang MM collected clinical data of the patients; Lai RM and Zheng Q wrote the manuscript; All authors approved the final version of the manuscript.

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Informed consent statement: Patients were not required to give informed consent to the study as the analysis used anonymous data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All the authors declare that they have no conflicts of interest related to the manuscript.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at bei0825@163.com.

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

Stages of care for patients with chronic hepatitis C at a hospital in southern Brazil

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Abstract

BACKGROUND

Hepatitis C virus (HCV) is defined as a public health problem by the World Health Organization (WHO) and since then has defined targets through the HCV elimination. The HCV cascade of care highlights the progress towards these goals and essential interventions that need to be delivered along this continuum care.

AIM

To document the treatment cascade for patients with HCV infection at the Hospital Nossa Senhora da Conceição (HNSC), defining the percentage of antibody-positive patients who collected molecular biology tests (polymerase

chain reaction), attended outpatient clinic assistance, underwent treatment, and achieved a virologic cure termed sustained virologic response (SVR).

METHODS

With the retrospective cohort design, patients diagnosed with HCV infection in the period between January 1, 2015 and December 31, 2020 were included. Data from HCV notification forms, electronic medical records, Computerized Laboratory Environment Manager System, and Medicine Administration System (evaluation of special medications) were collected in 2022 and all information up to that period was considered. The data were analyzed with IBM SPSS version 25, and Poisson regression with robust simple variance was performed for analysis of variables in relation to each step of the cascade. Variables with $P < 0.20$ were included in the multivariate analysis with $P < 0.05$ considered significant. Pearson's chi-square test was applied to compare the groups of patients who persisted in follow-up at the HNSC and who underwent follow-up at other locations.

RESULTS

Results were lower than expected by the WHO with only 49% of candidates receiving HCV treatment and only 29% achieving SVR, despite the 98% response rate to direct acting antivirals documented by follow-up examination. The city of origin and the place of follow-up were the variables associated with SVR and all other endpoints. When comparing the cascade of patients who remained assisted by the HNSC *vs* external patients, we observed superior data for HNSC patients in the SVR. Patients from the countryside and metropolitan region were mostly assisted at the HNSC and the specialized and continuous care provided at the HNSC was associated with superior results, although the outcomes remain far from the goals set by the WHO.

CONCLUSION

With the elaboration of the HCV cascade of care using local data, it was possible to stratify and evaluate risk factors associated with losses between each step of the cascade, to inform new strategies to guide elimination efforts in the future.

Key Words: Cascade of care; Elimination; Hepatitis C virus; Sustained virologic response

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Core Tip: Hepatitis C virus (HCV) is defined as a public health problem by the World Health Organization and since then has defined targets through the HCV elimination. The present study aimed to document the treatment cascade for patients with HCV infection at a hospital in southern Brazil. With the retrospective cohort design, patients diagnosed with HCV infection between 2015 and 2020 were included to create the HCV cascade of care described as five exposure columns according to the stages of HCV care. Variables were related to each step of the cascade to identify obstacles for patients to reach the last step. With the elaboration of the HCV cascade of care using local data, it was possible to stratify and evaluate risk factors associated with losses between each step of the cascade, to inform new strategies to guide elimination efforts in the future.

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INTRODUCTION

The World Health Organization (WHO) set the goal of diagnosing 90% of cases of viral hepatitis and treating 80% of diagnosed cases with the aim of reducing the incidence by 90% and mortality attributable to hepatitis by 65% by 2030[1]. The hepatitis C virus (HCV) care cascade represents the care that patients receive in the respective health services and consequently illustrates the basic indicators of the WHO targets[2]. In the first stage are people with HCV infection, in the second are the patients aware of the diagnosis of HCV, in the third, those who underwent treatment, and in the fourth stage, those who achieved cure with viral suppression from 12 wk to 24 wk after the end of treatment[2-4]. Other stages can be added, such as retention in care and after cure monitoring, but it is difficult to standardize the criteria, hindering the possibility of later comparison[3].

The elaboration of the treatment cascade facilitates the identification of barriers and groups of risks which we must work with[3,5]. The correlation between sociodemographic variables and results between stages is an important tool for the analysis of the HCV cascade of care[6]. Mental health problems, change in follow-up location, and restriction of information about the disease were detected as causes of failures in the stages of chronic HCV treatment in a study by health professionals[7]. Therefore, the construction of local cascades is necessary for the understanding of gaps in current practices and the elaboration of changes[8].

Brazil joined the Hepatitis C Elimination Plan in 2017[9]. Since then, the country has been developing and implementing guidelines of care and prevention to guarantee a greater access to diagnostic tests and the treatment for all patients with chronic HCV infection and acute hepatitis C and children with infection by HCV[9]. Retreatment is also possible, especially after the availability of new direct-acting antivirals (DAAs)[9]. Despite the measures instituted in Brazil, there are no data available for the elaboration of a local cascade of care, which determines the need to qualify national databases to monitor the hepatitis elimination policy[10].

The South region of Brazil is responsible for the highest detection rate of confirmed HCV infection in the country and also for the highest mortality rate, with higher rates than national data[11]. Within this region, the city of Porto Alegre, in 2020 was the second capital with the highest HCV detection rate, and in 2021 the first, even higher than the national rate [11]. The Hospital Nossa Senhora da Conceição (HNSC) in Porto Alegre is a tertiary hospital that has an infectology service which is a reference in treatment services for patients with human immunodeficiency virus (HIV) infection and viral hepatitis[12]. The objective of this study was to define the continuity of care or treatment cascade for patients with chronic HCV infection at the HNSC and to define sociodemographic variables that influence follow-up between each step of the cascade.

MATERIALS AND METHODS

This referred retrospective cohort study included patients diagnosed with chronic HCV infection between 2015 and 2020 at the HNSC. All hospitalized patients and outpatients above 16 years of age notified by the HNSC epidemiological center for viral hepatitis with positive anti-HCV or HCV-polymerase chain reaction (PCR) results detectable during this period were analyzed. Patients under 16 years of age were excluded, as they would be followed up at a pediatric hospital attached to the HNSC. Patients who died, who were not connected with the HNSC, or who were not located in the electronic medical records of the hospitals were excluded.

The Information System for Notifiable Diseases (SINAM) for Viral Hepatitis, electronic medical records from the HNSC, computerized Laboratory Environment Management System (GAL), and Medicine Administration System (AME) provided data such as age, gender, race, education level, history of pregnancy and drug use, profession, city of origin, institutionalization situation, and co-infection with HIV and hepatitis B virus (HBV). Specific data of the disease under study were also obtained, such as date of diagnosis of HCV infection (anti-HCV test), history of consultation with a specific outpatient clinic for the treatment of hepatitis, initial quantitative HCV-PCR and final quantitative HCV-PCR (after the 12th wk since the end of treatment), genotyping, history of cirrhosis or hepatocellular carcinoma, specific regimen, and date of treatment prescribed for HCV. Data were collected in 2022 and all information up to that period was considered.

Regarding the HCV cascade of care, five exposure columns were built according to the stages of HCV care. The first stage covers all people diagnosed with chronic HCV infection, that is, patients with positive anti-HCV or detectable quantitative HCV-PCR in the analyzed period. The second includes patients who underwent some quantitative PCR collection, and the third includes patients who underwent consultation at a specialized outpatient clinic for monitoring hepatitis C. The fourth step integrates all who underwent treatment with specific antivirals for chronic HCV infection according to the established protocol. The fifth step ends with all patients who achieved a sustained virological response, that is, those who obtained an undetectable quantitative HCV-PCR test after the 12th wk since the end of treatment. Patients who did not collect this exam after treatment were not included in the fifth step, being presented in the cascade as "missing" and subsequently analyzed within the group of those who were not cured. The percentages were then calculated using the "*n*" of the first step and the "*n*" of the previous step as denominator, thus obtaining two percentages for analysis, being represented using a series of unidirectional columns.

Using the IBM SPSS version 25 program, Poisson regression with robust simple variance was performed to estimate the incidence ratio (IR) at a 95% confidence interval (95%CI) for the variables of gender, age group, race, education, city of residence, place of follow-up, presence of cirrhosis, institutionalization, year of diagnosis, and co-infection with HIV/HBV related to each step of the cascade: PCR-HCV collection, bond, treatment, and sustained virologic response (SVR). All variables that had a value of $P < 0.20$ in the simple analyzes were included in the multivariable model, and in this model only variables with $P < 0.05$ were considered significant.

Two more cascades were also built, discriminating between patients who underwent treatment at the HNSC and those who underwent treatment at other locations after the diagnosis. The comparison of the sociodemographic characteristics between the groups, HNSC and external, was performed using Pearson's chi-square test and results with $P < 0.05$ were considered significant. The study was approved by the research ethics committee of the Hospitalar Conceição Group, under number 51462421.8.0000.5530, and informed consent was waived, subject to the patient's commitment to confidentiality.

RESULTS

By searching the HNSC viral hepatitis notification database between 2015 and 2020 at the HNSC, 2498 patients were identified. A total of 487 patients who died, with decompensated cirrhosis, hepatocellular carcinoma, renal failure, and sepsis as the main etiologies reported, were excluded. Another 1232 patients were also excluded because they had a diagnosis of other viral hepatitis, an HCV diagnosis prior to 2015, a false anti-HCV test result, no attachment to the HNSC, or being younger than 16 years old. A total of 779 patients diagnosed with HCV infection during the analyzed

period were included, but of these 70 had spontaneous cure and, as they did not require treatment, were disregarded for further analyses.

For the HCV cascade of care, 709 patients were analyzed, showing a sociodemographic profile of being predominantly male (54.3%), white (76.6%), and from Porto Alegre (44.7%), and just having had completed elementary education (67.4%). The mean age of the patients was 53 years. Only 22 patients had a history of pregnancy, 13% co-infection with the HIV, 10.3% co-infection with HBV, 17.8% had a history of cirrhosis, and only 2% a diagnosis of hepatocellular carcinoma. It was identified that 24.9% had a history of drug use and 33 patients of institutionalization. Regarding the genotype, 44.5% of the patients did not have an identified genotype and, among the available genotypes, genotype 1 was the most prevalent (60%), followed by genotype 3 (34.6%) and finally genotype 2 (5.3%).

Regarding the total of 709 patients, 534 (75.3%) collected quantitative PCR, 461 (65%) consulted at a specialized clinic, 344 (48.5%) underwent treatment for HCV infection, and 204 (28.7%) reached SVR. When considering the previous column as the denominator, the percentages would be, respectively, 75% with RT-PCR collection, 86% consulted, 75% treated, and 59% with SVR confirmed by post-treatment examination. Both percentages are represented in Figure 1. The results of the simple and multivariate analyzes of the variables in relation to each step of the cascade are described in Tables 1 and 2, respectively.

Patients with incomplete primary education had lower rates of HCV-PCR collections after the diagnosis of HCV infection in the simple analysis ($P < 0.20$), as well as patients who underwent diagnosis in 2018; however, these variables were not significant in multivariate analysis. Patients living in the metropolitan area and countryside regions, who consulted at an outpatient clinic at the HNSC, co-infected with HIV and HBV, with a history of cirrhosis, and who were diagnosed in 2016 and in 2020, had higher collections of PCR-HCV in the first analysis ($P < 0.20$). In the second analysis, patients from the metropolitan area and countryside regions, patients from the HNSC, and those diagnosed in 2020 were more likely to obtain HCV-PCR ($P < 0.05$).

Despite linkage being the subsequent step in the cascade, there were 22 patients who consulted and did not collect any HCV-PCR test. The variables that showed a difference in favor of creating a link to a specialized outpatient clinic were female gender, living outside the city of Porto Alegre, having cirrhosis, and consulting at the HNSC. The diagnoses in 2018 and in 2019, as well as having only elementary school, were factors contrary to consulting with specialists. In the multivariate analysis, only being from the countryside or metropolitan area and consulting the HNSC were significant ($P < 0.05$). Through the records in the GAL and AME, we identified that 121 patients were followed up in other places after the diagnosis in the hospital.

Being female, having a history of cirrhosis, living outside the city of Porto Alegre, and consulting at the HNSC were protective factors in the simple analysis for undertaking the treatment. As risk factors for not undergoing HCV treatment, co-infection with HIV, being institutionalized, being non-white, having had only completed elementary school, and having been diagnosed in 2019 were identified. Consulting at the HNSC and being from the countryside remained significant protective factors ($P < 0.05$). The mean time between diagnosis and initiation of treatment was approximately 2 years. The DAAs most used in treatment were sofosbuvir and ledispasvir (26.0%), sofosbuvir and velpatasvir (22.0%), sofosbuvir and daclatasvir (17.0%), and sofosbuvir, daclatasvir, and ribavirin (15.0%). Only eight patients (1.1%) underwent more than one treatment. Among the 22 pregnant women in the study, only nine underwent treatment, but timing of treatment before or after pregnancy was unknown. No history of vertical transmission was found in these cases, since the GAL system evaluated the newborns of the respective pregnant women.

Out of all 344 patients who underwent treatment, only 204 reached SVR; however, 136 patients had no record in the GAL of collection of HCV-PCR control after the 12th wk since the end of treatment. Only four had SVR failure, resulting in a documented DAA response rate of 98%. The risk factors in the simple analysis for not achieving documented SVR were being of non-white race and having only elementary education. As variables favorable to SVR, living in the metropolitan area, in the countryside, and consultation at the HNSC were found, in addition to having been diagnosed with HCV infection in 2015 and in 2016. In the multivariate analysis, only the places of residence (countryside and metropolitan area) and consultation at the HNSC were significant ($P < 0.05$).

Because significance was found in all stages of HCV care regarding the place of follow-up, we constructed two separate cascades of care: One for patients who remained under follow-up at the HNSC and another for patients who chose to be assisted in other places. As previously mentioned, 340 patients remained at the HNSC, of whom 70% underwent treatment and 49% achieved SVR, 1% showed failure, and 20% did not collect a PCR test after treatment. Among 121 outpatients, 88% underwent treatment and 31% had SVR, but 55% did not perform a control PCR collection and 2% had SVR failure (Figure 2). As variables favorable to SVR, living in the metropolitan area, in the countryside, and consultation at the HNSC were found, in addition to having been diagnosed with HCV infection in 2015 and in 2016. In the multivariate analysis, only the places of residence (countryside and metropolitan area) and consultation at the HNSC were significant ($P < 0.05$) (Table 3).

DISCUSSION

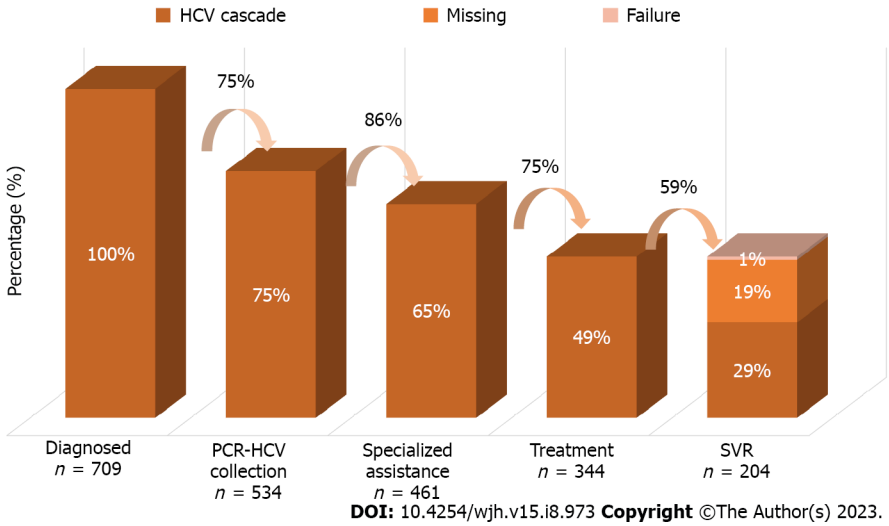
We conducted this study in our local hospital to identify the HCV care cascade and relevant characteristics for progressing from diagnosis to successful cure, to provide representative data from Rio Grande do Sul and mainly from the city of Porto Alegre[11,13]. The highest prevalence of HCV infection was found in patients who are male, white, and aged over 40 years, consistent with both data from the state of Rio Grande do Sul and national statistics[11,14]. Also, higher prevalence of HCV was observed in patients with only elementary education[11]. Regarding co-infection with HIV, we present data that are very close to those of the South region in 2021 (10.1%)[11]. Groups most vulnerable to

Table 1 Analysis of variables with absolute number and relative percentage (%) and Poisson regression with robust simple variance of each variable in relation to steps of cascade of care of patients diagnosed with hepatitis C virus infection at the Hospital Nossa Senhora da Conceição between 2015 and 2020

	Total (%)	PCR-HCV collection	*P* value	Specialized assistance	*P* value	Treatment	*P* value	SVR	*P* value
Total	709	534 (75.3%)		461 (65.0%)		344 (48.5%)		204 (28.7%)	
Gender									
Female	324 (45.7%)	238 (73.5%); 0.95 (0.87-1.04)	0.295	221 (68.2%); 1.09 (0.98-1.21)	0.101	175 (54.0%); 1.23 (1.05-1.43)	0.007	109 (62.3%); 1.10 (0.92-1.32)	0.253
Male	385 (54.3%)	296 (76.9%); 1.0		240 (62.3%); 1.0		169 (43.9%); 1.0		95 (56.2%); 1.0	
Age (yr)									
17-39	118 (16.6%)	90 (76.3%); 1.0		79 (66.9%); 1.0	0.420	52 (44.1%); 1.0		27 (51.9%); 1.0	
40-59	348 (49.1%)	267 (76.7%); 1.00 (0.89-1.13)	0.920	225 (64.7%); 0.96 (0.83-1.12)	0.646	170 (48.9%); 1.10 (0.88-1.39)	0.380	103 (60.6%); 1.16 (0.87-1.55)	0.294
> 60	243 (34.3%)	177 (72.8%); 0.95 (0.84-1.08)	0.479	157 (64.6%); 0.96 (0.82-1.12)	0.657	122 (50.2%); 1.13 (0.89-1.44)	0.284	74 (60.7%); 1.16 (0.86-1.57)	0.307
Race									
White	543 (76.6%)	405 (74.6%); 1.0		353 (65.0%); 1.0		269 (49.5%); 1.0		164 (61.0%); 1.0	
Non-white	154 (21.7%)	118 (76.6 %); 1.02 (0.92-1.13)	0.598	97 (63.0%); 0.96 (0.84-1.11)	0.649	65 (42.2%); 0.85 (0.69-1.04)	0.123	32 (49.2%); 0.80 (0.62-1.05)	0.113
Education									
Illiterate	39 (5.5%)	28 (71.8%); 0.88 (0.71-1.08)	0.238	26 (66.7%); 0.95 (0.74-1.22)	0.741	20 (51.3%); 0.96 (0.68-1.35)	0.834	13 (65.0%); 0.95 (0.66-1.36)	0.804
Elementary school	478 (67.4%)	344 (72%); 0.88 (0.80-0.97)	0.011	301 (63.0%); 0.90 (0.79-1.03)	0.134	219 (45.8%); 0.86 (0.71-1.03)	0.110	120 (54.8%); 0.80 (0.66-0.98)	0.031
High school and university	141 (19.9%)	115 (81.6%); 1.0		98 (69.5%); 1.0		75 (53.2%); 1.0		51 (68.0%); 1.0	
City of origin									
Porto Alegre	317 (44.7%)	215 (67.8%); 1.0		174 (54.9%); 1.0		121 (38.2%); 1.0		49 (40.5%); 1.0	
Metropolitan region	247 (34.8%)	190 (76.9%); 1.13 (1.02-1.25)	0.016	166 (67.2%); 1.22 (1.07-1.39)	0.003	126 (51.0%); 1.33 (1.11-1.60)	0.002	81 (64.3%); 1.58 (1.23-2.04)	0
Countryside	134 (18.9%)	118 (88.1%); 1.29 (1.17-1.43)	0	112 (83.6%); 1.52 (1.34-1.72)	0	91 (67.9%); 1.77 (1.48-2.13)	0	68 (74.7%); 1.84 (1.44-2.36)	0
Follow-up site									
HNSC	341 (48.1%)	320 (93.8%); 1.61 (1.47-1.76)	0	340 (99.7%); 3.03 (2.62-3.50)	0	237 (69.5%); 2.39 (2.00-2.84)	0	166 (70.0%); 1.97 (1.50-2.58)	0
External	121 (17.1%)	214 (58.2%); 1.0		121 (32.9%); 1.0		107 (29.1%); 1.0		38 (35.5%); 1.0	
Institutionalized									
Yes	33 (4.7%)	25 (75.8%); 1.0		18 (54.5%); 1.0		12 (36.4%); 0.74 (0.46-1.17)	0.198	8 (66.7%); 1.12 (0.74-1.70)	
No	676 (95.3%)	509 (75.3%); 1.00 (0.82-1.22)	0.952	443 (65.5%); 0.83 (0.60-1.14)	0.255	332 (49.1%); 1.0		196 (59.0%); 1.0	0.561
Co-infection with HBV									
Yes	73 (10.3%)	61 (83.6%); 1.12 (1.00-1.25)	0.041	43 (58.9%); 1.0		30 (41.1%); 0.83 (0.62-1.10)	0.208	18 (60.0%); 1.01 (0.74-1.37)	0.935
No	636 (89.7%)	473 (74.4%); 1.0		418 (65.7%); 0.89 (0.73-1.09)	0.282	314 (49.4%); 1.0		186 (59.2%); 1.0	0.935

Co-infection with HIV									
Yes	93 (13.1%)	75 (80.6%); 1.08 (0.97-1.20)	0.158	63 (67.7%); 1.04 (0.90-1.22)	0.541	35 (37.6%); 0.75 (0.57-0.98)	0.039	17 (48.6%); 0.80 (0.56-1.14)	0.222
No	616 (86.9%)	459 (74.5%); 1.08		398 (64.6%); 1.0		309 (50.2%); 1.0		187 (60.5%); 1.0	
Cirrhosis									
Yes	126 (17.8%)	112 (88.9%); 1.22 (1.13-1.33)	0	106 (84.1%); 1.38 (1.25-1.52)	0	82 (65.1%); 1.44 (1.23-1.69)	0	60 (73.2%); 1.33 (1.12-1.57)	0.001
No	583 (82.2%)	422 (72.4%); 1.0		355 (60.9%); 1.0		262 (44.9%); 1.0		144 (55.0%); 1.0	
Date of diagnosis									
2015	149 (21.0%)	111 (74.5%); 1.0		102 (68.5%); 1.0		76 (51%); 1.0		42 (55.3%); 1.0	
2016	136 (19.2%)	113 (83.1%); 1.11 (0.98-1.25)	0.076	101 (74.3%); 1.08 (0.93-1.25)	0.278	72 (52.9%); 1.03 (0.83-1.29)	0.744	51 (70.8%); 1.28 (0.99-1.64)	0.052
2017	121 (17.1%)	92 (76.0%); 1.02 (0.89-1.17)	0.771	84 (69.4%); 1.01 (0.86-1.19)	0.865	68 (56.2%); 1.10 (0.88-1.37)	0.393	45 (66.2%); 1.19 (0.91-1.56)	0.181
2018	121 (17.1%)	80 (66.1%); 0.88 (0.75-1.04)	0.140	72 (59.5%); 0.86 (0.72-1.04)	0.133	53 (43.8%); 0.85 (0.66-1.10)	0.244	30 (56.6%); 1.04 (0.75-1.39)	0.880
2019	128 (18.1%)	90 (70.3%); 0.94 (0.81-1.09)	0.440	67 (52.3%); 0.76 (0.62-0.93)	0.008	49 (38.3%); 0.75 (0.57-0.98)	0.038	22 (44.9%); 0.81 (0.56-1.17)	0.272
2020	54 (7.6%)	48 (88.9%); 1.19 (1.04-1.36)	0.009	35 (64.8%); 0.94 (0.75-1.18)	0.634	26 (48.1%); 0.94 (0.68-1.29)	0.723	14 (53.8%); 0.97 (0.64-1.46)	0.901

P value < 0.20 was considered significant. HNSC: Hospital Nossa Senhora da Conceição; HCV: Hepatitis C virus; HBV: Hepatitis B virus; SVR: Sustained virologic response.



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Figure 1 Cascade of care of hepatitis C virus infected patients diagnosed at the Hospital Nossa Senhora da Conceição between 2015 and 2020. HCV: Hepatitis C virus; SVR: Sustained virologic response.

infection by the HCV, such as people living with HIV, institutionalized people, and drug users, were considerably represented, corroborating the importance of focusing on testing and preventive actions for these particular subpopulations[9,14]. Genotypes 1 and 3 were the most prevalent, as expected according to national and local data[14].

According to the WHO, the second step of the HCV cascade of care would be represented by patients aware of their diagnosis, with a target of 90%[1]. However, in this study, we did not carry out this estimate and included patients from the diagnosis of chronic HCV infection which we performed at the HNSC. Overall, the WHO target of patients on treatment (80%) was not reached. But when analyzing the cascade broken down by place of follow-up, patients being followed up outside the HNSC reached the goal. This can be explained by a possible data collection bias, where all patients with prescriptions for treatment in the AME system from other locations were linked to other services. In addition, HNSC patients may have even started treatment due to the lack of clinical conditions, such as neoplasms or

Table 2 Poisson regression analysis with multivariable robust variance of significant variables in relation to steps of cascade of care of patients diagnosed with hepatitis C virus infection at the Hospital Nossa Senhora da Conceição between 2015 and 2020

	PCR-HCV collection	*P* value	Specialized assistance	*P* value	Treatment	*P* value	SVR	*P* value
Gender								
Female			0.06 (0.97-1.16)	0.148	1.20 (1.03-1.40)	0.014		
Male			1.00		1.00			
Race								
White					1.0		1.0	
Non-white					0.99 (0.81-1.21)	0.931	0.95 (0.72-1.25)	0.734
Education								
Illiterate	0.83 (0.67-1.03)	0.103	0.87 (0.73-1.04)	0.152	0.76 (0.53-1.09)	0.145	0.63 (0.35-1.12)	0.120
Elementary school	0.89 (0.81-0.97)	0.014	0.94 (0.85-1.04)	0.244	0.85 (0.72-1.02)	0.082	0.79 (0.65-0.95)	0.014
High school and university	1.0		1.0		1.0		1.0	
City of origin								
Porto Alegre	1.0		1.0		1.0		1.0	
Metropolitan region	1.11 (1.00-1.23)	0.033	1.12 (1.00-1.24)	0.034	1.19 (0.98-1.45)	0.074	1.54 (1.19-1.98)	0.001
Countryside	1.19 (1.08-1.33)	0.001	1.14 (1.03-1.25)	0.009	1.38 (1.14-1.68)	0.001	1.62 (0.24-2.12)	0.000
Follow-up site								
HNSC	1.58 (1.43-1.75)	0.000	3.00 (2.58-3.50)	0.000	2.19 (1.80-2.65)	0.000	1.59 (1.20-2.09)	0.001
External	1.0		1.0		1.0		1.0	
Institutionalized								
Yes					0.70 (0.42-1.17)	0.178		
No					1.0			
Co-infection with HBV								
Yes	1.11 (0.97-1.27)	0.121						
No	1.0							
Co-infection with HIV								
Yes	1.11 (0.97-1.27)	0.121			0.80 (0.59-1.08)	0.156		
No	1.0				1.0			
Cirrhosis								
Yes	1.02 (0.94-1.11)	0.504	0.96 (0.90-1.03)	0.327	1.07 (0.91-1.62)	0.363	1.18 (0.91-1.36)	0.275
No	1.0		1.0		1.0		1.0	
Date of diagnosis								
2015	1.0		1.0		1.0		1.0	1.000
2016	1.02 (0.90-1.16)	0.662	0.90 (0.80-1.01)	0.083	0.82 (0.65-1.03)	0.102	1.05 (0.80-1.37)	0.701
2017	0.97 (0.84-1.12)	0.711	0.93 (0.81-1.06)	0.311	0.96 (0.76-1.20)	0.730	1.06 (0.80-1.41)	0.652
2018	0.90 (0.77-1.05)	0.213	0.92 (0.79-1.07)	0.321	0.83 (0.64-1.07)	0.153	0.93 (0.67-	0.685

							1.30)	
2019	1.02 (0.88-1.19)	0.717	0.88 (0.76-1.03)	0.118	0.81 (0.62-1.05)	0.125	0.81 (0.57-1.16)	0.267
2020	1.27 (1.09-1.48)	0.002	1.03 (0.86-1.25)	0.698	1.02 (0.75-1.39)	0.879	0.96 (0.66-1.40)	0.862

P value < 0.05 was considered significant. HCV: Hepatitis C virus; SVR: Sustained virologic response; HNSC: Hospital Nossa Senhora da Conceição.

Table 3 Comparison between patients with hepatitis C virus infection who underwent follow-up at the Hospital Nossa Senhora da Conceição and external ones using Pearson's chi-square test

	HNSC	External	*P* value
Total	340 (73.8%)	121 (26.2%)	
Gender			0.654
Female	161 (47.2%)	60 (49.6%)	
Age (yr)			0.854
< 40	60 (17.6%)	19 (15.7%)	
40-59	167 (49.0%)	59 (48.8%)	
> 60	114 (33.4%)	43 (35.5%)	
Race			0.450
White	262 (79.2%)	91 (75.8%)	
Education			0.411
Illiterate	22 (7.1%)	4 (3.5%)	
Elementary school	218 (69.9%)	83 (73.5%)	
High school and university	72 (23.1%)	26 (23.0%)	
City of origin			0.006
Porto Alegre	121 (36.4%)	53 (43.8%)	
Metropolitan region	116 (34.9%)	51 (42.1%)	
Countryside	95 (28.6%)	17 (14.0%)	
Cirrhosis			0.000
Yes	100 (29.3%)	7 (5.8%)	

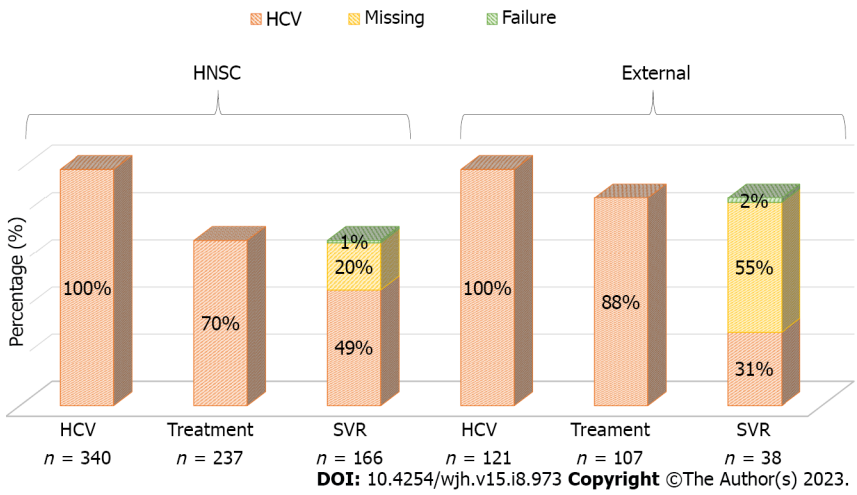
P value < 0.05 was considered significant. HNSC: Hospital Nossa Senhora da Conceição.

serious comorbidities. Not having genotyping or HCV-PCR test within a year are also bureaucratic reasons that interfere with the delay in starting HCV therapy, which is unfortunate given the possibility of simplified protocols with pangenotypic regimens[15,16].

About 90% of patients with HCV infection are cured with the new DAAs, resulting in a markedly decreased risk of liver-related morbidity and mortality and also a drastic reduction of onward transmission[5]. Despite the high response rate to DAAs, the SVR percentages are surprisingly low in this study, which represents the biggest "gap" among all the cascade steps. This is due to the large number of patients with missing data after treatment, making it impossible to confirm SVR. In addition to the collection of HCV-PCR control outside the Brazilian Unified Health System (SUS), another reason for the ignored data would be the loss to follow-up of the patients after the completion of the treatment, identifying the importance of implementing a strategy to enhance the rate of return of the patients after the exams[17]. The coronavirus disease 2019 pandemic had an important impact on the follow-up of these patients after treatment[18]. HNSC patients had a higher SVR and a lower number of ignored HCV-PCR tests, possibly due to less loss to follow-up.

RT-PCR collection

National data and data from the state of Rio Grande do Sul have shown a progressive decrease in reported cases of viral hepatitis with anti-HCV reagents and concomitant HCV-PCR reagents, which may demonstrate an increase in notifications of cases of serological cure, or even less access to confirmatory HCV-PCR tests[11,13]. Patients who were connected to the hospital collected significantly more PCR-HCV, which may have been facilitated by the logistics of collecting the



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Figure 2 Cascade care of hepatitis C virus infected patients diagnosed between 2015 and 2020 at the Hospital Nossa Senhora da Conceição and who underwent follow-up at the Hospital Nossa Senhora da Conceição and elsewhere (external). HNSC: Hospital Nossa Senhora da Conceição; HCV: Hepatitis C virus; SVR: Sustained virologic response.

test inside the hospital under study, after the diagnosis was made. This can also be explained by the greater severity of the patients, as patients with comorbidities, such as HBV and HIV infections and cirrhosis, were shown to have greater access to the test. Living in the countryside and in the metropolitan area were also significant protective factors in this analysis for HCV-PCR collection, as most of these patients have consultations at the HNSC, possibly due to less access to confirmatory HCV-PCR tests outside the capital Porto Alegre[13]. Patients ended up collecting more exams in 2020, demonstrating more accessibility to the exam that year or even greater concern for investigating the disease of patients throughout the pandemic.

Bond

Gaps occur at all stages of HCV care, with dropouts in care occurring before and after linking to specialized care[19]. Consultation at a specialized outpatient clinic is recommended in the treatment lines established in the country; however, more recent guidelines describe the intention of training non-specialist physicians[10,14]. HNSC patients may have found it easier to create a bond with an infectology or gastroenterology outpatient clinic because they were diagnosed in the same hospital. On the other hand, patients from the countryside and metropolitan area, who mostly have consultations at the HNSC, sought this service possibly due to a shortage or lack of specialized professionals in their cities of origin.

Treatment

Having hepatocellular carcinoma, decompensated cirrhosis, or other neoplasms interfere with the assessment of the patient's profile and with the recommendation of treatment[14]. Cirrhosis was shown to be a positive factor in the simple analysis, which is justified by the recent finding that having advanced cirrhosis was a necessary criterion for undertaking treatment[14]. On the other hand, co-infection with HIV and institutionalized individuals proved to be risk factors for not undergoing treatment, and it should be noted that they are vulnerable groups[14]. The non-white race in the simple analysis also proved to be a vulnerable group for not undergoing treatment, which reminds us of the need to consider race in the implementation of public policies[20]. In the final analysis, the female sex obtained higher data in the performance of treatment; however, this data contradicts the results seen previously where women, mainly young people, tend to face barriers to engaging in any form of health care[21]. Furthermore, HCV infection rates in women of childbearing potential have increased, making prenatal diagnosis a priority[22]. Being connected to the HNSC was associated with a significantly higher rate of undergoing treatment, which may be related to the maintenance of follow-up at a specialized outpatient clinic for gastroenterology and infectology services.

SVR

Having a low level of education, only elementary school, proved to be an obstacle to collecting HCV-RNA and achieving SVR, indicating the importance of education for the perception of their health status. Counterintuitively, it is possible that having more knowledge about the natural history of the disease is associated with greater stigma of HCV infection, another barrier to be addressed in the continuous care of patients. The patients at the HNSC were mostly from the countryside and metropolitan area and had cirrhosis inferring the need and demand for specialized care. Despite these results, the training of primary care professionals is able to increase the rate of treatment of patients, with HCV cure results in the decentralization of care similar to specialty outpatient clinics[22].

CONCLUSION

Among the limitations of the study is our inability to determine the true rate of SVR due to the high number of missing follow-up HCV-PCR data. Furthermore, other missing data from the HNSC medical records and external sites, important for a thorough analysis of sociodemographic variables, are also a considerable limitation of the referred study. This study identified that the sociodemographic characteristics of patients diagnosed with HCV infection at the HNSC are similar to regional and national data. It was also possible to stratify relevant risk factors for patients failing to proceed along the treatment cascade, thereby elucidating potential targeted strategies to improve care. According to the results of the hepatitis treatment at the HNSC, the importance of specialized care was highlighted. To make hepatitis treatment more accessible to patients in the countryside and metropolitan area, teams need to be properly trained. Given that we are far from the goals defined by the WHO necessary for elimination of HCV as a public health problem, it is critical to strengthen the treatment lines and facilitate care for patients not only at the HNSC, but also throughout the South region.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) is defined as a public health problem by the World Health Origination (WHO) and since then has defined targets through the HCV elimination.

Research motivation

The South region of Brazil is responsible for the highest detection rate of confirmed HCV infection in the country and also for the highest mortality rate, with higher rates than national data. Within this region, the city of Porto Alegre, in 2020 was the second capital with the highest HCV detection rate, and in 2021 the first, even higher than the national rate.

Research objectives

To define the continuity of care or treatment cascade for patients with chronic HCV infection at the Hospital Nossa Senhora da Conceição (HNSC) and to define sociodemographic variables that influence follow-up between each step of the cascade.

Research methods

With the retrospective cohort design, patients diagnosed with HCV infection in the period between January 1, 2015 and December 31, 2020 were included. Data from HCV notification forms, electronic medical records, Computerized Laboratory Environment Manager System and Medicine Administration System (evaluation of special medications) were collected in 2022 and all information up to that period was considered. The data were analyzed with IBM SPSS version 25, and Poisson regression with robust simple variance was performed for analysis of variables in relation to each step of the cascade. Variables with $P < 0.20$ were included in the multivariate analysis with $P < 0.05$ considered significant. Pearson's chi-square test was applied to compare the groups of patients who persisted in follow-up at the HNSC and who underwent follow-up at other locations.

Research results

Results were lower than expected by the WHO with only 49% of candidates receiving HCV treatment and only 29% achieving sustained virologic response (SVR), despite the 98% response rate to direct acting antivirals documented by follow-up examination. The city of origin and the place of follow-up were the variables associated with SVR and all other endpoints. When comparing the cascade of patients who remained assisted by the HNSC *vs* external patients, we observed superior data for HNSC patients in the SVR. Patients from the countryside and metropolitan region were mostly assisted at the HNSC and the specialized and continuous care provided at the HNSC was associated with superior results, although the outcomes remain far from the goals set by the WHO.

Research conclusions

This study identified that the sociodemographic characteristics of patients diagnosed with HCV infection at the HNSC are similar to regional and national data. It was also possible to stratify relevant risk factors for patients failing to proceed along the treatment cascade, thereby elucidating potential targeted strategies to improve care. According to the results of the hepatitis treatment at the HNSC, the importance of specialized care was highlighted. To make hepatitis treatment more accessible to patients in the countryside and metropolitan area, teams need to be properly trained.

Research perspectives

We have the perspective that other places carry out their HCV cascade of care for stratification of local risk factors, thus helping to eliminate hepatitis C.

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FOOTNOTES

Author contributions: Vaucher MB analyzed the data and wrote the manuscript; Silva CU performed the research; Varella IRS contributed to data analysis and helped perform the research; Kim AYS contributed analytic tools and reviewed the manuscript; and Kliemann DA designed the research study and reviewed the manuscript.

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ORIGINAL ARTICLE

Observational Study

Impact renaming non-alcoholic fatty liver disease to metabolic associated fatty liver disease in prevalence, characteristics and risk factors

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Abstract

BACKGROUND

Recently, a group of hepatologists proposed to rename non-alcoholic fatty liver disease (NAFLD) as metabolic associated fatty liver disease (MAFLD) with modified diagnostic criteria. It is important to note, however, that there are some differences between the diagnostic criteria used for NAFLD and MAFLD. Since the research on MAFLD is just beginning, however, evidence on its incidence and prevalence in the general population and in specific subpopulations remains limited.

AIM

To assess epidemiology of fatty liver in new definition and compare MAFLD with NAFLD. Exploring risk factors of MAFLD individuals.

METHODS

This was a retrospective, cross-sectional study. A total of 85242 adults were selected from the Chinese health management database in 2017–2022. The data of general information, laboratory indicators, lifestyle management and psychological status were obtained. MAFLD was diagnosed as ultrasound diagnosis of fatty liver and at least one between these three conditions: Overweight/obesity, type 2 diabetes mellitus (T2DM) or metabolic dysregulation. Metabolic factors were not considered in NAFLD diagnosis standard. The clinical characteristics of MAFLD and NAFLD were analysed using descriptive statistics. Continuous

variables normally distributed were expressed as means ± SD. Categorical variables were expressed as frequencies and proportions. Binary logistic regression was used to determine risk factors of the MAFLD.

RESULTS

The prevalence of MAFLD and NAFLD was 40.5% and 31.0%, respectively. The MAFLD or NAFLD population is more likely to be older (M: 47.19 ± 10.82 *vs* 43.43 ± 11.96; N: 47.72 ± 11.17 *vs* 43.71 ± 11.66), male (M: 77.21% *vs* 44.43%; N: 67.90% *vs* 53.12%) and high body mass index (M: 26.79 ± 2.69 *vs* 22.44 ± 2.48; N: 26.29 ± 2.84 *vs* 23.29 ± 3.12) than the non-MAFLD or non-MAFLD population. In multivariate analysis, general information (*e.g.*, ≥ 2 metabolic abnormalities OR = 3.38, (95%CI: 2.99-3.81), $P < 0.001$; diastolic blood pressure OR = 1.01, (95%CI: 1.00–1.01), $P = 0.002$), laboratory results [*e.g.*,total bilirubin (TBIL) OR = 0.98, (95%CI: 0.98-0.99), $P < 0.001$; serum uric acid(SUA) OR = 1.01, (95%CI: 1.01-1.01), $P < 0.001$], and lifestyle factors [*e.g.*, drink beverage OR = 0.32, (95%CI: 0.17-0.63), $P = 0.001$] were influence factors for MAFLD. Our study results offer new insight into potential risk factors associated with fatty liver disease, including SUA, TBIL and creatinine, all of which are related to chronic renal disease (CKD).

CONCLUSION

MAFLD is more prevalent than NAFLD, with two-fifths of individuals meeting the diagnosis criteria. MAFLD and NAFLD populations have different clinical characteristics. CKD may be related with MAFLD.

Key Words: Metabolic (dysfunction)-associated fatty liver disease; Non-alcoholic fatty liver disease; Epidemiology; Risk factors; Characteristics; Cross-section study

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Core Tip: This study explores the epidemiological characteristics, risk factors and draws reliable conclusions based on the new diagnostic criteria for metabolic associated fatty liver disease, using a large sample of data, and provides evidence for subsequent studies.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is associated with excessive lipid accumulation in the liver resulting from disordered hepatic lipid metabolism that is stimulated by non-alcohol-related factors[1]. In 2019, the global prevalence of NAFLD was approximately 30.6%[2]. In China, the prevalence is as high as 32.3%[3], making it the number one cause of chronic liver disease and abnormal liver biochemical indicators during routine physical examination. These findings indicate that NAFLD imposes a heavy disease burden on patients and society. As its disease mechanism has become better understood, the limitations of the NAFLD nomenclature have become more apparent. These include: (1) The lack of a uniform standard for calculating alcohol intake, which has led to an underestimation of the role of alcohol consumption in disease pathogenesis; and (2) a failure to recognize the influence of metabolic factors in disease etiology [4]. In 2020, Eslam *et al*[5] suggested renaming NAFLD as 'metabolic (dysfunction) associated fatty liver disease' (MAFLD). A diagnosis of MAFLD includes the presence of hepatic steatosis and one or more of the following features: (1) Overweight based on body mass index (BMI); (2) type 2 diabetes mellitus; or (3) lean or normal weight with evidence of metabolic dysregulation[6]. The new nomenclature aims to reflect the close relationship between fatty liver and overnutrition, sedentary lifestyle, and metabolic conditions such as type 2 diabetes, hypertension, dyslipidemia, and obesity[7]. Adopting a positive diagnosis like MAFLD recognizes the impact of metabolic conditions and fatty liver on the natural history of different liver diseases such as chronic viral hepatitis and alcohol-related liver disease[8].

It is important to note, however, that there are some differences between the diagnostic criteria used for NAFLD and MAFLD. Study indicates that[6] some NAFLD patients are excluded under the proposed MAFLD definition, based on disparate characteristics included in each definition. The rates of diabetes, hypertriglyceridemia, hypertension, and fibrosis risk are significantly higher among MAFLD than NAFLD patients. The proposed MAFLD definition challenges the current understanding of the prevalence and associated factors of fatty liver. Meanwhile, MAFLD is shown to be a better predictor of cardiovascular disease risk among asymptomatic individuals than NAFLD[9]. Since the research on MAFLD is just beginning, however, evidence on its incidence and prevalence in the general population and in specific subpopulations remains limited. The few studies are based on small sample sizes and do not directly compare the characteristics of NAFLD and MAFLD[10]. Thus, our study aims to conduct an updated analysis of the prevalence and factors associated with MAFLD. A more comparative analysis of the clinical characteristics of patients with NAFLD and MAFLD

is also performed in order to identify MAFLD-specific risk factors.

MATERIALS AND METHODS

Study design

A cross-sectional study was conducted by recruiting participants from the health management center of the general tertiary hospital of Southern China between August 1, 2017 and October 31, 2022. Patients who were ≥ 18 years of age, had received a fatty liver color Doppler ultrasonography result, blood lipid examination, exercise and dietary evaluation, and were voluntary participants in this study were included. Patients who lacked imaging or laboratory data for a MAFLD diagnosis, had incomplete Diet and Exercise Health Check survey responses, or were pregnant at the time of examination due to different waist circumference and BMI measurements caused by pregnancy, were excluded from the study. This study was reviewed and approved by the Central South University Ethics Review Board (IRB2022-S217). All patients provided their written informed consent to participate in the study.

Diagnostic criteria and group definitions

Definition of hepatic steatosis: Hepatic steatosis was defined in NHANES III participants using the Hepatic Steatosis Ultrasound Examination. Adult patients received a hepatic ultrasound at a mobile examination center using a Toshiba Sonolayer SSA-90A ultrasound machine (Toshiba America Medical Systems, Inc., Tustin, CA, United States)[11]. Board-certified radiologists used five different parameters to assess hepatic steatosis: parenchymal brightness, liver-to-kidney contrast, deep beam attenuation, bright vessel walls, and gallbladder wall definition. Ultrasonographic assessments were reported as normal, mild, moderate, or severe hepatic steatosis. Abiding by quality control procedures, reliability results (intra-rater and inter-rater) were calculated. The intra-rater and inter-rater reliabilities were 91.3% (kappa 0.77) and 88.7% (kappa 0.70), respectively[12].

Definition of MAFLD: MAFLD was defined[13] as the presence of hepatic steatosis by liver ultrasound plus one or more of the following conditions: (1) overweight/obesity (BMI>23 kg/m^2); (2) type 2 diabetes mellitus (T2DM); (3) at least two metabolic risk abnormalities. Metabolic risk abnormalities included: (1) A waist circumference ≥ 90 cm in males or ≥ 80 cm in females; (2) a blood pressure ≥ 130/85 mmHg or specific drug treatment; (3) plasma triglycerides ≥ 150 mg/dL (≥ 1.70 mmol/L) or specific drug treatment; (4) plasma high density lipoprotein cholesterol(HDL-C) < 40 mg/dL (< 1.0 mmol/L) for males and < 50 mg/dL (< 1.3 mmol/L) for females or specific drug treatment; (5) prediabetes [fasting glucose levels of 100–125 mg/dL (5.6–6.9 mmol/L) or HbA1c (5.7%–6.4%) 39–47 mmol/L]; (6) homeostasis model assessment of insulin resistance (HOMA-IR) score ≥ 2.5; (7) a plasma high sensitivity C-reactive protein level > 2 mg/L.

Definition of NAFLD: NAFLD was diagnosed according to the EASL-European Association for the Study of Diabetes-European Association for the Study of Obesity and American Association for the Study of Liver Diseases Clinical Practice Guidelines for the Management of NAFLD: (1) Fatty liver by abdominal ultrasonography; (2) alcohol consumption< £30 g/d for men and <£20 g/d for women; and (3) no competing etiologies for fatty liver or coexisting causes of chronic liver disease[14].

Demographic variables

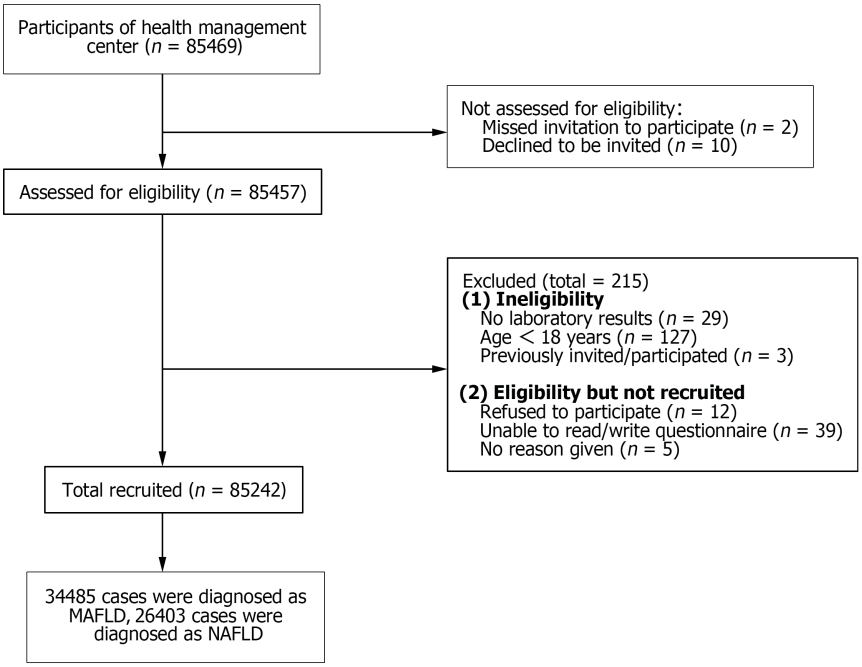
The following demographic variables were obtained from the patient electronic record database: age, gender, BMI, waist circumference, hip circumference, smoking, alcohol consumption, hypertension, diabetes, and actively acquisition of medical knowledge. BMI was calculated as the weight (in kilograms) divided by the square of the height (in meters). Overweight/obesity was defined as BMI > 23 kg/m^2. Waist and hip circumferences were determined in centimeters using a tape measure. Blood pressure was recorded in the sitting position using standardized equipment. Hypertension was defined as a systolic blood pressure (SBP) ≥ 130 mmHg and a diastolic blood pressure ≥ 85 mmHg or the use of antihypertensive medications. A diagnosis of diabetes was based on a history of diabetes, use of antidiabetic medications, and/or a fasting plasma glucose ≥ 7.0 mmol/L. Information on lifestyle and psychological factors was acquired from the patient self-report questionnaires.

Laboratory parameters

Laboratory measurements included total bilirubin (TBIL), aspartate aminotransferase (AST), alanine transaminase (ALT), albumin/globulin (A/G), fasting plasma glucose (FPG), glycated haemoglobin (HbA1c), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), platelet, creatinine, ≥ 2 metabolic abnormalities, blood urea nitrogen (BUN) and serum uric acid (SUA). All biochemical assessments were performed using standard laboratory methods. HDL-C, LDL-C, FPG, BUN, TC and TG were reported in millimoles per liter (mmol/L). The units of TBIL, SUA, total bile acid and creatinine were micromoles per liter(umol/L), HbA1c is expressed in percentage terms, and liver enzymes(AST,ALT) were reported in units per liter (U/L).

Statistical analysis

Continuous variables normally distributed were expressed as means ± SD. Categorical variables were expressed as frequencies and proportions. The prevalence of MAFLD and NAFLD was determined as the number of subjects with the corresponding conditions divided by the total number of subjects. Univariable and multivariable binary logistic regression analyses were also performed to determine factors associated with MAFLD. The univariate and multivariate



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Figure 1 Flow chart of the study population inclusion process. MAFLD: Metabolic associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease.

odd ratios (OR) were reported along with 95% confidence intervals (CI). All tests were two-tail and results with a P value < 0.05 were considered statistically significant. All analyses were conducted using SPSS 24 version.

RESULTS

Of the 85242 recruited participants (Figure 1), 26403 (31.0%) had NAFLD [8476 (32.10%) women, median age 47.72 ± 11.17 years], and 34485 (40.5%) met the criteria for MAFLD [7858 (22.79%) women; median age 47.19 ± 10.82 years] (Figure 2, Table 1). Total 23905 (28.0%) participants diagnosed with both MAFLD and NAFLD [7555 (31.60%) women; median age 47.85 ± 11.18 years]. Patients with MAFLD had a higher BMI than those without [26.79 ± 2.69 kg/m^2 *vs* 22.44 ± 2.48 kg/m^2, respectively]. 5.15% (1775/34485) of patients diagnosed with MAFLD have diabetes, and 79.85% (27536/34485) had two or more metabolic abnormalities. Meanwhile, 2498 patients met the definition of NAFLD but did not meet the MAFLD criteria (Figure 3). The general information , laboratory, lifestyle, and psychological characteristics of the study population are summarized in Table 1. All the patients were ethnic Chinese.

The prevalence of MAFLD was lower among individuals < 30 years of age (approximately 2.56%) and highest among those 50–59 years of age (Figure 4). Disease prevalence was significantly higher among men than women. Changes in age-related prevalence were similar for patients with NAFLD and MAFLD, however, there was a lower overall prevalence of NAFLD than MAFLD (Figure 5). The prevalence of both MAFLD and NAFLD increased with BMI and for patients with a BMI ≥ 25, the risk of NAFLD and MAFLD increased dramatically (Figure 6).

In univariate analysis, male sex, older age, higher BMI, higher diastolic blood pressure, higher waist circumference, lower hip circumference, and alcohol consumption, ≥ 2 metabolic abnormalities, medically knowledgeable, TG, HDL-C, TBIL, AST, ALT, A/G, glycated hemoglobin (HbA1c), SUA, platelet, creatinine, drink beverage, exercise frequency, exercise duration and physical labor intensity were associated with MAFLD. In contrast, systolic blood pressure, smoking, diabetes, TC, LDL-C, blood urea nitrogen (BUN), FPG, total bile acid, inappetence, night snacks, crapulent, food preferences, and psychological characteristics were not significantly associated with this disease (P > 0.05). In multivariate analysis, female sex (OR = 0.67, 95%CI: 0.57–0.80, P = 0.001), older age (OR = 1.01, 95%CI: 1.00–1.02, P = 0.001), higher BMI (OR = 1.45, 95%CI: 1.40–1.51, P < 0.001), diastolic blood pressure (OR = 1.01, 95%CI: 1.00–1.01, P = 0.002), waist circumference (OR = 1.12, 95%CI: 1.11–1.14, P < 0.001), hip circumference (OR = 0.95, 95%CI: 0.93–0.96, P < 0.001), metabolic abnormalities(OR = 3.38, 95%CI: 2.99–3.81, P < 0.001), actively acquire medical knowledge (OR = 1.14, 95%CI: 1.03–1.27, P = 0.014), TG (OR = 1.33, 95%CI: 1.27–1.40, P < 0.001), HDL-C (OR = 0.58, 95%CI: 0.47–0.71, P < 0.001), TBIL(OR = 0.98, 95%CI: 0.98–0.99, P < 0.001), AST OR = 1.01, 95%CI: 1.01–1.01, P < 0.001), ALT (OR = 1.02, 95%CI: 1.02–1.02, P < 0.001), HbA1c (OR = 1.52, 95%CI: 1.47–1.57, P < 0.001), higher SUA level (OR = 1.01, 95%CI: 1.01–1.01, P < 0.001), platelets (OR = 1.00, 95%CI: 1.00–1.00, P < 0.001), creatinine (OR = 0.99, 95%CI: 0.99–0.99, P < 0.001), drink beverages (OR = 0.32, 95%CI: 0.17–0.63, P = 0.001), exercise frequency (OR = 0.82, 95%CI: 0.71–0.95, P = 0.009), exercise duration (OR = 1.24, 95%CI: 1.04-1.47, P = 0.015), and labour intensity (OR = 0.78, 95%CI: 0.65–0.95, P = 0.013) remained as independent variables associated with MAFLD (Table 2).

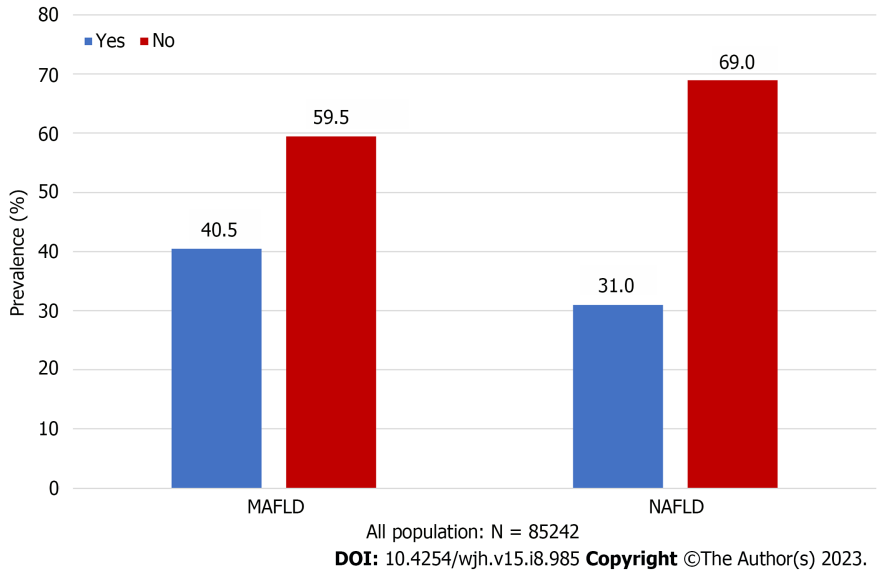
Table 1 Clinical characteristics of the study participants metabolic associated fatty liver disease & non-alcoholic fatty liver disease, with and without metabolic associated fatty liver disease and non-alcoholic fatty liver disease, *n* (%)

Characteristics	All	MAFLD	Not MAFLD	NAFLD	Not NAFLD	MAFLD & NAFLD
N	85242	34485 (40.5)	50757 (59.5)	26403 (31.0)	58839 (69.0)	23905 (28.0)
General information						
Age, yr		47.19 ± 10.82	43.43 ± 11.96	47.72 ± 11.17	43.71 ± 11.66	47.85 ± 11.18
Sex						
Male	49177	26627 (77.21)	22550 (44.43)	17927 (67.90)	31259 (53.12)	16350 (68.40)
Female	36065	7858 (22.79)	28207 (55.57)	8476 (32.10)	27589 (46.88)	7555 (31.60)
BMI, kg/m^2		26.79 ± 2.69	22.44 ± 2.48	26.29 ± 2.84	23.29 ± 3.12	26.64 ± 2.71
Systolic blood pressure, mmHg		128.50 ± 15.42	118.67 ± 15.13	127.40 ± 15.67	120.52 ± 15.68	128.21 ± 15.69
Diastolic blood pressure, mmHg		79.97 ± 11.06	72.17 ± 10.42	78.49 ± 10.96	73.91 ± 11.24	79.06 ± 10.96
Waist circumference, cm		90.41 ± 7.68	76.78 ± 8.01	88.57±8.03	79.51 ± 10.03	89.43 ± 7.74
Hip circumference, cm		97.81 ± 5.52	91.53 ± 5.03	96.92 ± 5.63	92.80 ± 5.83	97.42 ± 5.55
Smoke						
Never	57452	19713 (57.18)	37739 (74.36)	17589 (66.63)	39863 (67.76)	15824 (66.21)
Always	20951	11443 (33.19)	9508 (18.73)	6663 (25.24)	14288 (24.29)	6127 (25.64)
Smoke in the past	3106	1666 (4.83)	1440 (2.84)	1047 (3.97)	2059 (3.50)	956 (4.00)
Passive exposure to secondhand smoke	3720	1655 (4.80)	2065 (4.07)	1098 (4.16)	2622 (4.46)	992 (4.15)
Alcohol consumption						
Yes	27567	14899 (44.10)	12668 (25.31)	5546 (21.31)	22021 (38.09)	5042 (21.41)
No	56275	18882 (55.90)	37393 (74.69)	20478 (78.69)	35797 (61.91)	18506 (78.59)
Diabetes						
Yes	2596	1775 (5.15)	821 (1.62)	1287 (5.12)	1309 (2.22)	1287 (5.38)
No	82645	32710 (94.85)	49935 (98.38)	25116 (94.88)	57529 (97.78%)	22618 (94.61)
Inappetence						
Never	57298	24017 (69.66)	33281 (65.57)	18671 (70.73)	38627 (65.65)	16988 (71.08)
Occasionally	24989	9446 (27.40)	15543 (30.62)	6976 (26.43)	18013 (30.62)	6232 (26.08)
Often	2947	1016 (2.95)	1931 (3.80)	751 (2.84)	2196 (3.73)	680 (2.85)
Take the initiative to acquire medical knowledge						
Yes	48284	19268 (55.88)	29016 (57.17)	14989 (56.78)	33295 (56.59)	13534 (56.63)
No	36946	15210 (44.12)	21736 (42.83)	11409 (43.22)	25537 (43.41)	10366 (43.37)
Laboratory inspection						
TG, mmol/L		2.67 ± 2.40	1.32 ± 1.00	2.38 ± 2.07	1.63 ± 1.67	2.47 ± 2.14
TC, mmol/L		5.24 ± 1.04	4.89 ± 0.92	5.19 ± 1.00	4.96 ± 0.97	5.19 ± 1.01
LDL-C, mmol/L		2.88 ± 0.89	2.86 ± 0.78	2.94 ± 0.87	2.84 ± 0.80	2.92 ± 0.88
HDL-C, mmol/L		1.18 ± 0.24	1.42 ± 0.30	1.19 ± 0.24	1.38 ± 0.31	1.18 ± 0.23
TBIL, μmol/L		13.38 ± 5.15	13.52 ± 5.28	13.24 ± 5.15	13.56 ± 5.26	13.21 ± 5.16
AST, U/L		26.83 ± 18.10	22.55 ± 17.75	25.76 ± 13.67	23.74 ± 19.70	26.00 ± 14.07
ALT, U/L		35.73 ± 27.93	21.28 ± 21.60	33.86 ± 25.81	24.09 ± 24.57	34.59 ± 26.44
A/G		1.76 ± 0.29	1.76 ± 0.31	1.75 ± 0.28	1.77 ± 0.31	1.74 ± 0.28
FPG, mmol/L		5.96 ± 1.68	5.32 ± 1.77	5.88 ± 1.62	5.44 ± 1.81	5.93 ± 1.65

HbA1c (%)		5.91 ± 0.96	5.52 ± 0.63	5.90 ± 0.95	5.59 ± 0.72	5.92 ± 0.95
BUN, mmol/L		4.97 ± 1.23	4.71 ± 1.31	4.95 ± 1.23	4.75 ± 1.31	4.97 ± 1.23
SUA, μmol/L		385.23 ± 85.79	312.61 ± 79.14	372.25 ± 85.03	328.43 ± 87.85	375.02 ± 85.10
Platelets ($\times 10^9$/L)		227.84 ± 54.33	225.02 ± 55.02	229.55 ± 54.92	224.64 ± 54.61	229.55 ± 55.11
Total bile acid, μmol/L		4.36 ± 5.89	3.96 ± 5.25	4.24 ± 5.13	4.07 ± 5.71	4.29 ± 5.25
Creatinine, μmol/L		77.32 ± 16.64	78.80 ± 43.80	75.62 ± 17.34	72.46 ± 41.16	75.84 ± 17.52
≥ 2 metabolic abnormalities						
Yes	38399	27536 (79.85)	10863 (21.40)	19018 (72.03)	19381 (32.94)	19018 (79.56)
No	46843	6949 (20.15)	39894 (78.60)	7385 (27.97)	39458 (67.06)	4887 (20.44)
Lifestyle management						
Do you often eat late night snacks						
Never	56798	23097 (66.99)	33701 (66.40)	19221 (72.81)	37577 (63.87)	17377(72.70)
Occasionally	25624	10204 (29.59)	15420 (30.38)	6579 (24.92)	19045 (32.37)	5971 (24.98)
Often	2811	1179 (3.42)	1632 (3.22)	600 (2.27)	2211 (3.76)	554 (2.32)
Crapulent						
Yes	5750	3175 (9.21)	2575 (5.07)	1799 (6.81)	3951 (6.72)	1706 (7.14)
No	79484	31305 (90.79)	48179 (94.93)	24600 (93.19)	54884 (93.28)	22195 (92.86)
Food preference						
Light	35389	12278 (35.61)	23111 (45.54)	10875 (41.20)	24514 (41.67)	9665 (40.44)
Briny	26194	12755 (36.99)	13439 (26.48)	8694 (32.93)	17500 (29.74)	8014 (33.53)
Unclear	23649	9446 (27.40)	14203 (27.98)	6829 (25.87)	16820 (28.59)	6221 (26.03)
Drink beverage						
Never	46065	18399 (82.24)	27666 (82.51)	13920 (81.90)	32145 (82.27)	12602 (81.58)
Occasionally	9198	3653 (16.33)	5545 (16.54)	2830 (16.65)	6368 (16.30)	2624 (16.99)
Often	806	320(1.43)	320 (0.95)	246 (1.45)	560 (1.43)	222 (1.44)
Exercise frequency						
1-2 times/wk	21380	8441 (39.52)	12939 (41.37)	6477 (39.69)	14903 (41.04)	5820 (39.48)
3-5 times/wk	21162	8672 (40.60)	12490 (39.94)	6503 (39.85)	14659 (40.36)	5887 (39.93)
> 5 times/wk	10093	4247 (19.88)	5846 (18.69)	3338 (20.46)	6755 (18.60)	3036 (20.59)
Exercise training						
Yes	52829	21444 (62.20)	31385 (61.84)	16404 (62.15)	36425 (61.91)	14825 (62.03)
No	32400	13033 (37.80)	19367 (38.16)	9991 (37.85)	22409 (38.09)	9073 (37.97)
Exercise duration						
< 30 min	12701	4950 (23.17)	7751 (24.78)	4085 (25.03)	8616 (23.72)	3662 (24.84)
30-60 min	30669	12575 (58.87)	18094 (57.85)	9475 (58.06)	21194 (58.36)	8568 (58.12)
> 60 min	9266	3836 (17.96)	5430 (17.36)	2758 (16.90)	6508 (17.92)	2513 (17.05)
Labour intensity						
Light physical labor	77907	31651 (91.78)	46256 (91.13)	24186 (91.60)	53721 (91.30)	21844 (91.38)
Moderate physical labor	6282	2463 (7.14)	3819 (7.52)	1940 (7.35)	4342 (7.38)	1808 (7.56)
Heavy physical labor	1053	371 (1.08)	682 (1.34)	277 (1.05)	776 (1.32)	253 (1.06)
Psychological states						
Irritability						

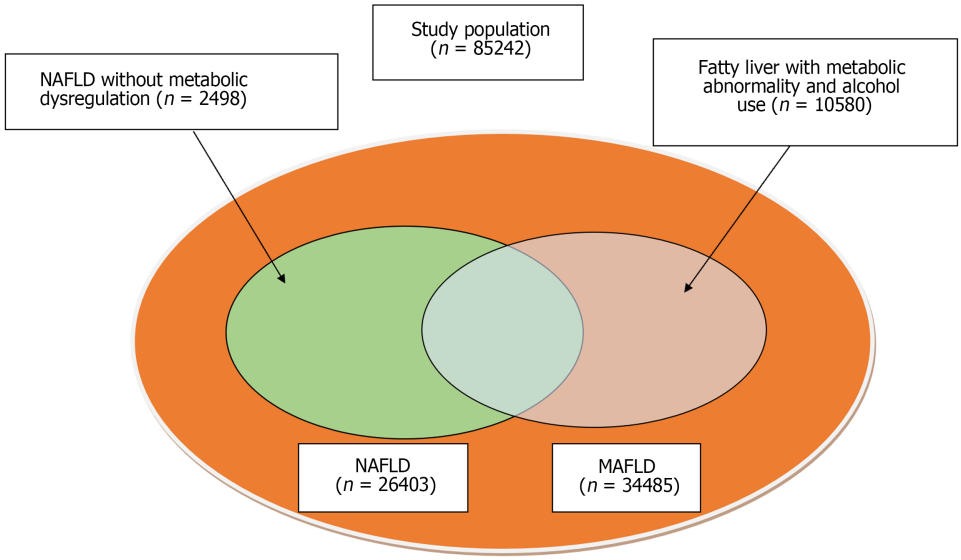
Never	43964	18836 (54.63)	25128 (49.51)	14745 (55.85)	29219 (49.67)	13428 (56.18)
Occasionally	35294	13631 (39.53)	21663 (42.68)	10152 (38.46)	25142 (42.74)	9103 (38.09)
Often	5973	2012 (5.84)	3961 (7.80)	1502 (5.69)	4471 (7.60)	1370 (5.73)
Tense and unrelaxed						
Never	54907	22753 (65.99)	31154 (61.38)	17813 (67.47)	36094 (61.35)	16156 (67.59)
Occasionally	26438	10081 (29.24)	16357 (32.23)	7379 (27.95%)	19059 (32.39)	6638 (27.77%)
Often	4890	1647 (4.78)	3243 (6.39)	1208 (4.58)	3682 (6.26)	1108 (4.64)
Anxious						
Never	55837	23594 (68.43)	32243 (63.53)	18363 (69.56)	37474 (63.69)	16670 (69.75)
Occasionally	25399	9578 (27.78)	15821 (31.17)	7059 (26.74)	18340 (31.17)	6337 (26.51)
Often	3999	1307 (3.79)	2692 (5.30)	977 (3.70)	3022 (5.14)	894 (3.74)
Depress						
Never	59871	25192 (73.06)	34679 (68.32)	19610 (74.28)	40261 (68.43)	17811 (74.52)
Occasionally	22155	8306 (24.09)	13849 (27.29)	6040 (22.88)	16115 (27.39)	5410 (22.63)
Often	3210	982 (2.85)	2228 (4.39)	750 (2.84)	2460 (4.18)	681 (2.85)
Sleep						
Well	33017	14188 (41.15)	18829 (37.10)	11027 (41.77)	21990 (37.38)	10043 (42.02)
Moderate	43242	16974 (49.23)	26268 (51.76)	12894 (48.84)	30348 (51.58)	11627 (48.65)
Bad	8974	3318 (9.62)	5656 (11.14)	2478 (9.39)	6496 (11.04)	2231 (9.33)

BMI: Body Mass Index; SUA: Serum uric acid; TG: Triglycerides; TC: Total cholesterol; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; TBIL: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine transaminase; A/G: Albumin/globulin; FPG: Fasting plasma glucose; BUN: Blood urea nitrogen; HbA1c: Glycated hemoglobin.



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Figure 2 Prevalence of metabolic associated fatty liver disease and non-alcoholic fatty liver disease. MAFLD: Metabolic associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease.



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Figure 3 Participants with metabolic associated fatty liver disease, non-alcoholic fatty liver disease, and those excluded by the two definitions. MAFLD: Metabolic associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease.

DISCUSSION

This study found that the prevalence of fatty liver disease was higher when the MAFLD definition was used for diagnosis rather than the NAFLD definition (40.5% *vs* 31.0%, respectively). In addition, a higher number of factors were associated with MAFLD, including general information (*e.g.*, 8 items such as metabolic abnormalities, diastolic blood pressure), laboratory (*e.g.*, 9 items such as TBIL, SUA), and lifestyle (*e.g.*, 4 items such as drink beverage) characteristics. In contrast, psychological factors were not significantly correlated with MAFLD. Among these significant indicators, we have an interesting finding that three indicators are associated with CKD. Participants with CKD may have elevated SUA levels [15], low TBIL levels[16] and abnormal creatinine values, which may suggest that there is an association between CKD and MAFLD, the exact mechanism need to further analysis.

MAFLD prevalence

The prevalence of MAFLD in this study was 40.5%. Several studies have assessed the epidemiology of MAFLD, however, the reported prevalence of this condition varies. While the study[17] demonstrated a lower prevalence of MAFLD (25%-37.3%), a meta-analysis of 2667052 individuals estimated that the global prevalence[18] of this disease was 50.7%. A study using 2017-2018 NHANES data[19] indicated that MAFLD prevalence was 39.1%, a finding similar to that reported here. Consistent with the our study, prior reports have also found that MAFLD[20] is more prevalent in males. Reported[21] variations in the prevalence of MAFLD may be the result of ethnic disparities and environment factors. Differences in the methods used to estimate steatosis (liver ultrasound, elastography, diagnostic scores) may account for some of the heterogeneity.

NAFLD prevalence

The prevalence of NAFLD (31.0%) was lower than the prevalence of MAFLD in this study. A total of 23905 participants had overlapping diagnostic criteria for NAFLD and MAFLD. While 2498 patients had NAFLD without metabolic dysregulation, 10580 patients had fatty liver with metabolic abnormalities and alcohol use. A recent study by Lee *et al*[22] identified a similar number of cases using the MAFLD and NAFLD criteria on population-based data (*n* = 8962813) from National Health and Nutrition Examination surveys (37.3% *vs* 28.0%, respectively), a result similar to our findings. It is probable that the high MAFLD prevalence in the current study was primarily caused by the high prevalence of overweight and metabolic dysfunction.

Comparison of MAFLD and NAFLD disease characteristics

Regardless of age, the prevalence of MAFLD and NAFLD was much higher in males than females, a finding consistent with a study by Ito *et al*[23]. This may be because males are more prone to poor lifestyle habits, such as smoking and alcohol consumption. The current study also found that the peak prevalence of MAFLD occurred earlier among men (40–49 years) than women (50–59 years), a finding reported previously[24]. Women enter menopause and begin to lose estrogen after they are ≥ 50 years of age. Estrogen is thought to suppress visceral fat accumulation and increase subcutaneous fat accumulation. A higher BMI is linked to a higher prevalence of MAFLD and NAFLD. Thus, individuals with high BMI should be appropriately educated about these conditions.

Table 2 Univariate and multivariate logistic regression analysis of factors associated with metabolic associated fatty liver disease, *n* (%)

Variable	Category	Univariate analysis		Multivariable analysis	
		Odds ratio (95%CI)	*P* value	Odds ratio (95%CI)	*P* value
General information					
Sex	Male(ref)				
	Female	0.69 (0.58-0.84)	**< 0.001**	0.67 (0.57-0.80)	**0.001**
Age		1.01 (1.01-1.02)	**0.001**	1.01 (1.00–1.02)	**0.001**
BMI, kg/m^2		1.46 (1.41-1.52)	**< 0.001**	1.45 (1.40-1.51)	**< 0.001**
Systolic blood pressure, mmHg		1.00 (0.99-1.00)	0.360	None	
Diastolic blood pressure, mmHg		1.01 (1.00-1.02)	**0.007**	1.01 (1.00–1.01)	**0.002**
Waist circumference, cm		1.12 (1.11-1.14)	**< 0.001**	1.12 (1.11-1.14)	**< 0.001**
Hip circumference, cm		0.95 (0.93-0.96)	**< 0.001**	0.95 (0.93-0.96)	**< 0.001**
Smoke	Never (ref)		0.409	None	
	Always	1.18 (0.90-1.56)	0.236	None	
	Smoke in the past	1.07 (0.80-1.43)	0.667	None	
	Passive exposure to secondhand smoke	1.15 (0.80-1.65)	0.464	None	
Alcohol consumption	No(ref)				
	Yes	0.89 (0.78-1.01)	**0.061**	1.11 (0.99-1.26)	0.082
Diabetes	No(ref)				
	Yes	1.16 (0.91-1.49)	0.231	None	
≥ 2 metabolic abnormalities	No(ref)				
	Yes	3.38 (3.00-3.82)	**< 0.001**	3.38 (2.99-3.81)	**< 0.001**
Take the initiative to acquire medical knowledge	No(ref)				
	Yes	1.14 (1.02-1.26)	**0.017**	1.14 (1.03-1.27)	**0.014**
Laboratory inspection					
TG, mmol/L		1.31 (1.15-1.49)	**< 0.001**	1.33 (1.27-1.40)	**< 0.001**
TC, mmol/L		1.09 (0.81-1.45)	0.578	None	
LDL- C, mmol/L		1.00 (0.74-1.35)	0.997	None	
HDL-C, mmol/L		0.50 (0.33-0.73)	**< 0.001**	0.58 (0.47-0.71)	**< 0.001**
TBIL, μmol/L		1.01 (1.00-1.02)	**0.044**	0.98 (0.98-0.99)	**< 0.001**
AST, U/L		0.98 (0.97-0.98)	**< 0.001**	1.01 (1.01-1.01)	**< 0.001**
ALT, U/L		1.02 (1.02-1.03)	**< 0.001**	1.02 (1.02-1.02)	**< 0.001**
A/G		1.85 (1.53-2.25)	**< 0.001**	0.99 (0.92-1.08)	0.884
BUN, mmol/L		0.98 (0.94-1.02)	0.388	None	
FPG, mmol/L		0.97 (0.91-1.04)	0.426	None	
HbA1c (%)		1.35 (1.20-1.52)	**< 0.001**	1.52 (1.47-1.57)	**< 0.001**
Total bile acid, μmol/L		1.00 (1.00-1.01)	0.395	None	
SUA, μmol/L		1.00 (1.00-1.01)	**< 0.001**	1.01 (1.01-1.01)	**< 0.001**
Platelets (×10^9/L)		1.00 (1.00-1.00)	**< 0.001**	1.00 (1.00-1.00)	**< 0.001**
Creatinine, μmol/L		0.99 (0.99-1.00)	**< 0.001**	0.99 (0.99-0.99)	**< 0.001**
Lifestyle management					

Inappetence	Never(ref)		0.597	None	
	Occasionally	1.13 (0.84-1.52)	0.409	None	
	Often	1.16 (0.86-1.57)	0.322	None	
Do you often eat late night snacks	Never(ref)		0.529	None	
	Occasionally	1.33 (0.78-2.28)	0.288		
	Often	1.37 (0.79-2.35)	0.260		
Crapulent	No(ref)				
	Yes	0.93 (0.73-1.20)	0.586	None	
Food preference	Light (ref)		0.868	None	
	Briny	0.97 (0.85-1.10)	0.617		
	Unclear	0.99 (0.85-1.14)	0.874		
Drink beverage	Never (ref)		**0.015**		**0.004**
	Occasionally	2.66 (1.37-5.18)	**0.004**	1.01 (0.85-1.20)	0.917
	Often	2.69 (1.36-5.32)	**0.004**	0.32 (0.17-0.63)	**0.001**
Exercise frequency	1-2 times/wk (ref)		**0.048**		**0.025**
	3-5 times/wk	1.20 (1.03-1.40)	**0.017**	0.95 (0.84-1.08)	0.429
	> 5 times/wk	1.14 (1.00-1.30)	**0.049**	0.82 (0.71-0.95)	**0.009**
Exercise training		0.39 (0.04-3.58)	0.404	None	
Exercise duration	< 30 min (ref)		**0.049**		**0.045**
	30-60 min	0.81 (0.68-0.96)	**0.017**	1.07 (0.94-1.22)	0.283
	> 60 min	0.87 (0.76-1.00)	**0.045**	1.24 (1.04-1.47)	**0.015**
Labor intensity	Light physical labor (ref)		**0.049**		**0.026**
	Moderate physical labor	0.80 (0.66-0.97)	**0.024**	0.78 (0.65-0.95)	**0.013**
	Heavy physical labor	0.79 (0.51-1.21)	0.273	0.77 (0.50-1.18)	0.226
Psychological states					
Irritability	Never (ref)		0.637	None	
	Occasionally	1.01 (0.89-1.15)	0.851		
	Often	1.14 (0.87-1.50)	0.346		
Tense and unrelaxed	Never (ref)		0.806	None	
	Occasionally	0.95 (0.820-1.11)	0.351		
	Often	0.96 (0.69-1.35)	0.828		
Anxious	Never (ref)		**0.076**		0.091
	Occasionally	0.92 (0.78-1.08)	0.290	0.96 (0.85-1.07)	0.447
	Often	1.38 (0.91-2.08)	0.127	1.31 (1.00-1.73)	0.052
Depress	Never (ref)		0.211	None	
	Occasionally	1.09 (0.92-1.29)	0.320		
	Often	0.77 (0.49-1.21)	0.264		
Sleep	Well (ref)		0.221	None	
	Moderate	0.98 (0.82-1.16)	0.791		
	Bad	0.89 (0.74-1.07)	0.225		

BMI: Body Mass Index; SUA: Serum uric acid; TG: Triglycerides; TC: Total cholesterol; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; TBIL: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine transaminase; A/G: Albumin/globulin; FPG: Fasting plasma glucose; BUN: Blood urea nitrogen; HbA1c: Glycated hemoglobin.

Independent risk factors for MAFLD

General information: This study found that as hip circumference increase (OR = 0.95, 95CI%: 0.93–0.96), the risk of MAFLD decreases, a finding consistent with Lin *et al*[25]. Indeed, fat accumulation on the hips may be beneficial to metabolic health and reduce the risk of metabolic-related diseases[26]. The risk of MAFLD was also 1.14 times higher among those who actively acquired medical knowledge than those who did not. This may be because individuals who are willing to actively acquire knowledge are more likely to attend medical check-ups for early detection and diagnosis. Meanwhile, people who aren't willing to acquire medical knowledge lack an understanding of self-health management and may be less likely to attend medical check-ups. This could cause an illusion of low MAFLD prevalence.

Laboratory indicators: After correcting for sex, age and BMI confounders, multivariate logistic regression analysis found that TG, HDL-C, TBIL, AST, ALT, glycated hemoglobin, SUA, platelets, and creatinine were associated factors for MAFLD. The risk of MAFLD increases by 1.33 times for each unit increase in TG value, which is consistent with the findings of previous studies[27]. Therefore, regular screening of TG levels and attention to dynamic changes in TG should be performed during routine medical examinations to facilitate screening of people at risk of MAFLD. A high HDL-C level indicates that the body is using cholesterol well and is a sign of good health. The OR value of 0.58 in our study, which suggests that elevated HDL-C may be an important protective factor for MAFLD. ALT and AST are indicators of hepatocellular damage, with ALT being the most sensitive. A number of studies have shown that ALT is an independent risk factor for the development of MAFLD in both obese and non-obese people[28]. In this study, ALT and AST were significantly increased in patients with MAFLD, and they were independent risk factors for the development of MAFLD. The increase in free fatty acids in the liver cells of MAFLD patients led to an increased susceptibility of the liver to inflammatory reactions and the production of oxygen free radicals, which led to hepatocyte degeneration and necrosis, resulting in an increase in serum ALT and AST.

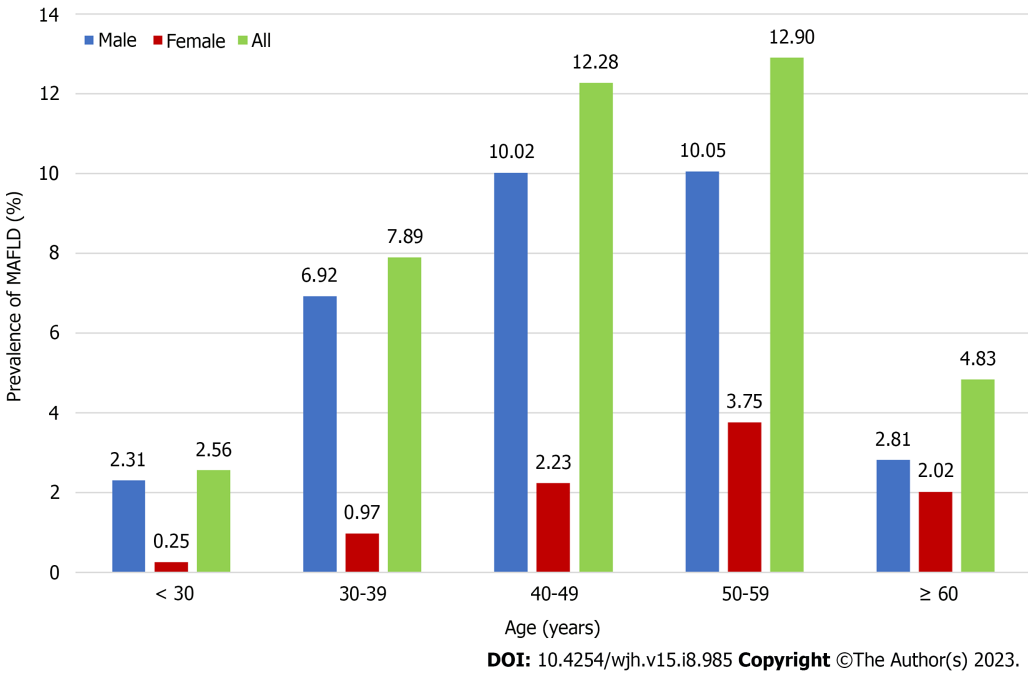
Platelets[29] are elevated during inflammation, and previous studies have found a linear correlation between platelet count and the severity of liver fibrosis[30] in individuals with MAFLD. In our study, we also found that platelet count was mild correlated with MAFLD, consistent with the results of Zeng *et al*[31], indicating that platelet count may be used as a reference indicator of MAFLD development and the resulting liver fibrosis. And our study found that high glycated hemoglobin values were also strongly associated with a high risk of MAFLD (OR = 1.52, 95%CI: 1.47-1.57), suggesting that glycated hemoglobin is an important reference indicator for screening for MAFLD, and there is previous evidence that patients with MAFLD have significantly higher glycated haemoglobin values compared to the healthy population [32].

In addition to above indicators, we have an interesting finding, SUA, TBIL and creatinine have significance in the multifactorial regression analysis of this study. Participants with CKD may have elevated SUA levels, low TBIL levels and abnormal creatinine values, which may suggest that there is an association between CKD and MAFLD, the exact mechanism need to further analysis. Longitudinal studies[33] have also shown an increased incidence of CKD among NAFLD patients. Despite these findings, however, there is little awareness about CKD in NAFLD, and evidence on the relationship between MAFLD and CKD is even rarer. The current study found that SUA was significantly correlated with MAFLD. While the mechanisms remain unclear, there are a few hypotheses. First, SUA may act as an oxidant and elevated levels may increase oxidative stress, thereby promoting the development of MAFLD. Second, SUA[34] induces adipogenesis through the production of endoplasmic reticulum, activating fatty acid synthase and acetyl coenzyme A carboxylase and leading to the accumulation of fat in hepatocytes. Indeed, low TBIL[35] and creatinine levels may be associated with MAFLD risk. The descend creatinine levels in MAFLD are consistent with the findings of Liu *et al*[36]. The reduction in creatinine associated with MAFLD may be the result of sarcopenia, which is linked to low skeletal muscle mass and reduced function. MAFLD patients maybe follow lower skeletal muscle mass, especially in lean MAFLD patients. There are differing views on the relationship between TBIL and the risk of MAFLD. Our study showed a mild negative correlation. This may be because TBIL activates toll-like receptor 4 signaling and promotes inflammation.

Lifestyle indicators: Previous studies[37] have shown that consuming sugary beverages may increase the risk of MAFLD, while drinking coffee and tea may reduce the risk. The current study found that individuals who regularly consumed beverages were 0.32 times more likely to develop MAFLD than those who never drank beverages. This may be because coffee and tea, which contain biologically active compounds with anti-oxidant and anti-fibrotic potential, were the most consumed beverages in this population[38]. Our study found that the risk of developing MAFLD when exercising > 5/wk was only 0.82 times that of exercising 1-2/wk. Meanwhile, prior studies have indicated that < 2/wk maybe no effect[39]. However, these findings do not necessarily mean that more frequent exercise is beneficial. It is also important to consider frequency in relation to exercise intensity and length. The risk of MAFLD was found to be 1.24 times higher following exercise lasting > 60 min than exercise lasting < 30 min, suggesting that the benefit of exercise doesn't increase after a certain length, perhaps due to fatigue that reduces long-term adherence. Finally, labor intensity was a protective factor, with moderate labor intensity is 0.78 times risk incidence of MAFLD than light labor intensity. This finding is consistent with a study by Chen *et al*[40] and suggests that moderate physical labor is beneficial to health.

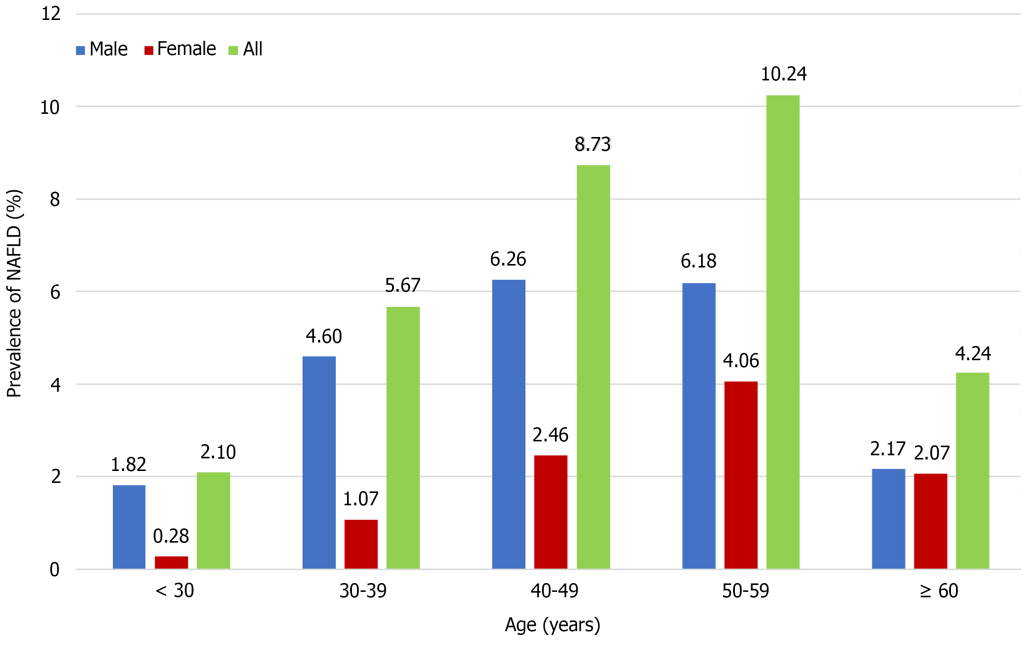
Limitations

To our knowledge, this is the first largest sample study to assess the new nomenclature of MAFLD in the Mid-South region of China. The study has some limitations, however. First, lifestyle information was self-reported by the participants, which may cause recall bias. Second, all the participants were recruited from one medical facility so the findings may not be generalizable to the Chinese population. Additional studies are needed to assess the prevalence and features of MAFLD in other regions of the country. Third, this study lacked histological information on steatosis and fibrosis diagnoses. While ultrasound imaging is highly sensitive and specific for liver fibrosis and steatosis, this technique



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Figure 4 Prevalence of metabolic associated fatty liver disease by gender and age group. MAFLD: Metabolic associated fatty liver disease.



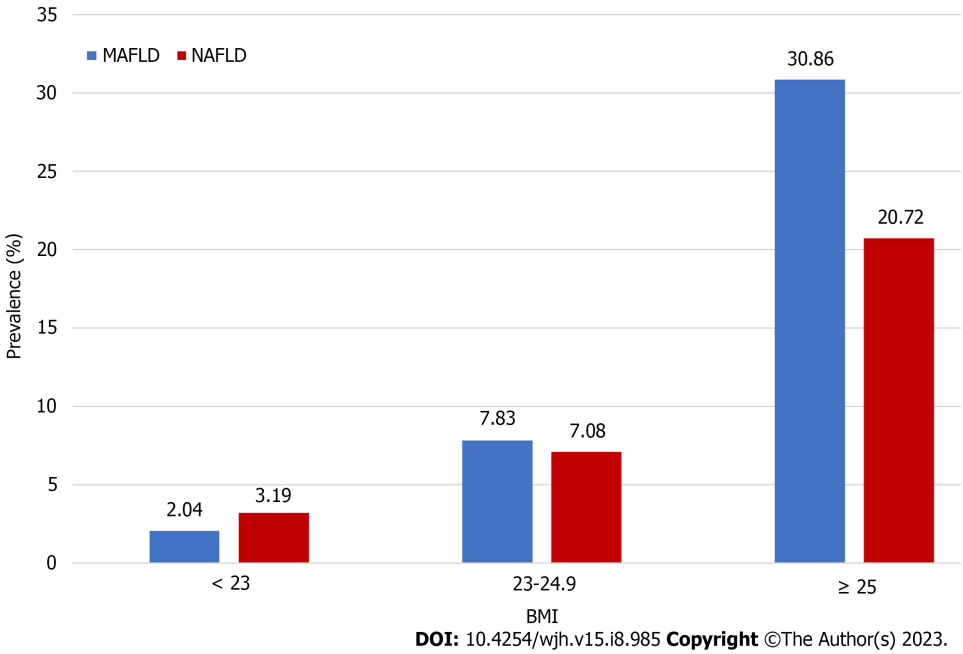
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Figure 5 Prevalence of non-alcoholic fatty liver disease by gender and age group. NAFLD: Non-alcoholic fatty liver disease.

is not the gold standard for diagnosis. In addition, vibration controlled transient elastography (VCTE) also been recommended for a wide range of studies related to NAFLD[41-45], and VCTE has good diagnostic performance in assessing steatosis. However, there are certain shortcomings that limit its use and make it less widespread than ultrasound, such as high dependence on operator experience, limited sampling range, large overlap in liver fibrosis staging data, and inconsistent delineation of Cut off values.

CONCLUSION

This study found that MAFLD was significantly more prevalent than NAFLD in our study population. In addition to the usual risk factors, our results suggest that CKD may be related with MAFLD. More research is needed to determine the



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Figure 6 Non-alcoholic fatty liver disease and metabolic associated fatty liver disease prevalence by body mass index zone. MAFLD: Metabolic associated fatty liver disease; NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

potential mechanisms underlying the occurrence of MAFLD and to develop interventions to prevent and treat this disease.

ARTICLE HIGHLIGHTS

Research background

Metabolic associated fatty liver disease (MAFLD) is renamed from non-alcoholic fatty liver disease (NAFLD), but there are differences in diagnostic criteria. Since the research on MAFLD is just beginning, however, evidence on its incidence and prevalence in the general population and in specific subpopulations remains limited.

Research motivation

MAFLD proposal is not only a change in nomenclature. On one hand, MAFLD includes patients with concomitant liver diseases and secondary causes of fatty liver. On the other hand, patients with hepatic steatosis but not fulfilling the metabolic criteria are not classified as MAFLD. How these criteria affect our understanding of the epidemiology of MAFLD is unclear. The clinical characteristics and risk factors between MAFLD and NAFLD has not been adequately explored. We aimed to further clarify a possible link and difference between the two diagnostic criteria.

Research objectives

We sought to assess the impact of the new definition on the epidemiology of fatty liver disease and compare MAFLD with NAFLD in a general population. Potential risk factors of MAFLD-diagnosed individuals were also explored.

Research methods

A total of 85242 adults were selected from the Chinese health management database in 2017–2022. Specifically, the participants were divided into MAFLD group, NAFLD group and MAFLD & NAFLD group for analysis and comparison. Several elements were included such as prevalence, disease characteristics, and risk factors.

Research results

We found a higher prevalence of MAFLD than NAFLD. There are differences in clinical features between MAFLD, NAFLD and MAFLD & NAFLD. In addition to the common risk factors, we identified CKD may be related with MAFLD.

Research conclusions

MAFLD was more prevalent than NAFLD in the study population, with two-fifths of individuals meeting the diagnosis criteria. Compared to NAFLD, MAFLD has its own disease characteristics and risk factors. Intervention program should address the risk factors for MAFLD and regular screening for the disease is recommended.

Research perspectives

Some of the risk factors for MAFLD have been initially identified, but cross-sectional studies of causality are weak. In the future, multi-centre, multi-regional longitudinal studies could be conducted to elucidate disease characteristics, disease trajectory and risk factors in depth.

FOOTNOTES

Author contributions: All authors on this manuscript made significant contributions to the study; Xinjuan Huang and Chunxiang Qin were primarily involved in the study design; Huang XJ, Zhou BQ, and Yin M were responsible for the analysis and interpretation of data, as well as drafting the manuscript; Tan XY and Xia YQ were involved in the acquisition of data; all authors read and approved the final manuscript.

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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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SYSTEMATIC REVIEWS

Emerging therapeutic options for non-alcoholic fatty liver disease: A systematic review

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) has become a prevalent cause of chronic liver disease and ranks third among the causes of transplantation. In the United States alone, annual medical costs are approximately 100 billion dollars. Unfortunately, there is no Federal Drug Administration (FDA)-approved medication for its treatment. However, various clinical trials are investigating several therapeutic classes that could potentially treat NAFLD. It is valuable to have a compilation of the data available on their efficacy.

AIM

To assess the efficacy of cyclophilin inhibitors, fibroblast growth factor 21 analogs (FGF21), and dual and pan peroxisome proliferator-activated receptor (PPAR) agonists for treating NAFLD.

METHODS

A comprehensive literature search using keywords including cyclophilin inhibitor, FGF agonist, pan-PPAR agonists, dual-PPAR agonist, NAFLD, non-alcoholic steatohepatitis, and fatty liver was conducted on October 29, 2022, in PubMed, EMBASE, Cochrane Library, Scopus and Web of Science. Animal and human research, case reports, and published articles in English from all countries with patients aged 18 and above were included. Only articles with a National Institutes of Health (NIH) Quality Assessment score of five or higher out of eight points were included. Articles that were narrative or systematic reviews,

abstracts, not in English, focused on patients under 18 years old, did not measure outcomes of interest, were inaccessible, or had a low NIH Quality Assessment score were excluded. Each article was screened by two independent researchers evaluating relevance and quality. Resources were scored based on the NIH Quality Assessment Score; then, pertinent data was extracted in a spreadsheet and descriptively analyzed.

RESULTS

Of the 681 records screened, 29 met the necessary criteria and were included in this review. These records included 12 human studies and 17 animal studies. Specifically, there were four studies on cyclophilin inhibitors, four on FGF agonists/analogs, eleven on pan-PPAR agonists, and ten on dual-PPAR agonists. Different investigational products were assessed: The most common cyclophilin inhibitor was NV556; FGF agonists and analogs was Efruxifermin; pan-PPAR agonists was Lanifibranor; and dual-PPAR agonists was Saroglitazar. All classes were found to be statistically efficacious for the treatment of NAFLD, with animal studies demonstrating improvement in steatosis and/or fibrosis on biopsy and human studies evidencing improvement in different metabolic parameters and/or steatosis and fibrosis on FibroScan ($P < 0.05$).

CONCLUSION

The data analyzed in this review showed clinically significant improvement in individual histological features of NAFLD in both animal and human trials for all four classes, as well as good safety profiles ($P < 0.05$). We believe this compilation of information will have positive clinical implications in obtaining an FDA-approved therapy for NAFLD.

Key Words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Cyclophilin inhibitors; Fibroblast growth factor 21 analogs; Dual peroxisome proliferator-activated receptor agonists; Pan peroxisome proliferator-activated receptor agonists

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Core Tip: Non-alcoholic fatty liver disease (NAFLD) has become a significant global health issue. There is no medication approved by the Federal Drug Administration for the treatment of NAFLD. However, there are several therapeutic classes currently being studied in clinical trials. In this systematic review, we analyze the scientific data of cyclophilin inhibitors, fibroblast growth factor 21 analogs, and dual and pan peroxisome proliferator-activated receptor agonists for the treatment of NAFLD.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a prevalent chronic liver disease affecting approximately 30% of the world's population[1]. It is characterized by the buildup of more than 5% of fat in hepatocyte histology[1]. NAFLD encompasses a range of conditions, such as non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), and cirrhosis[2]. NAFL is defined as hepatic steatosis without inflammation, based on liver biopsy histology[2].

Approximately 20% of patients with NAFL will develop NASH, which is the presence of hepatic steatosis, lobular inflammation, and hepatocyte ballooning[1,2]. This persistent liver cell injury leads to progressive fibrosis and cirrhosis in approximately 10%–20% of patients, converting NAFLD into the quickest-growing cause of hepatocellular carcinoma (HCC)[1,3]. NAFLD is currently liver transplantation's third most common cause[1]. Unlike other causes of HCC, which start with fibrosis, up to one-third of patients with NASH and HCC are non-cirrhotic and are more advanced, making treatment difficult[3].

NAFLD is a liver condition that is closely linked with metabolic syndrome[1]. It is often seen in people who have type 2 diabetes, are insulin resistant, have high levels of triglycerides and cholesterol, and are obese[1]. The main risk factors for developing NAFLD are a diet high in fats and sugars and a sedentary lifestyle[1]. Some experts have started using metabolic-associate fatty liver disease to describe this condition because of its strong link with metabolic dysfunction. Still, for clarity purposes, we will stick with the NAFLD nomenclature throughout this review[4].

Approximately 70% of diabetics, overweight patients, and 90% of patients with dyslipidemia and morbid obesity will develop NAFLD[1,4]. NAFLD is also associated with systemic pathologies such as chronic kidney disease, cardiovascular disease, and reduced mineral density[1]. Cardiovascular disease is the most common cause of death in NAFLD patients; however, they also have an increased overall mortality rate compared to the general population[1]. It is of utmost concern that there is an increase in the prevalence of adolescents with NAFLD, leading to earlier end-stage liver disease[5].

In the United States alone, $100 billion of annual medical costs are attributed to NAFLD. Searching for an approved medical therapy for NAFLD is a pressured race[4]. The lack of an authorized agent could be secondary to the limited understanding of a multifactorial disease process and the absence of dependable non-invasive biomarkers[4]. Due to the acknowledgment of an increasing epidemic and the severity of NAFLD, several trials are ongoing to identify possible pharmacologic agents[3]. Most of the agents target the known metabolic associations with NAFLD, such as adipose tissue dysfunction, insulin resistance, de novo lipogenesis, lipid exportation in the liver, and imbalance between energy intake and expenditure[5].

There is growing interest in future combined medications targeting multiple critical pathways involved in developing NAFLD[5]. Precise identification of the drivers of this disease is crucial for developing new agents, and it is hoped that registered therapy for NAFLD will become available in the next few years[2]. Clinicians must be aware of the emerging agents for the treatment of NAFLD and the need for further human research to characterize better the efficacy, dosage, length of treatment, *etc.* This systematic review will delve into the scientific data behind four innovative therapeutic classes currently being studied for treating NAFLD: Cyclophilin inhibitors, fibroblast growth factor 21 analogs (FGF21), and dual and pan peroxisome proliferator-activated receptor (PPAR) agonists.

MATERIALS AND METHODS

This review analyzed animal and human research, case reports, and published articles in English from all countries with patients aged 18 and above. Only articles with a five or higher National Institutes of Health (NIH) Quality Assessment score were included. Articles that were narrative or systematic reviews, abstracts, not in English, focused on patients under 18 years old, did not measure outcomes of interest, were inaccessible, or had a low NIH Quality Assessment score were excluded.

A comprehensive literature search using broad search criteria was conducted in five databases: PubMed, EMBASE, Cochrane Library, Scopus, and Web of Science (October 29, 2022). Our search terms were as follows: (rencofilstat OR "cyclophilin inhibitor" OR "cyclophilin inhibition" OR lanifibranor OR "PPAR agonist" OR "peroxisome proliferator activated receptor agonist" OR "pan-ppar agonist" OR "pan-peroxisome proliferator activated receptor agonist" OR efruxifermin OR "FGF-21 inhibitor" OR "fibroblast growth factor-21 inhibitor" OR "fibroblast growth factor 21 inhibitor" OR "FGF21 inhibitor" OR "FGF21 inhibition" OR "FGF-21 inhibition") AND (NASH or "fatty liver" or "hepatic steatosis" or steatohepatitis OR NAFLD OR "non-alcoholic fatty liver disease" OR "Non-alcoholic Fatty Liver Disease" OR "Fatty Liver, Non-alcoholic" OR "Fatty Livers, Non-alcoholic" OR "Liver, Non-alcoholic Fatty" OR "Non-alcoholic Fatty Liver" OR "Non-alcoholic Fatty Livers" OR "Non-alcoholic Steatohepatitis" OR "Non-alcoholic Steatohepatitides" OR "Steatohepatitides, Non-alcoholic" OR "Steatohepatitis, Non-alcoholic" OR "liver, fatty" OR "steatosis of liver" OR "visceral steatosis" OR "steatosis, visceral" OR "steatoses, visceral" OR "visceral steatosis" OR "liver steatosis" OR "liver steatoses" OR "steatosis, liver" OR "steatoses liver").

For the study selection process, Covidence was used, a platform that facilitates the importation of citations and screening of titles, abstracts, and full text. Each article was initially screened by title and abstract by two independent researchers (J.T. and N.B.) to exclude studies irrelevant to our aim. Next, each article was screened by full text by the same two independent researchers (J.T. and N.B.) to exclude studies that were finally irrelevant to our aim. Once both researchers completed all screening stages, any conflicts were registered in Covidence, and the discrepancies were reviewed and resolved.

The following data were collected separately by J.T. and N.B. from all eligible studies and recorded in Excel: First author, digital object identifier, study design, number of participants, name of therapy, mechanism of action, side effects, and statistical data about liver enzymes, cholesterol panels, weight reduction, NAFLD activity score (NAS), Fibroscan controlled attenuation parameter (CAP) and kPa, fibrosis stage, fibrotic markers, and quality assessment scores. J.T. and N.B. resolved all discrepancies in the collected data. The quality of included studies was assessed by the NIH Quality Assessment tool. We included articles with a score greater than or equal to five out of eight points.

RESULTS

Records were identified from 5 databases: PubMed, EMBASE, Cochrane Library, Scopus, and Web of Science. One hundred twenty-two duplicate records were removed before the screening. Six hundred eighty-one records were screened, out of which eighty-two were excluded by an automation tool. Five hundred fifty-nine reports were sought for retrieval, out of which three hundred were not retrieved. Two hundred fifty-nine reports were assessed for eligibility, out of which two hundred and thirty were excluded. The most common reason for exclusion was review articles (156) followed by irrelevant articles (31), abstracts (18), duplicates (16), foreign language (5), and unable to be accessed (4). Twenty-nine of the two hundred and fifty-nine records assessed for eligibility were included (Figure 1). Most studies, including human and animal participants, were small (n < 100). Some studies enrolled patients with NAFLD and others with NASH. Articles were included when the NIH Quality Assessment Score was greater than or equal to five points. The majority of articles included were scored as six or seven points. Reasons for lower scores included unknown publication bias and no rating by two independent reviewers. Most studies did not report harmful outcomes.

Four studies evaluated cyclophilin inhibitors (Table 1), four evaluated FGF analogs (Table 2), eleven evaluated pan-PPAR agonists (Table 3), and ten evaluated dual-PPAR agonists (Table 4). Different investigational products were assessed; the most common for cyclophilin inhibitors was NV556, for FGF agonists/analogs was Efruxifermin (EFX), for

Table 1 Studies of cyclophilin inhibitors in the treatment of non-alcoholic fatty liver disease

Ref.	Human or animal	Study design	Number of participants	Key inclusion criteria	Investigational product/dose	Study end points	Key findings
Harrison *et al*[9], 2022	Human	Randomized, single-blind, placebo-controlled, phase 2a study; Duration: 4 wk	n = 49	Patients with presumed F2/F3 NASH	Rencofilstat Placebo *vs* Rencofilstat (75 or 225 mg daily)	Evaluate the effect of Rencofilstat on ALT, Pro-C3, liver steatosis, and fibrosis measured by FibroScan	ALT in the placebo *vs* 75 *vs* 225 mg group was 70.67 *vs* 42.5 *vs* 30.56 IU/L. Pro-C3 was reduced in stratified patients with Pro-C3 > 15 (P < 0.01). Fibrosis was 22 *vs* 14 *vs* 12 kPa. Steatosis was 351 *vs* 337 *vs* 329 dB/m
Kuo *et al* [6], 2019	Animal	Duration: 30 wk	n = 10	High-fat diet-induced NASH mouse model (n = 10)	CRV431: Control *vs* 50 mg/kg daily	Evaluate the effect of CRV431 on liver fibrosis measured by Sirius red staining in liver biopsy sections	Fibrosis levels were 37%–46% lower in the treatment *vs* control group (P < 0.05)
Kuo *et al* [8], 2019	Animal	Duration: 6 wk	n = 9	CCL4-induced liver fibrosis mouse model (n = 9)	CRV431: Control *vs* 50 mg/kg daily	Evaluate the effect of CRV431 on liver fibrosis measured by Sirius red staining in liver biopsy sections	Liver fibrosis was lowered by 43% in the treatment *vs* control group (P < 0.01)
Kuo *et al* [8], 2019	Animal	Duration: 6 wk	n = 8	High-fat diet-induced NASH mouse model	NV556: Control *vs* 50 mg/kg daily	Evaluate the effect of NV556 on liver collagen and fibrosis measured by Sirius red staining in liver biopsy sections	Fibrosis was reduced by 60% in the treatment *vs* control group (P = 0.0281)
Simón Serrano *et al*[7], 2019	Animal	Duration: 7 wk	n = 20	Choline-deficient high-fat diet-induced model of NASH in mice (n = 10 per group)	NV556: Control *vs* 100 mg/kg daily	Effect of NV556 on liver fibrosis and collagen production measured by Sirus red staining	Reduction of liver fibrosis by 25% (2% in control *vs* 1.5% in treatment group P < 0.01)

ALT: Alanine transaminase; AST: Aspartate aminotransferase; NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease; CCL4: Carbon tetrachloride; IU: International units.

pan-PPAR agonists was Lanifibranor, and for dual-PPAR agonists was Saroglitazar.

In terms of cyclophilin inhibitors, four animal studies demonstrated significant improvement in fibrosis on liver biopsy weeks after product use (P < 0.05). The randomized controlled trial (RCT) performed in humans (n = 49) noted similar results, with a reduction in ALT and Pro-C3 levels (P < 0.01), as well as steatosis and fibrosis as measured on FibroScan (P < 0.01).

The three animal studies using FGF analogs demonstrated significant improvement in both steatosis and fibrosis measured on liver biopsy (P < 0.05). The RCT performed in humans (n = 80) measured the change in hepatic fat fraction (HFF) on magnetic resonance imaging at 12 wk of treatment. It noted a greater than 50% reduction in HFF in all treatment dosage groups (P < 0.0001).

Eight animal studies using pan-PPAR agonists evidenced a significant reduction in steatosis on biopsy as measured by the decrease in triglyceride or lipid accumulation in hepatocytes (P < 0.05). There was also a reduction in fibrosis and collagen deposition on liver biopsy (P < 0.05). The human studies included three RCTs that examined the metabolic effects and/or steatosis markers (steatosis activity score) and concluded improved metabolic function, resolution of steatosis, and fibrosis improvement (P < 0.05).

In terms of dual-PPAR agonists, six animal studies reported improvement in steatosis, reduction in fibrosis or progression to fibrosis, and improvement in lipid metabolism and insulin sensitivity (P < 0.05). Two human in-vitro studies on hepatic cells were performed, which demonstrated a reduction in hepatic lipid accumulation, secretion of inflammatory chemokines, and profibrotic gene expression. Four additional human studies, including prospective design and RCTs, showed improved metabolic parameters such as insulin sensitivity, hemoglobin A1c, and lipid profiles (P < 0.05). Additionally, FibroScan results showed improved liver stiffness and steatosis (P < 0.05).

DISCUSSION

Cyclophilin inhibitors

Cyclophilins are thought to contribute to the development of liver fibrosis and cancer. Among these, Cyclophilin B is known to play a role in collagen production, leading to fibrosis. To treat NASH, several investigational products have

Table 2 Studies of fibroblast growth factor analogs/agonists in the treatment of non-alcoholic fatty liver disease

Ref.	Human or animal	Study design	Number of participants	Key inclusion criteria	Investigational product/dose	Study endpoints	Key findings
Harrison *et al*[10], 2021	Human	Randomized, double-blind, placebo-controlled, phase 2a BALANCED study. Duration: 16 wk	n = 80	Patients with biopsy-confirmed NASH (F1-F3)	Efruxifermin: Placebo *vs* EFX (28, 50, 70 mg)	Absolute change from baseline in HFF measured as MRI-proton density fat fraction at 12 wk of EFX	The mean relative change in HFF at week 12 was -63.2% - 70.9%, and -72.3%, respectively, in the treatment groups of 28, 50, and 70 mg (P < 0.0001)
Bao *et al* [12], 2018	Animal	Duration: 15 d	n = 10	Choline-deficient high-fat diet-induced model of NASH in mice (n = 5 per group)		Effect of PSTag600 on attenuation of the development of NASH measured by NAS and oil red O staining	Decrease in NAS in control *vs* treatment group of 5 *vs* 1 and area of oil red O of 26% *vs* 3%, respectively (P < 0.05)
Le *et al* [11], 2018	Animal	Duration: 4 wk	n = 8	MCD diet-induced NASH mouse model		Evaluate the attenuation of fibrosis with the administration of LY2405319 by measuring levels of a-SMA and GPR91 (cells and receptors involved in hepatic fibrogenesis) on liver biopsy after 8 wk	The expression of α-SMA and GPR91 in the liver of mice fed with MCD diet was increased. The treatment group had an attenuated increase of collagen type 1, α-SMA, and GPR91 protein levels (P < 0.05). LY2405319 intraperitoneal administration for 4 wk daily ameliorated hepatic steatosis and fibrosis that was induced by MCD diet
Puengel *et al*[13], 2022	Animal	Duration: 6 wk	n = 12	Choline-deficient high-fat diet-induced model of NASH in mice (n = 6 per group)	BMS-986171: Control *vs* 0.6 mg/kg twice weekly	Effect of BMS-986171 on liver steatosis and fibrosis measured NAS on biopsy	NAS of the control vs. treatment group was 5 *vs* 4 (P < 0.05), hepatic steatosis 2.5 *vs* 1.5 (P < 0.01), inflammation 3.5 *vs* 2.5 and ballooning 1.2 *vs* 0.75 (P < 0.001) respectively

NASH: Non-alcoholic steatohepatitis; n: Number; NAS: NAFLD activity score; EFX: Efruxifermin; HFF: Hepatic fat fraction; MRI: Magnetic resonance imaging; α-SMA: Alpha-smooth muscle actin; MCD: Methionine and choline-deficient.

been developed to target Cyclophilins[6]. The main cyclophilin inhibitors reviewed here are CRV431[6], NV556[7,8], and Rencofilstat[9].

Studies conducted on animals, mainly mice that were administered a cyclophilin inhibitor, have shown positive results in improving liver fibrosis during biopsy[6-8]. In particular, Kuo *et al*'s research indicated a reduction of over 37% in liver fibrosis with CRV431 treatment compared to control on various mouse models[6,8]. Likewise, NV556 also demonstrated a significant decrease in collagen production and liver fibrosis[7,8].

Due to these promising results, researchers conducted a phase 2a RCT with 49 patients who received Rencofilstat (75, 225 mg) or a placebo[9]. The results showed that patients with high baseline Pro-C3 levels (> 15) experienced a decrease in collagen biomarkers, which are predictors for collagen deposition (P < 0.01)[9]. This aligns with previous animal studies, suggesting that cyclophilin inhibitor treatment may reduce liver fibrosis. The patients generally tolerated Rencofilstat well, with only mild side effects reported, such as constipation, diarrhea, back pain, dizziness, and headache [9]. Animal and human studies have shown that various investigational products that inhibit cyclophilin effectively treat patients with NASH. These agents are also well-tolerated and have anti-fibrotic properties that are beneficial.

FGF21 analogs or agonists

FGF21 is an active component of organ metabolism. Different variants have been studied for treating fatty liver disease, diabetes, and obesity due to their effect on glucose and lipid metabolism[10]. The main FGF21 analogs and agonists reviewed here are LY2405319[11], PsTag600[12], BMS-986171[13], and EFX[10]. Multiple animal studies involving FGF21 analogs and agonists have demonstrated improved glucose metabolism and reductions in markers of liver injury and fibrosis[11,12]. Le *et al*[11] performed an animal study using LY2405319 (FGF21 analog), which attenuated increased collagen type 1, alpha-smooth muscle actin, and GPR91 protein levels[11]. These results align with research on PsTag600 (long-acting FGF21) and BMS-986171 (FGF21 agonist)[12,13].

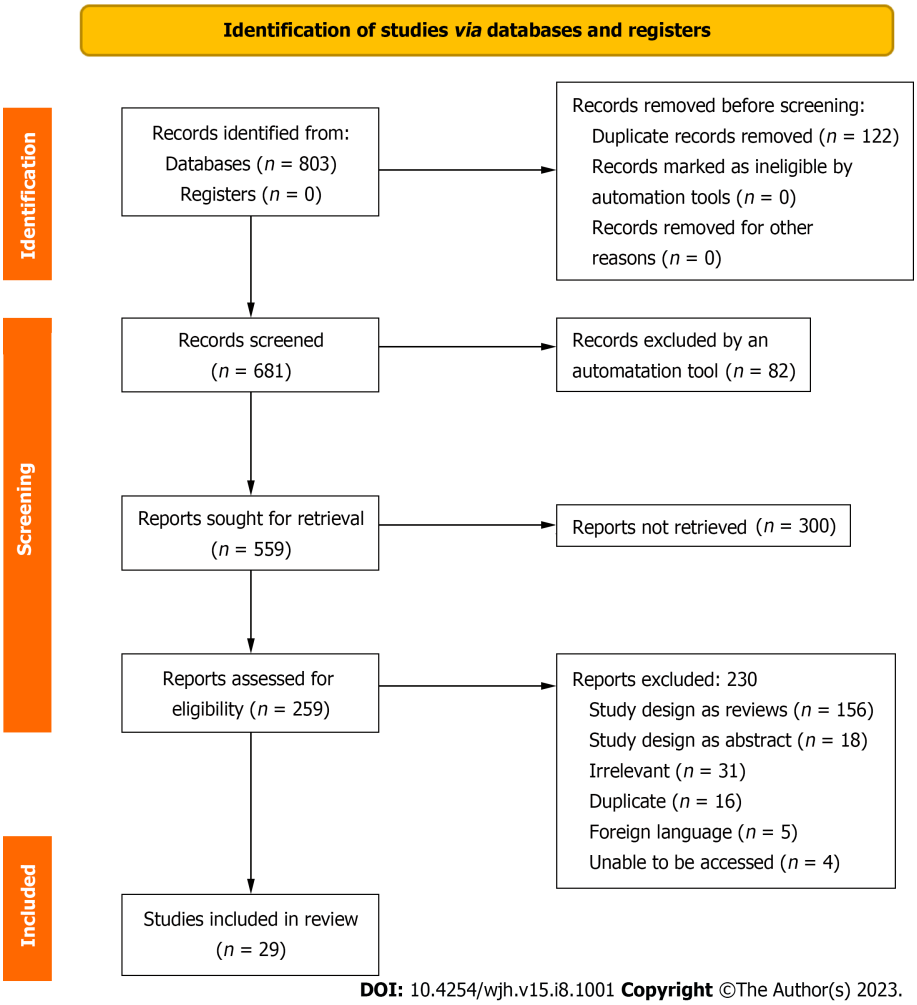
A phase 2a study included 80 patients treated with a placebo or EFX 28 mg, 50 mg, or 70 mg (FGF21 analog) for 12 wk [10]. The results indicated a significant decrease in HFF, with 78% of patients showing a positive response to NAS with an increase of at least 2 points and 48% of patients showing a resolution of NASH[10]. There was also a statistically

Table 3 Studies of pan peroxisome proliferator-activated receptor agonists in the treatment of non-alcoholic fatty liver disease

Ref.	Human or animal	Study design	Number of participants	Key inclusion criteria	Investigational product/dose	Study endpoints	Key findings
Abitbol *et al*[21], 2016	Human	Double-blind, randomized, placebo-controlled, parallel-group study. Duration: 4 wk		Patients with biopsy-confirmed NASH and type 2 diabetes on stable doses of metformin	IVA337 (Lanifibranor). Placebo *vs* IVA337 (400, 800, or 1200 mg daily)		Reduction in triglycerides by 32% and ALT by 10% ($P < 0.05$)
Cooreman *et al*[14], 2022	Human	Post-hoc analysis of the phase 2b NATIVE study. Duration: 24 wk	$n = 247$	Patients with non-cirrhotic biopsy-confirmed NASH	Lanifibranor Placebo *vs* Lanifibranor (800 or 1200 mg daily)	Effect of Lanifibranor on glycemic control and NASH markers. Efficacy in NASH was measured with SAF score and fibrosis staging	NASH resolution and fibrosis improvement in the treatment group *vs* placebo was 26% *vs* 7%, respectively, and a 41% reduction of HbA1c from baseline ($P < 0.001$)
Francque *et al*[18], 2021	Human	Randomized, double-blind, placebo-controlled, phase 2b trial. Duration: 24 wk	$n = 247$	Patients with noncirrhotic, highly active NASH (SAF ≥ 1 or higher for steatosis, hepatocellular ballooning, and lobular inflammation on liver biopsy)	Lanifibranor Placebo *vs* Lanifibranor (800 or 1200 mg daily)	Decrease of at least 2 points in the SAF score without worsening of fibrosis	48% of patients in the 800 mg group and 55% in the 1200 mg group had a decrease of at least 2 points in the SAF score *vs* 33% in the placebo group ($P = 0.007$)
An *et al* [22], 2017	Animal	Duration: 3 wk	$n = 5$	Genetically obese mice	MHY2013: Control *vs* 5 mg/kg daily	Reduction of hepatic steatosis measured *via* liver triglycerides on biopsy	Liver triglycerides were 10 mg/100 mg of protein in the control *vs* 7 mg/100 mg of protein in the treatment group ($P < 0.05$)
An *et al* [25], 2018	Animal	Duration: 3 wk	$n = 6$	Aged model mice	MHY2013: Control *vs* MHY2013 (1 or 3-5 mg/kg daily)	Evaluate the attenuation of hepatic lipid accumulation measured by liver biopsy	The ratio of liver weight/body weight was 0.035, 0.03, and 0.025 in control, 1 and 3-5 mg/kg groups, respectively ($P < 0.01$)
Barbosa-da-Silva *et al*[16], 2015	Animal	Duration: 4 wk	$n = 20$	High-fat diet mice ($n = 10$ per group)	Bezafibrate: Control *vs* 100 mg/kg daily	Effect of Bezafibrate on hepatic lipid metabolism measured by liver TG and steatosis on biopsy	Reduction in TG levels and liver steatosis of 30% and 50%, respectively, in the treatment group ($P < 0.0001$)
Boubia *et al* [19], 2018	Animal	Duration: 3 wk	$n = 16$	CCl4-induced liver fibrosis in mice ($n = 8$ per group)	Lanifibranor: Control *vs* 30 mg/kg daily	Efficacy of Lanifibranor in reducing fibrosis in NASH measured by hepatic collagen on biopsy	Reduction in hepatic collagen deposition from 0.6% of the area to 0.3% in the control *vs* treatment group ($P < 0.01$)
Lefere *et al* [15], 2020	Animal	Duration: 6 wk	$n = 16$	Choline-deficient high-fat diet-induced NASH mouse model ($n = 8$). Isolated hepatic macrophages ($n = 8$)		Effect on NAFLD measured by the NAFLD activity score, fibrosis by the Sirus red staining, and hepatic macrophages assessed by IHC	Reduction of NAFLD activity score from 6 to 2 in the treatment *vs* control group ($P < 0.0001$), collagen by 5% to 3% ($P < 0.01$), and liver macrophages from 22% to 8% ($P < 0.0001$)
Møllerhøj *et al*[20], 2022	Animal	Duration: 12 wk	$n = 13$	Gubra-Amylin NASH diet-induced obese mouse with biopsy-confirmed NASH	Lanifibranor: Control *vs* 30 mg/kg daily	Change in NAS and fibrosis stage measured on biopsy	At least a 2-point improvement in the steatosis score, and only 20% of hepatocytes had lipid droplets *vs* 80% in the control group ($P < 0.001$). 50% of mice had a 1-point improvement in fibrosis ($P < 0.05$)

Nagasawa *et al*[17], 2006	Animal	Duration: 5 wk	$n = 7$	Choline-deficient high-fat diet-induced NASH mouse model	Benzafibrate: Control *vs* Benzafibrate (50, 100 mg/kg daily)	Effect on hepatic lipid content and histopathological changes measured on biopsy by the number of activated hepatic stellate cells	Liver TG was 25, 20, and 55 mg/g in the 50, 100 mg/kg *vs* placebo groups, respectively ($P < 0.01$). The activated hepatic stellate cells were 11 number/15 fields *vs* 1 number/15 fields, respectively
Wettstein *et al*[24], 2017	Animal	Duration: 3 wk	$n = 20$	Choline-deficient high-fat diet-induced model of NASH in mice ($n = 10$ per group)	IVA337 (Lanifibranor) Control *vs* 30 mg/kg daily	Evaluate the effects of IVA337 on hepatic features associated with NASH measured by hepatic lipid droplet count and lobular inflammation foci count	Prevention of steatosis in 98% of mice and inflammation in 75% of mice ($P < 0.001$)

ALT: Alanine transaminase; NASH: Non-alcoholic steatohepatitis; n: Number; TG: Triglycerides; CCL4: Carbon tetrachloride; SAF: Steatosis activity fibrosis; NAFLD: Non-alcoholic fatty liver disease; NAS: NAFLD activity score; IHC: Immunohistochemistry.



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Figure 1 PRISMA flow diagram.

significant reduction in alanine transaminase (ALT), aspartate aminotransferase (AST), and total cholesterol levels[10]. Compared to Resmetirom, a selective thyroid hormone receptor-β agonist in phase 3 trials, FGF21 analogs/agonists showed similar reductions in HFF and fibrosis[10]. The side effects reported for EFX were mild and included diarrhea, nausea, vomiting, abdominal pain, frequent bowel movements, and fatigue[10]. In conclusion, FGF21 analogs and agonists have numerous benefits for NAFLD, including improved glucose and lipid metabolism, reduced markers of liver injury, and liver fibrosis. They effectively reduce hepatic steatosis and fibrosis, making them a promising treatment for NAFL and NASH.

Table 4 Studies of dual-pan peroxisome proliferator-activated receptor agonists in the treatment of non-alcoholic fatty liver disease

Ref.	Human or animal	Study design	Number of participants	Key inclusion criteria	Investigational product/dose	Study endpoints	Key findings
Boeckmans *et al*[34], 2019	Human		N/A	Hepatic cells generated from human skin-derived precursors with induced NASH	Elafibranor	Effect on hepatic steatosis and inflammatory chemokines	Reduction in hepatic lipid load, as well as the expression and secretion of inflammatory chemokines, which are responsible for the recruitment of immune cells
Boeckmans *et al*[33], 2021	Human	*In vitro* study. Duration: N/A	N/A	Hepatic cells generated from human skin-derived precursors with induced NASH	Elafibranor	Effect on hepatic steatosis, inflammatory chemokines, and pro-fibrotic gene expression	Attenuated lipid accumulation, inflammatory chemokine secretion, and pro-fibrotic gene expression
Cariou *et al* [27], 2013	Human	Multicenter, randomized, single-blind, placebo-controlled, crossover study. Duration: 8 wk	n = 22	Abdominally obese insulin-resistant males	GFT505: Placebo *vs* 80 mg daily	Effect on peripheral and hepatic insulin sensitivity with improvement in GIR	Improved peripheral insulin sensitivity with a 21% increase of the GIR (P = 0.048) and enhanced hepatic insulin sensitivity with a 44% increase in insulin suppression of endogenous glucose production (P = 0.006)
Chaudhuri *et al*[32], 2023	Human	Single-center, prospective, observational, open-label, single-arm study. Duration: 52 wk	n = 76	Patients with NAFLD and elevated ALT levels along with liver stiffness value ≥ 6 kPa and/or liver steatosis CAP > 290 dB/m	Saroglitazar 4 mg daily	Effect on liver stiffness and steatosis measured by LSM and CAP on FibroScan at baseline, 24 and 54 wk	There was significant improvement of LSM from baseline (11.03 ± 7.19 kPa) to 24-wk (9.29 ± 6.39 kPa) and 52-wk (8.59 ± 6.35 kPa) values, respectively (P < 0.001). There was a significant improvement in median CAP at 24 wk 281 dB/m, (P < 0.001) and 52 wk 287 dB/m, (P < 0.001) as compared with the baseline 328 dB/m
Hassan *et al* [29], 2019	Animal	Duration: 5 wk	n = 12	Mice with induced NASH by a high-fat emulsion diet (n = 6 per group)	Saroglitazar: Control *vs* 4 mg/kg daily	Histopathological effects of Saroglitazar by using light microscopy	In the control *vs* treatment group, steatosis score was 3 *vs* 0.5, hepatic ballooning was 2 *vs* 0.5, lobar hepatitis was 3 *vs* 1, and portal hepatitis was 3 *vs* 0.25, respectively (P < 0.05)
Padole *et al* [31], 2022	Human		n = 91	Patients with BMI > 23 kg/m^2 diagnosed with NAFLD (CAP > 248 dB/m)	Saroglitazar 4 mg daily	Change from baseline of liver biomarker, hepatic steatosis, and fibrosis in patients who lost > 5% of the weight	Patients with > 5% of weight loss had a median AST of 36 *vs* 40 at baseline (P = 0.038), ALT 44 *vs* 53 (P < 0.01), kPa 5.9 *vs* 6.8 (P = 0.336) and CAP 265 *vs* 311 (P = 0.128)
Rajesh *et al* [28], 2022	Human		n = 85	Patients with NAFLD (US, CT, or MRI) and type 2 diabetes mellitus, and dyslipidemia	Saroglitazar 4 mg daily	Evaluate the effect of Saroglitazar on liver function test, liver fibrosis score by FibroScan, lipid profiles, and HbA1c	From baseline, there was a reduction in ALT from 49 u/L to 48 (P < 0.05), fibrosis score 10 kPa to 6 (P < 0.0001), TG 359.89 to 103.04 (P = 0.0001), HbA1c 10.29% to 9.85% (P = 0.002)
Jain *et al* [30], 2018	Animal	Duration: 12 wk	n = 18	CDHFD-induced model of NASH in mice (n = 9 per group)	Saroglitazar: Control *vs* 3 mg/kg daily	Reversal of CDHFD-induced NASH after 8 wk	In control vs. treatment, respectively, steatosis score was 2.6 *vs* 0, ballooning 1.4 *vs* 0, inflammation 3 *vs* 1.1 (P < 0.1)
Jain *et al* [30], 2018	Animal	Duration: 12 wk	n = 16	CCL4-induced fibrosis model in mice (n = 8 per group)	Saroglitazar: Control *vs* 4 mg/kg daily	Reversal of CCl4-induced liver fibrosis after 4 wk	Saroglitazar protected mice from CCl4-induced liver fibrosis measured *via* Hematoxylin and Eosin stains
Staels *et al* [26], 2013	Animal	Duration: 7 wk	n = 16	Choline-deficient high-fat diet-	GFT505: Control *vs* 10 mg/kg	Evaluate the prevention of the	The percentage of animals with macrosteatosis in

				induced model of NASH in mice (*n* = 8 per group)	daily	development of NASH in CDHFD mice	control *vs* treatment was 100% to 0%, inflammation was 100% to 0%, and the percentage of fibrosis was 1.3% to 0.8% ($P < 0.01$)
Staels *et al* [26], 2013	Animal	Duration: 7 wk	*n* = 12	CCl4-induced liver fibrosis in mice (*n* = 6 per group)	GFT505: Control *vs* 30 mg/kg daily	Evaluate the prevention of the development of NASH in CCL4 mice	The fibrotic surface of control *vs* treatment was 8% *vs* 4% in CCL4 mice ($P < 0.001$)
Ye *et al*[23], 2003	Animal	Duration: 2 wk	*n* = 6	High fat-fed rats	Ragaglitazar: 3 mg/kg-1 daily	Evaluate the benefits of Ragaglitazar on insulin sensitivity and lipid metabolism.	Enhanced insulin suppressibility of hepatic glucose output by 79% ($P < 0.001$), decrease in liver TG from baseline of 23 μmol/g to 7 μmol/g ($P < 0.01$)

N/A: Not applicable; NASH: Non-alcoholic steatohepatitis; n: Number; TG: Triglycerides; CCL4: Carbon tetrachloride; NFS: NAFLD fibrosis score; CAP: Controlled attenuation parameter; HbA1c: Hemoglobin A1c; NAFLD: Non-alcoholic fatty liver disease; GIR: Glucose infusion rate; CDHFD: Choline-deficient high-fat diet; BMI: Body mass index; ALT: Alanine transaminase; AST: Aspartate aminotransferase; US: Ultrasound; MRI: Magnetic resonance imaging; CT: Computerized tomography.

Pan-PPAR agonists

There are three different isoforms of PPAR, α, γ, δ[14]. PPARα mainly regulates genes that participate in lipid transport, beta-oxidation, gluconeogenesis, and ketogenesis[15]. PPARγ regulates adiponectin, glucose metabolism, adipocyte differentiation, and lipogenesis[15]. PPARδ limits inflammation and regulates hepatic fatty acid oxidation[15]. Single PPAR agonists have had unwanted adverse effects and less effective results, for which investigational products that act on several isoforms have been attractive[15].

The main pan-PPAR agonists reviewed here are Benzafibrate[16,17], Lanifibranor[14,15,18-21], and MHY2013[22,23]. Multiple animal studies involving pan-PPAR agonists have demonstrated increased plasma adiponectin, improvement in hepatic steatosis, and markers of liver injury[15,17,19,20,22-24]. In alignment with the mechanism of pan-PPAR agonists, MHY2013[22,25] and Lanifibranor[19,20,24] also led to a decrease in hepatic steatosis, hepatic inflammation, serum triglycerides, profibrotic and fibrotic genes. In addition to the previously mentioned effects of Lanifibranor, Møllerhøj *et al*[20] revealed that Lanifibranor resulted in progressive weight loss, a 23% decrease at eight weeks and a 30% decrease at 12 wk [20].

In line with results from animal studies, a study of 45 patients using Lanifibranor (400 mg, 800 mg, or 1200 mg) or placebo for four weeks revealed an increase in adiponectin, a decrease in triglycerides, and ALT[25]. Shortly after, a more significant phase 2b trial was performed on 247 patients with NASH that were randomly assigned to Lanifibranor (800 or 1200 mg) or a placebo daily for 24 wk[18]. Participants had at least a 2-point decrease in the Steatosis, Activity, and Fibrosis score[18]. A comparison of pan-PPAR agonists *vs* single agents revealed that pan-PPAR agonists were more potent in counteracting fibrosis by combining specific mechanisms of single PPAR agonists[15]. Lanifibranor was generally well tolerated with mild reported side effects, including diarrhea, nausea, peripheral edema, anemia, and weight gain[18]. Based on initial data, pan-PPAR agonists are more effective in improving the histological features of fatty liver disease with fewer adverse side effects than single PPAR agonists. This makes them a desirable option for the treatment of fatty liver disease.

Dual-PPAR agonists

Like pan-PPAR agonists, these agents act on two isoforms of PPAR, allowing for a more targeted effect. Saroglitazar has already been Federal Drug Administration (FDA)-approved for diabetic dyslipidemia and hypertriglyceridemia and has been shown to improve NAFLD, which piqued interest.

The main dual-PPAR agonists reviewed here are Ragaglitazar (α/γ)[23], GFT505 (α/δ)[26,27], Saroglitazar (α/γ)[28-32] and Elafibranor (α/δ)[33,34]. Multiple animal studies involving dual-PPAR agonists have demonstrated promising results, including reduced triglycerides and liver injury markers[23,29]. Ragaglitazar revealed an 88% reduction in triglycerides, increased adiponectin, counteracted an increase in visceral fat mass, and enhanced insulin suppressibility of hepatic glucose output[23]. These outcomes correlate with results seen with GFT505[26] and Saroglitazar[20,29]. Furthermore, Saroglitazar completely normalized AST and ALT, reduced serum TNF-α level by 47.6% and leptin by 58.6%[29].

Human research showed promising results in line with the aforementioned animal studies. GFT505 80 mg/day revealed a statistically significant reduction of fasting plasma triglycerides, LDL, and liver enzyme levels[27]. However, the most studied investigational product is Saroglitazar. A more extensive study in 85 patients revealed reduced ALT and triglycerides[28]. Furthermore, a study of Saroglitazar in 91 patients showed that 57 patients (63%) could reduce ≥ 5% of their weight[31]. There has been discussion regarding pan-PPAR agonists *vs* dual agents; Boeckmans *et al*[33] compared Elafibranor *vs* Lanifibranor (pan-PPAR agonist), which identified Elafibranor as having higher anti-NASH properties[33]. In general, dual-PPAR agonists are safe and effective in treating NAFLD and obesity. Research suggests that Elafibranor may be more effective than pan-PPAR agonists in treating these conditions.

CONCLUSION

NAFLD has become one of the most common causes of chronic liver disease globally. It's troubling that no FDA-approved treatments are currently available for this condition. Patients are limited to lifestyle changes and managing any concurrent diseases associated with fatty liver. However, there are promising developments in the form of investigational products that are being studied through clinical trials. These products include cyclophilin inhibitors, FGF21 agonists, and pan and dual PPAR agonists. The data analyzed in this review show clinically significant improvement in individual histological features of NAFLD in both animal and human trials for all four classes. These agents were generally well tolerated, with minimal side effects. We believe this compilation of information will have positive clinical implications in obtaining an FDA-approved therapy for NAFLD. However, more extensive trials are needed to further determine their efficacy, proper dosage, duration of therapy, and potential side effects for patients with NAFLD, including those with hepatic steatosis and fibrosis.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) has become a global health issue with significant medical costs. The lack of a Federal Drug Administration (FDA)-approved medication for the treatment of NAFLD has prompted the investigation of several potential therapeutic classes. It is valuable to have a compilation of the data available on their efficacy.

Research motivation

Due to the absence of an approved medication by the FDA for the treatment of NAFLD, several therapeutic classes have been investigated in clinical trials. It is important to understand the mechanisms and statistical significance of the agents being investigated, as NAFLD is extremely prevalent.

Research objectives

To assess the efficacy of cyclophilin inhibitors, fibroblast growth factor 21 analogs (FGF21), and dual and pan peroxisome proliferator-activated receptor (PPAR) agonists as possible therapeutic classes for treating NAFLD.

Research methods

We searched PubMed, EMBASE, Cochrane Library, Scopus, and Web of Science using keywords including cyclophilin inhibitor, FGF agonist, pan-PPAR agonists, dual-PPAR agonist, NAFLD, non-alcoholic steatohepatitis, and fatty liver. Articles with a National Institutes of Health Quality Assessment score of five or higher were included. Each article was screened by two independent researchers evaluating relevance and quality. Pertinent data were extracted in a spreadsheet and descriptively analyzed.

Research results

We identified 29 studies that met the necessary criteria and were included in this review. These records included 12 human studies and 17 animal studies. Specifically, there were four studies on cyclophilin inhibitors, four on FGF analogs, 11 on pan-PPAR agonists, and ten on dual-PPAR agonists. All classes were found to be efficacious for the treatment of NAFLD with statistical significance ($P < 0.05$).

Research conclusions

We found that cyclophilin inhibitors, fibroblast growth factor 21 analogs, and dual and pan PPAR agonists are not only statistically efficacious for the treatment of NAFLD but also generally well tolerated. We recommend more extensive human clinical research to further delineate therapy's efficacy, dosage, and duration.

Research perspectives

It is to be expected that additional human clinical trials of the therapeutic classes assessed in this review, as well as additional novel agents, will be conducted in the near future. An FDA-approved agent for the treatment of NAFLD is of utmost importance.

FOOTNOTES

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MINIREVIEWS

Noninvasive prognostic models, imaging, and elastography to predict clinical events in primary sclerosing cholangitis: A review

Mark W Russo

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Abstract

Surrogate endpoints are needed to estimate clinical outcomes in primary sclerosing cholangitis (PSC). Serum alkaline phosphatase was among the first markers studied, but there is substantial variability in alkaline phosphatase levels during the natural history of PSC without intervention. The Mayo risk score incorporates noninvasive variables and has served as a surrogate endpoint for survival for more than two decades. Newer models have better test performance than the Mayo risk score, including the primary sclerosing risk estimate tool (PREsTo) model and UK-PSC score that estimate hepatic decompensation and transplant free survival, respectively. The c-statistics for transplant-free survival for the Mayo risk model and the long-term UK-PSC model are 0.68 and 0.85, respectively. The c-statistics for hepatic decompensation for the Mayo risk model and PREsTo model are 0.85 and 0.90, respectively. The Amsterdam-Oxford model included patients with large duct and small duct PSC and patients with PSC-autoimmune hepatitis overlap and had a c-statistic of 0.68 for transplant-free survival. Other noninvasive tests that warrant further validation include magnetic resonance imaging, elastography and the enhanced liver fibrosis score. Prognostic models, noninvasive tests or a combination of these surrogate endpoints may not only serve to be useful in clinical trials of investigational agents, but also serve to inform our patients about their prognosis.

Key Words: Cholestatic; Survival; Mortality; Predict; Cirrhosis; Decompensation

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Core Tip: Several noninvasive prognostic models have been validated that improve upon serum alkaline phosphatase and the Mayo risk score or include subgroups of patients not validated by these tests. The UK-PSC score has superior test performance compared to the Mayo risk score for short and long term transplant free survival. The Primary sclerosing risk estimate tool (PREsTo) has excellent test performance for risk of hepatic decompensation. The Amsterdam-Oxford model includes patients with small duct primary sclerosing cholangitis (PSC) and PSC-autoimmune hepatitis overlap. Elastography and magnetic resonance imaging show promise as prognostic tools.

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a cholestatic liver disease associated with diffuse inflammation of the biliary treat that may lead to cirrhosis, complications from portal hypertension and cholangiocarcinoma. The diagnosis is typically established on cholangiogram obtained during endoscopic retrograde cholangiography or magnetic resonance cholangiography and less commonly from findings on liver biopsy. The estimated prevalence of PSC varies by geographic location and ranges from 0.1-13.6 per 100000 with higher rates seen in Scandinavian countries and the United States[1]. The median transplant free survival is 21 years and there is no effective medical therapy that improves upon this outcome [2].

The etiology of PSC is not known but proposed mechanisms include dysregulation of the immune system, alterations in bile duct transporters that result in accumulation of toxic bile salts, gut microbiome interactions and immune mediated injury to the biliary epithelium, or environment triggers in genetically susceptible individuals[3].

There have been a number of drugs that have been evaluated for the treatment of PSC[4-6]. Because it would take years or even decades to evaluate the effect of a medication on liver related events on survival surrogate endpoints are needed. A consensus group suggested noninvasive surrogate endpoints are needed for clinical trials, which are preferred to more invasive surrogate endpoints such as liver biopsy or ERCP[7]. Serum alkaline phosphatase (ALP) and the Mayo model, which initially included liver histology, were among the first surrogate endpoints for PSC[8,9]. Since a prior review on this topic noninvasive prognostic models have been developed and validated with excellent test performance[10]. The most common clinical outcomes that surrogate endpoints have been associated with include liver transplant free survival, hepatic decompensation and cholangiocarcinoma. A review of the topic of noninvasive prognostic tests and models is timely because there are a number of molecules under development and noninvasive surrogate tests are recommended as endpoints in clinical trials[7].

MATERIALS AND METHODS

The focus of this invited review is to discuss noninvasive surrogate endpoints for patients with PSC and how they compare to ALP and Mayo Risk Score. Key words or search terms used to identify relevant articles published in English from January 1, 2000 to January 1, 2023 that were entered into PUBMED, OVID and EMBASE included "primary sclerosing cholangitis" and "biomarkers", "primary sclerosing cholangitis" and "prognostic score", "primary sclerosing cholangitis" and "model" and "prognosis", "primary sclerosing cholangitis" and "elastography". The references of articles were reviewed for additional relevant articles.

SERUM MARKERS

ALP and total bilirubin

A reduction or normalization of ALP has been associated with improved outcomes in patients with PSC. In a study of 86 patients with PSC, 38 (44%) achieved ALP normalization within 12 months of diagnosis[11]. Normalization of ALP was not associated with ursodeoxycholic acid (UDCA) therapy or therapeutic endoscopic retrograde cholangiography. Persistent ALP normalization was associated with a 79% lower risk of death, hepatobiliary neoplasia, or liver transplantation. In a separate study, patients with PSC who achieved an ALP less than 1.5 times the upper limit of normal had lower rate of a composite outcome (liver decompensation, liver transplantation, liver related deaths, cholangiocarcinoma) compared to those who did not have a reduction in ALP, 6% *vs* 38%, $P = 0.0002$[12]. Among 692 patients with PSC, an ALP > 1.3 × upper limit of normal (ULN) at 1 year of follow-up was associated with a 2-fold greater risk of liver transplant or PSC related death (death from end stage liver failure, cholangiocarcinoma or liver surgery)[13]. A reduction in ALP is associated with improved transplant free survival in patients with PSC with or without dominant strictures[14].

Among UDCA treated PSC patients who did and did not have ALP levels decrease by 40% or more after 1 year, 12 year survival was 90% and 47%, respectively, $P = 0.001$[15]. Patients in the placebo group had better survival if they had a 40% reduction or more in ALP after 1 year compared to those who did not have a decline in ALP. In a study that included patients who were and were not treated with UDCA no patient with persistently normal ALP reached a clinical endpoint (cholangiocarcinoma, liver transplantation or death) compared to 33% with persistent ALP abnormalities[16].

Patients with PSC have substantial variability in ALP levels over 5 years with 65% and 34% of patients achieving an ALP < 1.5 × ULN or normalizing ALP, respectively[17]. Despite variability in ALP levels, an ALP that declined to < 1.5 × ULN was independently associated with death, liver transplantation, hepatic decompensation or cholangiocarcinoma. However, others have shown that ALP reductions of 40% or more from baseline are seen in 15%-18% of patients with PSC at 2 years that are not associated with disease progression[18].

Total bilirubin

Total bilirubin is associated with lower survival in patients with PSC, but studies demonstrating this association have included patients with advanced disease, thus limiting its usefulness in patients with early stage PSC[19,20].

Enhanced liver fibrosis score and test

Enhanced liver fibrosis (ELF) score (R&D systems, Orion diagnostics, Espoo Finland) and ELF test (Siemens Medical Solutions Diagnostics Inc., Tarrytown, NY, United States) are derived from algorithms that include tissue inhibitor of metalloproteinase I, hyaluronic acid, and propeptide of type III procollagen. The association between transplant free survival and ELF score was derived and validated in 167 and 138 PSC patients, respectively. ELF score was independent of Mayo Risk Score and had a c-statistic of 0.82 for transplant free survival. A score of 10.6 or higher was associated with lower transplant free survival independent of Mayo Risk Score[21]. The ELF test was better than the ELF score at identifying the group at low risk for clinical endpoints. In a clinical trial that randomized patients with PSC to simtizumab or placebo, an ELF test ≥ 9.8 was associated with PSC-related progression events (ascites, spontaneous bacterial peritonitis, variceal hemorrhage, hepatic encephalopathy, ascending cholangitis, cholangiocarcinoma, hepatocellular carcinoma, liver transplantation, and death)[22]. Among those with an ELF test ≥ 9.8, 34% experienced a clinical event compared to 11% of those with scores below this threshold.

Among patients with PSC and cholangiocarcinoma, the ELF test was higher compared to those with PSC alone, 11.4 and 9.9, respectively $P < 0.001$[23]. In multivariable analysis an ELF test ≥ 9.8 was associated with a diagnosis of cholangiocarcinoma in patients with PSC (OR = 4.91, 95%CI: 1.19-20.21, $P = 0.021$).

NONINVASIVE PROGNOSTIC MODELS

Mayo risk score

The Mayo Risk Score was developed and validated in 405 patients and 124 patients, respectively with PSC from five centers[24]. The earlier Mayo Model for PSC required liver biopsy because histologic stage of PSC is a variable in the model[8,9]. The Mayo Risk Score includes age, total bilirubin, aspartate aminotransferase, variceal bleeding (yes/no), and albumin (Table 1). In the Mayo risk score study median follow up was 36 mo and the outcome was overall survival up to 4 years. Newer models that have been compared to the Mayo Risk Score will be discussed in further detail below.

Amsterdam-Oxford model

The Amsterdam-Oxford model was developed and validated among 956 patients with PSC from 44 Dutch hospitals or referral centers[25]. Large duct PSC was diagnosed in 91% of patients, 4% had PSC-autoimmune hepatitis overlap, 71% had inflammatory bowel disease, and 80% of the derivation cohort was taking UDCA. Median follow-up was 110 mo and the primary outcome was a composite outcome of liver transplant or PSC-related death (death from end-stage liver failure, death from liver surgery, death from cholangiocarcinoma or death from colorectal carcinoma).

The Amsterdam-Oxford model includes PSC subtype, age at diagnosis, albumin, platelets, aspartate aminotransferase, ALP, and total bilirubin (Table 2). The c-statistics for the primary outcome at 3 years of follow-up in the validation cohort were 0.66 with similar c-statistics at 1 and 2 years of follow-up (Figure 1).

A study from three centers in Italy, Belgium and The Netherlands evaluated the test performance of the Amsterdam-Oxford model and compared it to the Mayo Risk Score[26]. The cohort included 534 patients of which 3% had small duct PSC, 10% had PSC-autoimmune hepatitis overlap, 60% had inflammatory bowel disease and 92% were on UDCA therapy. The primary outcome was transplant free survival. The c-statistics for Amsterdam-Oxford model and Mayo Risk Score at 5 years of follow-up in the validation cohort were 0.76 and 0.79, respectively.

Primary sclerosing cholangitis risk estimate tool

Primary sclerosing cholangitis risk estimate tool (PREsTo) was developed and validated in 787 patients with PSC from centers in North America and Norway[27]. Patients with small duct PSC or PSC-autoimmune hepatitis overlap were excluded and approximately 70% of patients had inflammatory bowel disease. The number of patients on UDCA was not provided. Median follow-up was 6 and 4 years for the derivation and validation cohorts, respectively. The primary outcome was hepatic decompensation defined as variceal hemorrhage, hepatic encephalopathy, or ascites.

The authors employed artificial intelligence and used gradient boosting machines, a machine learning technique, to identify variables associated with hepatic decompensation. Variables included in the PREsTo model include total

Table 1 Noninvasive prognostic tests for primary sclerosing cholangitis

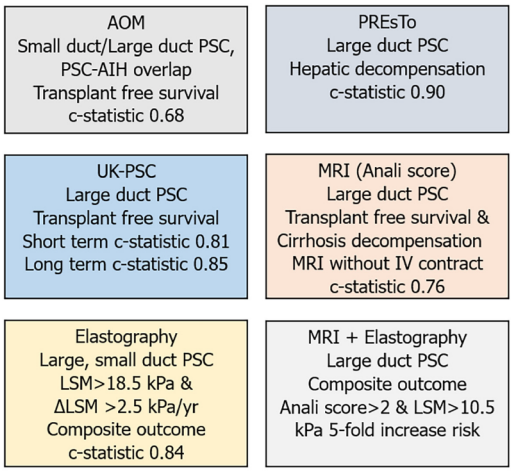
Serum markers	Models	Elastography	Imaging
Alkaline phosphatase; Total bilirubin; Enhanced liver fibrosis score	Mayo Risk Score; UK-PSC score; Amsterdam-Oxford Model; PREsTo score	Vibration controlled transient elastography; Magnetic resonance elastography	Magnetic resonance imaging-Anali score

PSC: Primary sclerosing cholangitis; PREsTo: Primary sclerosing risk estimate tool.

Table 2 Variables in noninvasive prognostic models for primary sclerosing cholangitis

	MRS	AOM	PREsTo	UK-PSC$_{ST}$	UK-PSC$_{LT}$
Age	√	√			
PSC subtype		√			√
Albumin	√	√	√	√	
ALP		√	√		√
AST	√		√		
Hemoglobin			√	√	
Platelets		√	√	√	√
Sodium			√		
Total bilirubin	√	√	√	√	√
Variceal bleed	√				√

AOM: Amsterdam-Oxford Model; ALP: Alkaline phosphatase; AST: Aspartate transaminase; MRS: Mayo Risk Score; PSC: Primary sclerosing cholangitis; PREsTo: Primary sclerosing risk estimate tool.



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Figure 1 Noninvasive prognostic models and test performance for primary sclerosing cholangitis. AOM: Amsterdam-Oxford Model; PREsTo: Primary sclerosing risk estimate tool; MRI: Magnetic resonance imaging; PSC: Primary sclerosing cholangitis; LSM: Liver stiffness measurements.

bilirubin, ALP, albumin, alanine aminotransferase, platelets, sodium, and hemoglobin (Table 1). Total bilirubin, albumin and ALP had the highest relative importance in the PREsTo model. In the validation cohort the c-statistic was 0.90 for PREsTo for 5-year risk of decompensation compared to c-statistics of 0.72, 0.85, and 0.65 for model for end stage liver disease score, Mayo Risk Score, and ALP < 1.5 × ULN, respectively.

UK-PSC score

The UK-PSC score was developed and validated in 1452 patients with PSC from 155 sites throughout the United Kingdom[28]. All patients had large duct PSC, 73% had inflammatory bowel disease, and 57% were on UDCA. Median follow-up ranged from 6-14.8 years in the validation and derivation cohorts. The primary outcome was transplant-free survival.

A short-term model for 2-year outcome and long-term model for 10-year outcomes were developed. The variables in the short-term UK-PSC score include total bilirubin, albumin, hemoglobin and platelet count (Table 2). The long-term model includes baseline and year 2 total bilirubin, platelet count, ALP, disease type (presence or absence of extrahepatic biliary disease) and history of variceal bleed (yes/no). In the validation cohort the c-statistics for short term UK-PSC model, Mayo Risk Score, model for end stage liver disease score were 0.81, 0.73, and 0.78, respectively. The c-statistics for long term UK-PSC model, Mayo Risk Score and aspartate aminotransferase platelet ratio index were 0.85, 0.69, and 0.70, respectively (Figure 1).

IMAGING

Magnetic resonance imaging

Features on magnetic resonance imaging (MRI) with cholangiography have been associated with outcomes in patients with PSC called the Anali score developed by Ana Ruiz and Lionel Arrive[29]. Based 289 MRI images from 64 patients with a median follow-up of 4 years a model for findings on MRI without and with contrast were developed to predict radiologic progression. The Anali score without gadolinium includes dilatation of intrahepatic bile ducts, dysmorphy, and portal hypertension while the score with gadolinium includes dysmorphy and parenchymal enhancement heterogeneity. Dysmorphy was defined as significant atrophy of either the right or left hepatic lobe and/or marked lobulations of the liver surface and/or increase in the caudate/right lobe ratio. The c-statistics for the Anali scores with and without gadolinium were 0.83 and 0.80, respectively (Figure 1)[29].

The MRI derived Anali score was validated in a study that included 338 patients with large duct PSC from France, Canada, Italy and the United Kingdom equally divided between a derivation and validation cohort[30]. The primary endpoint was transplant free survival or cirrhosis decompensation. The c-statistics for the primary outcome for the Anali score with and without gadolinium in the validation cohort were 0.73 and 0.76, respectively[30].

Transient elastography and magnetic resonance elastography

Liver stiffness measurements (LSM) obtained by vibration controlled transient elastography at baseline and during follow-up are associated with outcomes. A prospective study that included patients with large duct, small duct PSC (9%) or PSC-autoimmune hepatitis (3%) overlap reported adverse outcomes associated with baseline LSM and change in LSM [31]. Adverse outcomes were defined as a composite of death, liver transplantation, ascites, hepatic encephalopathy, gastrointestinal bleeding related to portal hypertension, cholangiocarcinoma or hepatocellular carcinoma. All patients were on UDCA and 68% had inflammatory bowel disease. The LSMs with highest accuracy for adverse outcomes were LSM > 18.5 kPa and change in LSM > 4 kPa/yr.

The group that developed PREsTo demonstrated an increase in magnetic resonance elastography (MRE) score is associated with hepatic decompensation (ascites, variceal hemorrhage or hepatic encephalopathy)[32]. In this study of 204 patients with PSC of which 82% had inflammatory bowel disease and 34% were on UDCA reported an increase of LSM > 0.34 kPa/yr had a c-statistic of 0.79 for hepatic decompensation. Combining a LSM > 4.32 kPa at baseline and an increase > 0.34 kPa/yr had a c-statistic of 0.93 for hepatic decompensation.

MRI and vibration controlled transient elastography

In a retrospective study that included 162 patients with PSC from 3 centers, Anali score without gadolinium and vibration controlled transient elastography (VCTE) were combined to risk stratify patients at risk for liver transplantation or cirrhosis decompensation[33]. Patients were categorized into three groups: Anali score ≤ 2 and LSM < 10.5 kPa, Anali score > 2 or LSM > 10.5 kPa, or Anali score > 3 and LSM score > 10.5 kPa. An Anali score > 2 and LSM > 10.5 kPa was associated with a 5-year risk of liver transplantation, death or cirrhosis decompensation of 38% compared to 8% for those with an Anali score ≤ 2 and LS ≤ 10.5 kPa, $P < 0.001$.

DISCUSSION

Despite the development and validation of several prognostic models and the evolution of imaging and elastography, ALP has persevered as a surrogate marker for disease progression in PSC. ALP or changes in ALP remain as an outcome in clinical trials of investigational agents for PSC[34-39]. The simplicity and availability of ALP make it an attractive biomarker in clinical practice. However, there is variability in ALP that occurs over time without any intervention, and it has inferior test performance compared to more recently validated prognostic models. Despite these limitations, ALP, it remains a variable in Amsterdam-Oxford model, PREsTo and UK-PSC$_{LT}$ models (Table 2). The ELF score has been associated with survival or cholangiocarcinoma but requires further validation. Furthermore, ELF is associated with added cost compared to noninvasive prognostic models where variables and lab data are usually already available.

The Mayo Risk Score has stood the test of time, but a criticism has been that the study cohort included a large number of patients with advanced PSC and the time span for the model is limited to 4 years. The PREsTo and UK-PSC scores provide estimates for outcomes at 5 and 10 years of follow-up. The test performance of UK-PSC and PREsTo models are better compared to the Mayo Risk Score.

Each of the models has its role in informing our patients with PSC about their prognosis (Figure 1). The UK-PSC model provides short-term and long-term estimates of transplant free survival. The PREsTo score provides risk of hepatic

decompensation over 5 years. The Amsterdam-Oxford model provide transplant free survival and included patients with small duct PSC and PSC-autoimmune hepatitis overlap, although there were very small numbers in each group.

MRI and VCTE are attractive as prognostic tools because they are frequently obtained during clinical care. The Anali score can be readily obtained from MRI of the abdomen with or without gadolinium because imaging is commonly obtained as part of clinical practice. Results from VCTE or MRE, including baseline measurements as well as annual changes can provide prognostic information, although data on VCTE and MRE are limited to those derived from retrospective studies.

A number of novel biomarkers involved in inflammation, fibrosis or the gut barrier have been studied that are not commercially available but may warrant further study, including third generation anti-neutrophil cytoplasmic antibodies to serine protease-3[40-44]. Future studies could combine results for the prognostic models that include clinical and laboratory data with scores from MRI and elastography (*e.g.* UK-PSC or PREsTo+Anali score+elastography score). As the test performance of these noninvasive prognostic models improve they may not only serve as surrogate endpoints in clinical trials, but they can also be used to inform our patients about their prognosis. Other cutting-edge techniques, including artificial intelligence may be employed to identify findings on imaging associated with disease progression, survival or patients at risk for cholangiocarcinoma[44].

CONCLUSION

In conclusion, a number of noninvasive prognostic models for PSC are available that can be used in clinical trials as surrogate endpoints or as tools to inform patients about their disease progression. The models can be tailored to a specific trial endpoint, such as PREsTo score for hepatic decompensation or UK-PSC score for transplant-free survival. In the future, combining these models with results from elastography may improve test performance. Other areas warranting further investigation include novel molecular diagnostics, composition of the gut microbiome and its association with clinical outcomes as well as exploring the role of artificial intelligence in identifying imaging findings associated with disease progression or cholangiocarcinoma.

FOOTNOTES

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MINIREVIEWS

Toxicity of targeted anticancer treatments on the liver in myeloproliferative neoplasms

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Abstract

The liver has a central role in metabolism, therefore, it is susceptible to harmful effects of ingested medications (drugs, herbs, and nutritional supplements). Drug-induced liver injury (DILI) comprises a range of unexpected reactions that occur

after exposure to various classes of medication. Even though most cases consist of mild, temporary elevations in liver enzyme markers, DILI can also manifest as acute liver failure in some patients and can be associated with mortality. Herein, we briefly review available data on DILI induced by targeted anticancer agents in managing classical myeloproliferative neoplasms: Chronic myeloid leukemia, polycythemia vera, essential thrombocythemia, and myelofibrosis.

Key Words: Myeloproliferative neoplasms; Chronic myeloid leukemia; Myelofibrosis; Polycythemia vera; Essential thrombocythemia; Hepatotoxicity; Drug-induced liver injury

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Core Tip: Drug-induced liver injury (DILI) comprises a range of unexpected reactions that occur after exposure to any type of medication. Patients diagnosed with classical myeloproliferative neoplasms (MPNs) (chronic myeloid leukemia, polycythemia vera, essential thrombocythemia or primary myelofibrosis) are often prescribed pharmacological agents that can lead to DILI. Herein, we examine the hepatotoxic potential of kinase inhibitors used in the treatment of classical MPNs with a focus on DILI diagnosis, management and prevention. In most cases, DILI can be successfully managed with dose interruptions or reductions and use of hepatoprotective agents, however, in some cases drug cessation may be warranted.

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INTRODUCTION

A brief overview of myeloproliferative neoplasms

Haematopoietic stem cells exhibit pluripotency and have the capacity to self-renew, resulting in myeloid or lymphoid cell lines which differentiate into mature blood cells. Overproduction of terminal myeloid cell lines in the bone marrow due to certain mutations in hematopoietic stem cells gives rise to a group of disorders known as myeloproliferative neoplasms (MPNs). MPNs are broadly classified into three categories: Philadelphia-positive MPNs, such as chronic myeloid leukemia (CML), classical Philadelphia-negative MPNs, such as polycythemia vera (PV), primary myelofibrosis (PMF) and essential thrombocythemia (ET), and non-classical Philadelphia-negative MPNs which include chronic neutrophilic leukemia, chronic eosinophilic leukemia-not otherwise specified and MPN-unclassifiable[1,2].

Epidemiology of MPNs

The incidence rate of CML has increased, whereas its age-standardized incidence rate decreased to 0.84 per 100000 individuals in 2019 from 0.96 in 1990. In addition, a slight increase in the incidence of CML cases has been observed in males *vs* females[3]. According to a systematic review of 20 studies from Europe, North America, Asia, and Australia which assessed the incidence rate of PV, the annual pooled incidence rate was 0.84 per 100000 individuals. There was no significant difference in the crude annual incidence between males and females[4]. Ten studies from Europe and North America reported the annual pooled incidence rate of 1.03 per 100000 inhabitants, with a higher pooled annual incidence in males compared to females[4]. PMF has the lowest incidence among classical Philadelphia-negative MPNs, with an annual pooled incidence of 0.47 per 100000 subjects and a higher incidence in males than females[4].

Pathophysiology of MPNs

CML is characterized by a reciprocal translocation between chromosomes 22 and 9, resulting in the fusion of the Abelson Murine Leukemia (ABL) 1 gene with the Breakpoint Cluster Region (BCR) gene. This generates a chimeric protein with constitutively active tyrosine kinase activity, which promotes cell growth and signaling through various downstream pathways[5]. The World Health Organization has divided the progression of CML into 2 phases primarily based on blast cell counts in the peripheral blood or bone marrow: Chronic phase and blast phase (≥ 20% myeloid blast cells in the bone marrow or peripheral blood or elevated numbers of lymphoid blast cells in the bone marrow or peripheral blood or evidence of extramedullary proliferation of blast cells), with the majority of patients presenting in the chronic phase[6]. There has been an increase in the life expectancy of CML patients, similar to that of the general population. This can be attributed to the fact that most newly diagnosed cases of CML occur in the chronic phase of the disease and due to the availability of new and effective therapies[7].

Classical Philadelphia-negative MPNs include PV, which primarily involves excess proliferation of red blood cells, ET, with thrombocytosis in the peripheral blood and overactive megakaryocytes in the bone marrow, and PMF, which involves fibrosis of the bone marrow and other diagnostic criteria. The pathogenesis of classical Philadelphia-negative

MPNs requires constitutive activation of the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway due to mutations in a variety of genes, out of which, JAK2V617F gain of function mutation is the most frequent, being present in > 95% of PV cases and > 50% of PMF and ET cases[8,9]. The remaining cases of PV are linked to JAK2 exon 12 mutations, while most of the remaining cases of PMF and ET have detectable MPL or CALR mutations[10]. Both ET and PV have a relatively favorable prognosis, with ET carrying the most favourable prognosis and PMF carrying the worst prognosis[11].

DRUG-INDUCED LIVER INJURY: BRIEF OVERVIEW

The liver is susceptible to the harmful effects of ingested medications (drugs, herbs, and nutritional supplements) because of its central role in metabolism[12,13]. Drug-induced liver injury (DILI) comprises a range of unexpected reactions occurring after exposure to various medications. Even though most cases consist of mild, temporary elevations in liver enzyme markers, DILI can result in acute liver failure (ALF). Thus, DILI may emerge as a significant cause of liver disease and sometimes lead to increased mortality rates[14-16].

The pathogenesis of DILI is complex and not fully understood. It can vary significantly between different individuals and based on the drugs that cause liver injury, which explains the wide range of phenotypic traits in clinical presentation and severity[17]. DILI results from a combination of genetic, non-hereditary, and environmental variables, and is often attributed to an allergic immune response[18].

The potential for multiple clinical presentations and the lack of specific biomarkers or biochemical tests often make the diagnosis difficult and delayed. Consequently, DILI must always be considered in patients who are prescribed medications and exhibit unexplained liver injury[17,19]. Moreover, DILI is the leading cause of drug withdrawal from the marketplace which can result in changes in drug costs and challenges in medication availability[20].

The pathophysiology of DILI is a complex, multistep process involving both direct injury and different inflammatory responses induced by either the drug itself, its metabolites, or the immune system. It denotes a combination of various host-related, environmental, and drug-related factors. If ALF does not occur, patients usually fully recover after an episode of DILI if the responsible medication is discontinued[12,18,21].

Among the main pathophysiological processes involved in the pathogenesis of DILI, one must highlight oxidative stress, interference with bile acids' transportation, alteration of mitochondrial biogenesis, and triggering of innate immune responses, necrosis, or even apoptosis[15,18].

Liver toxicity is further categorized as direct, indirect, or idiosyncratic based on the underlying mechanism of action of the chemical compound that leads to DILI. Direct hepatotoxicity is caused by agents which produce immediate and direct injury to the liver. This is a common, predictable, dose-dependent injury with a short latency period (1 to 5 d). It causes elevations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations, induces minimal or no symptoms, is associated with normal total bilirubin levels, and usually disappears once the drug is stopped or the dose is lowered[22,23].

Indirect hepatotoxicity is defined as "a condition caused by the medication's actions rather than from its inherent hepatotoxic effects or immunogenicity". This best translates to "what the drug does rather than what the drug is". This can either result in induction of a new liver condition or an exacerbation of a preexisting condition, *e.g.*, induction of immune-mediated hepatitis, reactivation of viral hepatitis or progression of fatty liver disease[12,22].

Idiosyncratic hepatotoxicity is caused by agents that have no direct hepatotoxic effect. It is an unpredictable condition, less common (< 1 of every 10000 exposed individuals), is not dose-related, has a longer latency period (up to several weeks), and a more variable clinical presentation[12,22,24].

A rapid diagnosis of DILI is crucial since one of the primary treatment interventions for hepatotoxicity is drug withdrawal. Moreover, establishing a DILI diagnosis can support the prevention of further adverse reactions through regulatory decisions such as prescription warnings or the removal of pharmaceuticals from the market[12,13,25].

In most cases, a diagnosis of DILI is one of exclusion since there is no specific test available for this entity. It is imperative to eliminate other causes of liver injury, *e.g.*, infectious hepatitis, acute alcoholic hepatitis, or ischaemic hepatitis. Suspicion of DILI arises from the discovery of alterations in standard liver function tests, *i.e.*, AST, ALT, total and direct bilirubin levels, serum albumin, alkaline phosphatase (ALP), or international normalized ratio[13].

Clinical manifestations, such as fatigue, nausea, malaise, right upper quadrant pain, pruritus, and jaundice, are non-specific and commonly encountered in various acute and chronic liver diseases. Liver imaging (abdominal ultrasonography, magnetic resonance cholangiography or computed tomography) is often used to exclude the presence of biliary obstruction and focal lesions. Liver biopsies are completed in less than half of suspected cases, and usually in instances where the evolution of the liver injury is not reversed after a suspected medication has been discontinued[22,26].

Currently, over 18 different histological aspects of DILI have been proposed, all of which are associated with varying degrees of inflammation, bile accumulation, ductopenia, steatohepatitis, macro- and micro-vesicular fatty depositions in the liver, pigment deposition, fibrosis or vascular congestion and obliteration[12,19].

DILI can also be classified by its biochemical pattern based on ALT and ALP levels. A pattern of hepatocellular damage is defined by an "elevation in ALT greater than 2 to 5 times the upper limit of normal (ULN) and/or by an ALT/ALP ratio also greater than 5". A pattern of cholestatic damage is defined by an "elevation in ALP greater than 3 times the ULN and/or an ALT/ALP ratio less than 2". A pattern of mixed hepatocellular/cholestatic damage is defined by "an increase in ALT greater than 2 to 5 times the ULN and an increase in ALP greater than 3 times the ULN and/or an ALT/ALP ratio between 2 and 5". These patterns have been proposed by the "American Association for the Study of Liver Diseases" and are summarized in Table 1[18,27,28].

Table 1 Biochemical classification of drug-induced liver injury

	Hepatocellular DILI	Mixed DILI	Cholestatic DILI
AASLD criteria for diagnosis of DILI	Elevation of ALT ≥ 3 times ULN and ALT/ALP ratio ≥ 5 times	ALT ≥ 3 times ULN, ALP ≥ 2 times ULN and ALT/ALP ratio < 5 but >2 times ULN	ALP ≥ 2 times ULN and ALT/ALP ratio of ≤ 2 times ULN
R value criteria for different patterns of DILI	R = (ALT/ULN)/(ALP/ULN) > 5	R = (ALT/ULN)/(ALP/ULN) < 5 and > 2	R = (ALT/ULN)/(ALP/ULN) < 2

ALP: Akaline phosphatase; ALT: Alanine Aminotransferase; DILI: Drug-induced liver injury; ULN: Upper limit of normal.

Management

While in some patients, DILI can spontaneously resolve without active treatment, in most cases, the hallmark of DILI treatment is the withdrawal of the offending drug. Clinical and biochemical alterations are expected to improve over several days or weeks. Since patients who develop jaundice are more likely to progress to ALF, these subjects usually require strict monitoring and hospitalization, particularly if DILI exhibits a hepatocellular phenotype. Treatment of DILI is usually supportive, with no other specific medications showing any significant benefit. However, there are many agents used for supportive purposes, *e.g.*, corticosteroids (empirically used by many clinicians), cholestyramine (administered to patients with acute liver injury caused by leflunomide), carnitine (an antidote for valproate-induced liver injury) or N-acetyl cysteine (NAC, a treatment for acetaminophen toxicity), silymarin, L-arginine, L-ornithine L-aspartate and/or vitamin E[13,29-32].

Prognosis

DILI typically resolves after discontinuing the offending drug and/or administering hepatoprotective agents. However, in rare cases, DILI may progress to ALF, with clinical features such as jaundice, ascites, encephalopathy, coagulopathy, and a mortality rate of 60% to 90% without liver transplantation[14,17].

Different scoring systems for predicting the prognosis of DILI have been proposed. The most validated and the one used by The Food and Drug Administration (FDA) during the drug development process to identify pharmacological agents that can potentially induce severe liver injury is "Hy's law". This was developed by Hyman Zimmerman in the 1960s, according to which 10% of the patients who develop jaundice will develop ALF. Other scores for predicting the severity of DILI are the Model for End-Stage Liver Disease score, King's college criteria score, and Acute Liver Failure Study Group index [12,13,19].

Older age, higher drug dosages, presence of liver disorders or cardiovascular comorbidities, African American ethnicity, and female sex have all been linked to an elevated risk of DILI and more severe forms. Still, there is little empiric data available to support that these variables are indeed risk factors for DILI or have an impact on its prognosis [33].

MAIN THERAPEUTIC AGENTS USED IN MPNS

The development of tyrosine kinase inhibitors (TKIs) following the discovery of the BCR-ABL chimeric gene has drastically improved the success rate of CML treatment. TKIs have improved the 10-year survival rate from 20% to 80%-90%[34]. Commonly used TKIs for the treatment of chronic phase CML involve first-generation TKIs (*e.g.*, imatinib), second-generation TKIs (*i.e.*, dasatinib, nilotinib, and bosutinib), and third-generation TKIs (*i.e.*, ponatinib). Imatinib was the first TKI to be approved by the FDA[35]. The IRIS trial first showed the high effectiveness of imatinib in increasing the survival rate of newly diagnosed CML patients compared to interferon-alpha plus cytarabine[34]. It is a fairly safe drug as long as patients are closely monitored. Second-generation TKIs exhibit rapid molecular responses and have been used in cases of resistance/intolerance to imatinib[36].

The discovery of the involvement of the JAK/STAT pathway in the pathogenesis of classical Philadelphia-negative MPNs paved the way for the TKIs to inhibit the JAK/STAT pathway. Ruxolitinib was the first targeted drug developed that inhibits both JAK1 and JAK2 and is approved for use in intermediate and high-risk myelofibrosis (MF) based on the COMFORT trials and in cases of PV resistant or intolerant to hydroxyurea based on the RESPONSE trial[37].

Fedratinib is another TKI inhibiting JAK2 and FMS-like tyrosine kinase 3 and is approved for treating intermediate or high-risk PMF or secondary MF. Diarrhea, nausea, and anemia are common side effects associated with this therapy. Renal function, liver enzymes, lipase, and amylase may require frequent monitoring during the treatment[38].

Momelotinib is a recently FDA-approved JAK1/JAK2 inhibitor that antagonizes the activin A receptor type 1. It is used to treat patients with MF with moderate/severe anemia[14]. It is similar to ruxolitinib but with the added advantage of improving anemia[12]. The most common side effects associated with it include diarrhea, peripheral neuropathy, dizziness, nausea, and thrombocytopenia[39].

In high-risk patients suffering from PV and ET, cytoreductive therapy with hydroxyurea and interferon alpha are first-line choices used to reduce the rate of thrombotic events. Hydroxyurea is a potent ribonucleotide reductase inhibitor causing inhibition of DNA synthesis and cell death[40].

Interferons, especially pegylated interferon α (peg-IFNα) and ropeg interferons, are increasingly employed as effective alternatives to cytoreduction with hydroxyurea in patients with ET and PV. Studies have reported a decrease in the JAK2V617F allele burden following the prescription of interferon-based therapy, which does not occur with hydroxyurea. Interferon is also used along with ruxolitinib in patients with low to intermediate-risk MF. Peg-IFNα and ropeg interferons are associated with a lower rate of adverse effects than standard interferons α used in the past[41].

EPIDEMIOLOGY OF DILI IN PATIENTS DIAGNOSED WITH MPNS

DILI is mainly characterized by increased liver enzyme concentrations due to damage induced to hepatocytes. Hepatotoxicity in CML subjects on TKI therapy presents as low-grade elevation of ALT and/or AST levels in about 25%-35% of cases, and high-grade elevation in about 2% of patients. The use of newer-generation TKIs (*e.g.* bosutinib, nilotinib, and ponatinib), has been associated with higher risks of liver toxicity[42].

DILI was a rare event in the 5-year follow-up of the phase 3 DASISION (Dasatinib Versus Imatinib Study in Treatment-Naïve CML Patients) trial, which compared the two aforementioned pharmacological agents. Therapy discontinuation was only required in the subgroup who received 400 mg of imatinib daily due to increases in ALT or AST concentrations (n = 1 each out of 258 individuals) and in one case of toxic hepatitis (n = 1). However, no subjects (n = 0 out of 258 individuals) discontinued treatment with 100 mg of daily dasatinib due to DILI[43].

In the ENESTnd trial, which compared the daily administration of 400 mg nilotinib (n = 277 patients) and 300 mg nilotinib (n = 279 patients) to each other, and to 400 mg imatinib (n = 280 patients) for the management of newly diagnosed CML, the investigators identified multiple cases of liver toxicity. Elevations in total bilirubin (n = 171, 62% *vs* n = 149, 53% *vs* n = 27, 10%), ALT (n = 203, 73% *vs* n = 186, 66% *vs* n = 57, 20%) and AST (n = 134, 48% *vs* n = 112, 40% *vs* n = 65, 23%) concentrations were more likely to occur in the 400 mg nilotinib and 300 mg nilotinib *vs* 400 imatinib group, respectively. In contrast, an increase in ALP (n = 76, 27% *vs* n = 59, 21% *vs* n = 92, 33%) value was more common in patients who received imatinib. However, grade 3/4 adverse events were rare and occurred predominantly in individuals who were prescribed nilotinib 400 mg or 300 mg *vs* imatinib 400 mg; all grades elevations occurred in total bilirubin (n = 21, 8% *vs* n = 10, 4% *vs* n = 1, < 1%), ALT (n = 25, 9% *vs* n = 11, 4% *vs* n = 7, < 2%) and AST (n = 8, 3% *vs* n = 4, 1% *vs* n = 3, 1%) values, respectively. Whereas a grade 3/4 increase in ALP only occurred in 1 case (< 1%) of imatinib-treated patients[44].

The NOVEL trial evaluated the safety and efficacy of nilotinib in 85 patients with imatinib intolerant/resistant CML. Their findings demonstrated that non-hematological adverse events occurring in correlation with the use of nilotinib manifested as elevations in ALT (n = 18, 21.2%), bilirubin (n = 12, 14.1%) and/or AST (n = 7, 8.2%) values. However, grade 3/4 elevations were rare and only 2 and 1 patients, respectively, experienced them in AST (2.4%) and/or ALT (1.2%) concentrations. In NOVEL, one patient with imatinib-induced liver toxicity required a switch of therapy to nilotinib, which resulted in DILI resolution. Serious DILI-related adverse events such as jaundice and chronic hepatitis have been reported[45].

The BYOND trial explored the benefits of 500 mg once daily of bosutinib for CML individuals (n = 163 subjects) who exhibited resistance and/or intolerance to other TKIs. DILI manifested only as elevations in AST (all grades n = 32, 19.6%) and/or ALT concentrations (all grades n = 42, 25.8%). However, grade 3/4 increases in ALT (n = 23, 14.1%) and/or AST (n = 7; 4.3%) were not common[46].

In the clinical trial which evaluated the benefits of bosutinib prescription in 119 CML subjects who failed to achieve satisfactory responses to imatinib and dasatinib and/or nilotinib, increases in AST/ALT values were noted in 16% of cases (13% classified as therapy-related), with only 6% of grade 3 adverse events and none severe/grade 4 side effects being noted. Elevations in these biochemical markers manifested early after drug initiation (approximately 81 d) and lasted approximately 29 d. DILI was successfully managed with dose interruptions, reductions, and/or use of hepatoprotective agents in 6, 5, and 1 CML case(s), respectively. Grade 3/4 DILI was more likely in CML individuals who received imatinib in the first six months following CML diagnosis and in subjects who exhibited elevated basophil counts[47].

Data from the CML registry in Belgium suggests that ponatinib-induced DILI cases are rare. Liver toxicity was uncommon in the 33 CML patients who received ponatinib and occurred in < 10% of treated individuals. Hepatocellular injury, hepatitis, and cholestasis were noted in 1 case each[48].

The PEARL study evaluated the safety and efficacy of ponatinib in CML subjects who experienced failure of 2 or more TKIs. Grade 1/2 non-hematological adverse events (including DILI) were highlighted in 19 (40%) of the 48 CML individuals enrolled. The investigators noticed no liver-related grade 3/4 adverse events[49].

Asciminib is a recently introduced TKI for managing CML, including T315I-mutated cases. This pharmacological agent inhibits the BCR-ABL1 protein in an allosteric manner, leading to an inactive conformation of its target. In a phase 1 trial of asciminib in heavily pretreated CML individuals, this novel medication led to elevation in ALT (n = 16, 10.7%; grade 3/4, n = 4, 2.7%), AST (n = 15, 10%; grade 3/4, n = 3, 2%) and gamma-glutamyltransferase (n = 12, 8%; grade 3/4, n = 3, 2%) concentrations, however, grade 3/4 liver-related adverse events occurred in < 3% of asciminib-treated subjects[50].

In the STAMP trial that investigated 40 mg of asciminib twice daily *vs* 500 mg of bosutinib once daily in individuals diagnosed with CML with ≥ 2 previous TKI therapies, DILI was more frequently noticed in the bosutinib group, *i.e.*, 27.6% (n = 21) and 21.1% (n = 16) of subjects experienced elevations in ALT and AST concentrations, respectively, *vs* 3.8% (n = 6) each in the asciminib group. Grade 3/4 adverse effects were more likely to present in bosutinib-prescribed subjects (14.5%, n = 11 for ALT; 6.6% n = 5 for AST) *vs* asciminib-treated subjects (3.8%, n = 6 for ALT and AST each). Significant elevations in ALT values necessitated treatment cessation in bosutinib-treated CML patients[51].

Ruxolitinib appears to be a safe option in terms of liver toxicity. Based on the findings of a double-blind, placebo-controlled trial assessing ruxolitinib in the management of MF classified as intermediate-2 or high-risk, DILI was not mentioned amongst the most common adverse side effects (experienced by at least 10% of the 155 enrolled subjects) caused by ruxolitinib[52].

Ruxolitinib was associated with an increase in ALT concentrations in around 6% of the MF patients and with high-grade elevations in only 1% of the individuals enrolled in the JUMP trial[53].

In the RuxoBeat trial, which investigated the benefits of ruxolitinib therapy in newly-diagnosed PV, 7 of 28 treated subjects (25%) experienced changes in biochemical markers, including DILI. However, only 3 cases of grade 3 adverse events were reported, out of which 2 consisted of elevations in AST/ALT concentrations and required reduction of the prescribed dose. However, no patient necessitated interruption of ruxolitinib[54].

Similarly, the assessment results conducted by Vannucchi *et al*[55] only indicate minor changes in ALT/AST values following ruxolitinib administration in individuals diagnosed with PV.

The EXPAND trial assessed the safety and efficacy of ruxolitinib in individuals with MF and thrombocyte counts between 50000/mmc and 100000/mmc. In this investigation, a total of 5 cases of elevations in bilirubin values were noticed: 1 of the 18 patients who had platelets < 75000/mmc but > 50000/mmc and 4 of the 20 patients who had platelets < 100000/mmc but > 75000/mmc. However, of these, only two grade 3 or more increases in bilirubin levels were highlighted, all were reported in patients with thrombocyte counts < 100000/mmc but > 75000/mmc[56].

In a clinical trial that explored the co-administration of ruxolitinib and interferon alpha-2 for the management of PV and MF, grade 1/2 liver-related adverse events, *i.e.*, an increase in AST and lactate dehydrogenase values, were noted in 7 (14%) and 17 (34%) of the 50 patients receiving therapy, with no cases of grade 3-4 side effects occurring in neither subjects with PV nor MF[57].

The JAKARTA trial compared fedratinib *vs* placebo elevations of liver enzymes were detected in 40%-60% of the subjects; however, ≥ 3 grade elevations in these serum markers were not common. In JAKARTA, 96 subjects were assigned to receive 400 mg of fedratinib daily, 97 to receive 500 mg/day of the same drug, and 95 individuals received placebo pills. All grade elevations in ALT (fedratinib 400 mg: 53%; fedratinib 500 mg: 46%; placebo: 17%) and AST (fedratinib 400 mg: 60%; fedratinib 500 mg: 48%; placebo: 29%) concentrations were frequently detected in the fedratinib subgroups *vs* placebo, whereas bilirubin levels were more likely to increase in the placebo group (fedratinib 400 mg: 31%; fedratinib 500 mg: 28%; placebo: 40%)[58]. However, grade 3-4 increases in ALT values were only noted in 3% of the fedratinib 400 mg and 500 mg subgroups each *vs* 0% in the placebo group. Grade 3-4 increases in AST values were only noted in 2% of the fedratinib 400 mg and 500 mg subgroups each *vs* 1% in the placebo group, whereas grade 3-4 elevations in bilirubin concentrations occurred in 2% of the placebo group and fedratinib 400 mg subgroups and in 1% of the fedratinib 500 mg subgroup, respectively[58].

Momelotinib therapy in MF was also associated with cases of liver toxicity, *i.e.*, grade 3-4 increases in ALT concentrations in 4% of patients and in AST and ALP concentrations in 2% of patients each[59]. Grade 1-2 increases in AST (21%), ALT (19%), and bilirubin (13%) concentrations were also detected[59]. In an integrated assessment of momelotinib based on data derived from phase 3 randomized clinical trials, Verstovsek *et al*[60] also highlighted that of the 725 individuals with MF who received the drug, nine subjects (1.2%) experienced notable elevations in ALT values requiring dose interruption/reduction or momelotinib discontinuation. When momelotinib was studied for PV and/or ET, no occurrences of liver damage were reported in either cohort[61].

Ropeg interferons are relatively safer drugs than previously used interferons with respect to liver toxicity. When peg-IFNα-2a was tested in the management of PV (n = 40) and ET (n = 39), grade 3 increases in liver function tests were reported in 5% (n = 2) and 8% (n = 3) of the PV and ET subgroups, respectively. No grade 4 side effects were reported [62]. In a trial exploring the safety and benefits of peg-IFNα-2b in PV and ET, elevations in liver enzymes were among the most frequent non-hematological side effects. In total, 2 subjects required peg-IFNα-2b discontinuation due to elevations in AST and ALT values[63].

DILI IN MPNS

Data on risk factors for DILI in patients living with MPNs are scarce. However, several studies have identified potential risk factors associated with an increased risk of imatinib/TKI-induced hepatotoxicity, namely[64-66]: (1) Use of proton pump inhibitors (3.8- fold increased risk): Imatinib is both a substrate and an inhibitor of the ABCG2 which is a drug efflux pump expressed on various body tissues, including the liver. Thus, the inhibition of this pump leads to increased drug concentrations in liver cells, thus increasing the risk of hepatotoxicity. Moreover, proton pump inhibitors are hepatotoxic on their own; (2) Presence of liver disease or HBV carrier state (8-fold elevated risk): Imatinib is metabolized by the liver; therefore, liver impairment or HBV carrier state may increase its plasma levels due to ineffective drug metabolism; (3) Drug dose > 400 mg (2.3-fold increased risk): Higher plasma levels of imatinib can enhance the risk of liver toxicity; (4) Body weight of < 55 kg (2.2-fold increased risk): The dose of imatinib is chosen based on the phase of the disease and not based on body surface area; (5) Concomitant use of acetaminophen: Acetaminophen itself is hepatotoxic; (6) Use of alcohol: Alcohol acts as a cytochrome oxidase enzyme inducer, thereby increasing the levels of toxic metabolites; and (7) Use of hepatotoxic drugs.

The relative risk of DILI seems higher with the prescription of 2nd & 3rd generation TKIs compared to 1st generation pharmacological agents (imatinib)[67]. The average duration from drug initiation to DILI development with TKIs was 2-6 mo, whereas with the use of ruxolitinib, it was 1-6 mo[68,69]. In most cases, the diagnosis of DILI was established after the virology panel results for hepatitis B, human immunodeficiency virus, cytomegalovirus and Epstein-Barr virus

infections came in negative. Autoimmune antibody testing, abdominal ultrasound, liver biopsy, and toxicology screening (alcohol, illicit drugs, acetaminophen) were also performed in conjunction with liver function tests to exclude other causes of liver injury[70,71]. Liver biopsy is usually not preferred to provide diagnostic information regarding liver injury but is considered for the staging of fibrosis[72]. In some instances, genetic testing for hereditary conditions, such as hemochromatosis or Wilson's disease, maybe required[73]. Pharmacogenomics assessments for mutations/polymorphisms in human leukocyte antigen genes, drug-metabolizing enzymes, ATP-binding cassette and/or solute carrier transporters, may also be required to understand why certain individuals develop DILI[74].

However, investigations on DILI in subjects with MPNs remain scarce and the pathogenesis of DILI induced by targeted anticancer agents warrants further consideration in future studies. For example, researchers could investigate the impact of oxidative stress, immunity, and bile acid metabolism on the emergence of DILI in individuals with MPNs. As such, DILI still remains a diagnosis of exclusion, with the recommended biochemical criteria being as follows[72,73]: (1) ALT values ≥ 5 ULN; (2) AST values ≥ 3 ULN; (3) ALP values ≥ 2 ULN; and (4) total bilirubin ≥ 2 ULN.

A common tool used for the diagnosis of DILI is the Roussel Uclaf Causality Assessment Method. It is based on 7 factors, including the onset of reaction, clinical response after withdrawal or continuation of the drug, response to re-administration of the drug, temporal relationship, risk factors, concomitant drug use, and absence of any non-drug etiologies[75].

Management of DILI in MPNs

In most cases, discontinuation of the offending drug in individuals with clinically established hepatotoxicity and/or administration of hepatoprotective agents has been found to normalize liver enzymes within a few weeks. In cases of severe liver injury, resolution has been achieved by treatment with high-dose steroids for a few weeks. For example, imatinib therapy can be resumed with dose reductions or with co-administration of low doses of steroids if hepatotoxicity occurs[76]. The European LeukemiaNet has established guidelines for the management of TKI-induced liver injury[77] (Figure 1).

Since CML patients often require lifelong treatment, Lopina *et al*[78] have suggested a novel score-based decision algorithm (Table 2) for restarting TKIs after acute imatinib-induced liver injury and for the choice of 2nd generation TKIs. The score takes into account: (1) The grade of hepatotoxic reaction; (2) the grade of response to the use of imatinib, *i.e.*, the presence of early molecular response (EMR) to imatinib therapy at 3 mo (3-month BCR-ABL1 ≤ 10% according to the international scale). This is not applicable if imatinib treatment was prescribed for < 3 mo; (3) the grade of response to the use of imatinib, *i.e.*, the presence of EMR to imatinib therapy at 6 mo (6-month BCR-ABL1 < 1% according to the international scale). This is not applicable if hepatotoxicity developed in <6 mo of imatinib therapy; (4) the presence of a liver offender (concomitant use of another drug that probably caused drug interactions); and (5) the presence of viral hepatitis reactivation identified by polymerase chain reaction.

The approach to restart imatinib can be based on the score listed above[78] as follows: (1) Score = 0 points: Withdraw the drug and switch to 2nd generation TKIs; (2) Score = 1 point: It is preferred to withdraw imatinib if the patient requires treatment for > 6 mo; and (3) Score ≥ 2 points: Restart imatinib after resolution of DILI.

The choice of a 2nd generation TKI is based on the presence of comorbidities and/or BCR-ABL1 kinase domain mutations[78].

Other cases of DILI in CML patients require special consideration. For example, reactivation of hepatitis B infection often undergoes spontaneous resolution but treatment with antiviral agents (tenofovir and entecavir) is sometimes needed. Moreover, liver transplantation can be successful in imatinib-induced fulminant liver failure[70].

In patients who develop hepatotoxicity while on ruxolitinib, abrupt drug discontinuation should be avoided as it can lead to potentially fatal withdrawal symptoms. Therefore, dose reduction should be completed gradually. Tremblay *et al* [79] recommends liver biopsy for adaptive management in patients with evidence of hepatocellular damage potentially caused by the use of ruxolitinib.

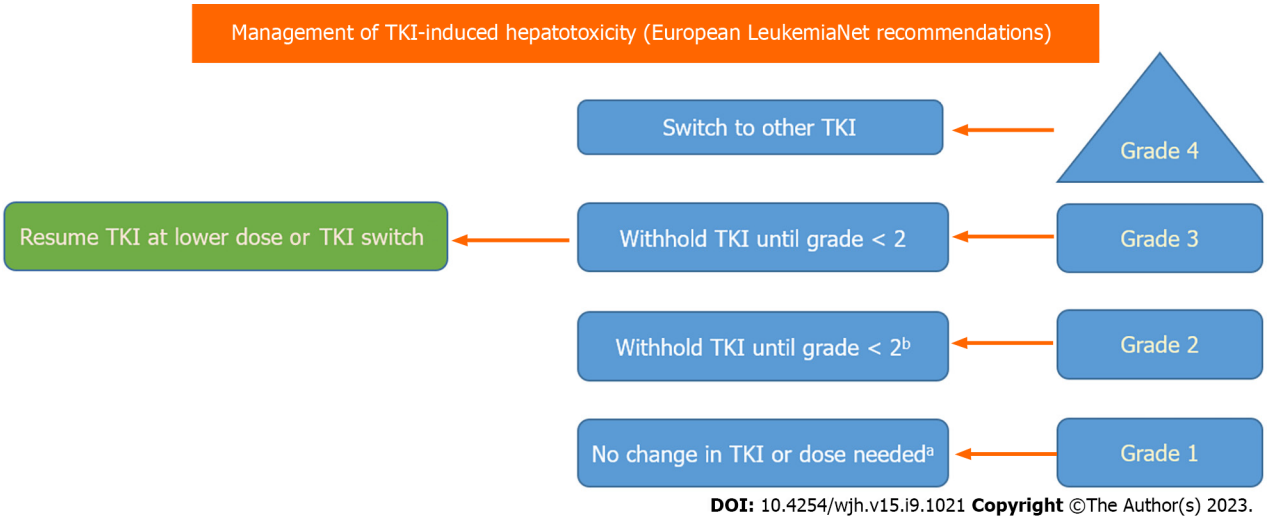
Prevention of DILI in MPNs

The relatively limited number of particular treatments and antidotes that are currently available restricts the medical therapy of acute DILI. The primary therapeutic strategy for DILI remains discontinuing the alleged harmful substance [80]. When NAC is administered within 4 to 16 h after an acute acetaminophen overdose, hepatotoxicity is effectively avoided. NAC is less helpful for ALF caused by non-acetaminophen drugs[81]. First-line prevention measures include avoiding the use of potentially hepatotoxic medications in patients with an underlying chronic liver disease or who have been identified as having a genetic, or other risk factors for developing DILI. Other measures include monitoring ALT, AST, and other liver-associated enzymes (ALP, bilirubin, *etc.*) to detect hepatotoxicity for particular medications early on. In some developed countries, regulating the availability of potentially dangerous amounts of acetaminophen has proven effective in reducing overdoses[82]. To prevent purposeful and inadvertent overdoses, improvements in abeling and patient education are still required in countries with unlimited access to acetaminophen. The significance of the gut microbiota in preventing DILI will likely continue to be understood, allowing for the development of new therapeutic strategies. Its ability to guard against acetaminophen-induced and other types of acute DILI is currently being investigated[83]. Thus, in patients diagnosed with MPNs who are started on potentially hepatotoxic agents, we recommend checking liver function tests before therapy initiation and regularly during treatment. Moreover, the management of each case should be tailored to the comorbidities and concurrent medications of the patient, especially in subjects who suffer from MPNs and exhibit a high burden of cardiometabolic disorders[84]. Thus, DILI can be avoided in some instances. Further research should focus on identifying new hepatoprotective agents that could enable clinicians to overcome DILI and avoid drug cessation or dose reductions/interruptions which aid in the resolution of liver toxicity but might impact the treatment of the hematological malignancy.

Table 2 Novel score for the decision of restarting or withdrawing imatinib in chronic myeloid leukemia[78]

Factors	Imatinib restart[1]	Imatinib withdrawal
Grade of hepatotoxic reaction		
Grade 1	+	-
Grade 2	+[2]	-
Grade 3	+/-[3]	+/-
Grade 4 or presence liver transplantation or imatinib-induced liver cirrhosis or viral hepatitis reactivation	-	+
Presence of EMR to imatinib at 3 mo = BCR-ABL1^{IS} ≤ 10%		
Yes	+	-
No	-	+
Presence of EMR to imatinib at 6 mo = BCR-ABL1^{IS} < 1% (if applicable[4])		
Yes	+	-
No	-	+
Use of another drug that might cause liver toxicity		
Yes	+	-
No	-	+
Diagnosis of viral hepatitis established by PCR		
Yes	+	-
No	-	+

[1]Decide whether to restart imatinib only after resolution of acute hepatitis and normalization of liver function tests.
[2]Restart imatinib at a reduced or at the same dose.
[3]Restart imatinib if liver toxicity resolves in ≤ 1 month and there is no sign of recurrence.
[4]Do not take into consideration this factor if liver toxicity develops ≤ 6 months after imatinib initiation.
+: 1 point/yes; -: 0 points/no; ABL: Abelson Murine Leukemia; BCR: Breakpoint Cluster Region; EMR: Early molecular response; PCR: Polymerase chain reaction.



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Figure 1 Management of tyrosine kinase inhibitor-induced hepatotoxicity according to European LeukemiaNet recommendations. [a]Adverse events may require specific treatment; [b]Alternatively, continue TKI for 1 wk with appropriate management of adverse event. If no resolution is achieved, withhold TKI until liver toxicity is grade < 2 and monitor weekly. TKI: Tyrosine kinase inhibitor.

CONCLUSION

Data on liver toxicity induced by targeted anticancer therapy in MPNs is scarce; however, the use of TKIs has been linked to hepatotoxicity and/or DILI in CML, PV, ET, and MF in clinical trials and real-world data. Minor liver injury can be managed with drug discontinuation and/or dose reductions/interruptions and the administration of hepatoprotective agents. Careful consideration must be given in cases of severe hepatotoxicity.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Liver transplant in patients with primary sclerosing cholangitis: A retrospective cohort from Northeastern Brazil

Louyse Teixeira de Souza Freitas, Elodie Bomfim Hyppolito, Victor Leonardo Barreto, Luiz Humberto Jataí Castelo Júnior, Bianca Carneiro de Melo Jorge, Frederico César Tahim de Sousa Brasil Háteras, Malthus Barbosa Marzola, Clébia Azevedo Lima, Raquel Mendes Celedonio, Gustavo Rêgo Coelho, Jose Huygens Parente Garcia

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Abstract

BACKGROUND

Primary sclerosing cholangitis (PSC) manifests within a broad ethnic and racial spectrum, reflecting different levels of access to health care.

AIM

To evaluate the clinical profile, complications and survival rates of patients with PSC undergoing liver transplantation (LTx) at a Brazilian reference center.

METHODS

All patients diagnosed with PSC before or after LTx were included. The medical records were reviewed for demographic and clinical variables, including outcomes and survival. The level of statistical significance was set at $P < 0.05$.

RESULTS

Our cohort represented 1.6% (n = 34) of the 2113 patients receiving liver grafts at our service over the past two decades. Most were male (n = 19; 56%). The average age (40 ± 14 years) was similar for men and women (P = 0.347). The mean follow-up time from diagnosis to LTx was 68 mo. Most patients had the classic form of PSC. Three women had PSC/autoimmune hepatitis overlap syndrome, and one patient had small-duct PSC. Alkaline phosphatase levels at diagnosis and pre-LTx model for end-stage liver disease. scores were significantly higher in males. Inflammatory bowel research (IBD) was investigated by colonoscopy in 26/34 (76%) and was present in most cases (18/26; 69%). IBD was less common in women than in men (44.4% *vs*. 55.6%) (P = 0.692). Cholangiocarcinoma (CCA) was diagnosed in 2/34 (5.9%) patients by histopathology of the explant (survival: 3 years 6 mo, and 4 years 11 mo). Two patients had complications requiring a second LTx (one after 7 d due to hepatic artery thrombosis and one after 17 d due to primary graft dysfunction). Five patients (14.7%) developed biliary stricture. The overall median post-LTx survival was 66 mo. Most deaths occurred in the first year (infection n = 2, primary liver graft dysfunction n = 3, unknown cause n = 1). The 1-year and 5-year survival rates of this cohort were 82.3% and 70.6%, respectively, matching the mean overall survival rates of LTx patients at our center (87.1% and 69.43%, respectively) (P = 0.83).

CONCLUSION

Survival after 1 and 5 years was similar to that of other LTx indications. The observed CCA survival rate suggests CCA may be an indication for LTx in selected cases.

Key Words: Primary sclerosing cholangitis; Epidemiology; Liver transplantation; Survivor; Clinical associations; Pathological features

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Core Tip: We present a case series of liver transplantation (LTx) patients from the largest center in Northeastern Brazil, with epidemiological features different from what is expected for primary sclerosing cholangitis (PSC) (*e.g.*, early manifestation and proportion of female patients). The finding of two cases of cholangiocarcinoma (CCA) with good survival is relevant to the discussion on the eligibility of selected cases of CCA for LTx. The survival of PSC patients was similar to that of LTx patients with other etiologies.

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic, progressive autoimmune disease causing inflation, stenosis and dilation of the intra- and extrahepatic bile ducts[1]. Clinically characterized mainly by fatigue and pruritus[2], PSC may lead to cholangiocarcinoma (CCA) and cirrhosis. Around 70% of PSC patients have inflammatory bowel disease (IBD), especially ulcerative colitis, with elevated risk of colorectal cancer[3]. Currently available clinical treatments do not alter the natural history of PSC, and liver transplantation (LTx) is the only curative treatment available[4], although some studies have reported a post-LTx relapse rate of as much as 25%[5]. Intractable pruritus, recurrent cholangitis, hepatocarcinoma and decompensated cirrhosis are some of the classic indications for LTx[1], but the ideal moment for transplantation can be difficult to determine. The purpose of this study was to evaluate the clinical profile, complications and survival rates of PSC patients submitted to LTx at a Brazilian referral center.

MATERIALS AND METHODS

In this retrospective observational cohort study, we included all LTx patients diagnosed with PSC before or after transplantation. The diagnosis was based on clinical and laboratory findings confirmed by magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiopancreatography (ERCP). Between May 2012 and May 2022, the LTx team at our service (Hospital Universitário Walter Cantídio, Federal University of Ceará, partnered with Hospital São Carlos) performed 2113 procedures; 34 of which (1.6%) were due to PSC. The study variables were age, sex, clinical manifestations, association with IBD and other comorbidities, time between diagnosis of PSC and LTx, cause of

LTx, PSC classification, laboratory findings, treatments and complications prior to LTx, time of ischemia, Child–Pugh and model for end-stage liver disease (MELD) scores, immunosuppression, PSC relapse following LTx, rejection, and death.

Histopathological diagnosis of small-duct PSC was considered when liver biopsy was performed prior to LTx or in the explant biopsy. Small-duct PSC was defined as cholestasis associated with a compatible liver biopsy, in the absence of biliary stricture on ERCP or MRCP. Autoimmune hepatitis (AIH) was diagnosed using the International AIH Group Score[6].

PSC relapse was defined as the presence on ERCP or MRCP of biliary stricture post-LTx at a site other than the anastomosis. The follow-up time was defined as the time of outpatient follow-up until the moment of inclusion in the study, or death.

The study protocol was approved by the Research Ethics Committee of the Federal University of Ceará and filed under #CAAE 98627218.6.2018.5045.

The level of statistical significance was set at 5% ($P < 0.05$). Non-normally distributed data were analyzed with the Mann–Whitney U test, while the χ^2 test and Fisher's exact test were used for categorical variables. Cumulative survival rates at the 95% confidence interval were estimated with Kaplan–Meier survival analysis.

RESULTS

Male sex was slightly predominant (n = 19; 56%). The average age was 40 ± 14 years, with no significant difference between men (38 ± 14 years) and women (43 ± 13 years) (P = 0.347). The mean MELD score was 24.1 ± 4.7 for men and 19.9 ± 8.1 for women (P = 0.011). The average time from onset of symptoms to diagnosis was 23 mo (range: 0–128 mo). The mean follow-up time from diagnosis to LTx was 68 mo (range: 0–196 mo). Classic PSC was the most frequently observed clinical form. Three women had AIH–PSC overlap syndrome, and one patient had small-duct PSC. All patients were symptomatic at diagnosis (Table 1).

Nearly all patients (n = 27; 93%) were treated with ursodeoxycholic acid (UDCA) and half (n = 14; 52%) used prednisone. All users of prednisone had overlap with AIH, with a predominance of the female sex (75%; P = 0.031). Endoscopic treatment was administered significantly more often to men (88%) than to women (12%) (P = 0.010). Alkaline phosphatase levels at diagnosis and pre-LTx MELD scores were significantly higher in males. The baseline and pre-LTx laboratory findings are shown in Table 2.

IBD was investigated by colonoscopy in 26 (76%) of 34 patients, and was present in most cases (18/26; 69%). The development of IBD was less common in women (44.4%) than in men (55.6%) (P = 0.692).

The mean age of PSC patients at the time of IBD diagnosis was 35 ± 14 years (median: 32 years). PSC and IBD were diagnosed simultaneously in two (11%) patients. PSC was diagnosed before IBD (range: 1–6.8 years; median: 3 years) in 6/18 (33%), and after IBD (range: 0.5–32 years; median 9.8 years) in 10/18 (56%). Patients without IBD (MELD: 24.6 ± 5.3) were significantly more severe at the time of LTx than patients with some form of IBD (19.3 ± 4.7) (P = 0.033). Table 3 shows the patients' clinical variables according to the presence/absence of IBD.

Diabetes mellitus (DM) was the most frequent comorbidity (n = 7; 22%), followed by systemic arterial hypertension and alcoholism (n = 6; 19%), dyslipidemia and smoking (n = 4; 12%), obesity (n = 1; 3.1%) and others (n = 3; 9%). DM was more frequent in patients without IBD (n = 4; 80%) than in patients with IBD (n = 1; 20%) (P = 0.030). Although frequently associated with PSC, ankylosing spondylitis and seronegative arthritis were not observed in this series. Information on densitometry was available for only four (12.5%) patients, although seven (21%) patients were undergoing treatment for osteoporosis.

Two techniques were used for bile duct reconstruction: end-to-end anastomosis (65%) and Roux-en-Y hepaticojejunostomy (35%). The former was preferred in patients with macroscopically normal common bile ducts.

CCA was diagnosed in two (5.9%) of 34 patients upon the histopathological examination of the explant, with the following characteristics.

Case 1: 47-year old man. Explant with nodule measuring 3.0 cm 2.5 cm 2.5 cm, with periductal and neural involvement, involvement of the liver hilum and intrahepatic bile ducts, vascular invasion and compromised margins (pT2bN2). The patient was peremptorily treated with capecitabine for 6 mo after LTx, but after 2 years and 4 mo experienced a recurrence of the neoplasm in the inferior vena cava, pancreas and lung. At this point, immunosuppression was reduced and 10 sessions of systemic chemotherapy with gemcitabine/cisplatin were administered but without response. Following that, the liver hilum and chest were submitted to radiotherapy. After 3 years and 6 mo, the patient presented neoplastic obstruction of the biliary tract for which a metallic prosthesis was inserted. The patient continues to use oral capecitabine and presents an excellent overall condition and quality of life, despite the relapse, with a survival of 4 years and 11 mo.

Case 2: 40-year old man. Intraoperative diagnosis of nodule, later confirmed in the explant to be an adenocarcinoma with biliary pattern measuring 2.8 cm 2.5 cm, with infiltration of the liver parenchyma, lymphovascular and perineural invasion, and compromised margins (pT2bN2). After 1 year, the patient experienced a recurrence of the neoplasm in the hepatic artery and lung. Chemotherapy with capecitabine for 6 mo and local radiotherapy were administered. The patient developed biliary obstruction for which a metallic prosthesis was inserted. Currently, the patient is clinically well, with a survival of 3 years and 10 mo.

As for complications of LTx, two patients required a second transplant, one after 7 d due to hepatic artery thrombosis and one after 17 d due to primary graft dysfunction. Five (14.7%) patients developed biliary stricture (end-to-end, n = 3; Roux-en-Y, n = 2), treated with ERCP and percutaneous drainage, respectively. Two patients had post-LTx relapse of PSC, with the appearance of intrahepatic biliary stricture confirmed on MRCP at 11 years and 7 mo (survival: 14 years

Table 1 Clinical and socioepidemiological variables of patients with primary sclerosing cholangitis submitted to liver transplantation between 2012 and 2022

Variables	*n*	Total[1]	Females (*n* = 14[1])	Males (*n* = 19[1])	*P* value[2]
Age at LTx, yr	32	40 ± 14 (36)	43 ± 13 (39)	38 ± 14 (35)	0.347
Age at first symptom, yr	30	32 ± 14 (30)	35 ± 13 (36)	30 ± 14 (29)	0.498
Age at IBD diagnosis, yr	17	35 ± 14 (32)	37 ± 18 (42)	33 ± 12 (30)	0.370
Months between 1st symptom and 1st consultation	26	18 ± 37 (2)	16 ± 33 (3)	19 ± 39 (2)	0.616
Months between onset of symptoms and diagnosis	31	80 ± 235 (23)	148 ± 357 (56)	31 ± 41 (13)	0.155
Baseline clinical symptoms					
Jaundice	32	29 (91%)	11 (85%)	18 (95%)	0.552
Pruritus	32	25 (74%)	6 (24%)	19 (76%)	
Fever + shivering	32	14 (44%)	5 (38%)	9 (47%)	0.618
Weight loss	32	18 (56%)	9 (69%)	9 (47%)	0.221
Fatigue	32	13 (41%)	6 (46%)	7 (37%)	0.598
PSC classification					
Classic PSC	31	30 (97%)	12 (92%)	18 (100%)	0.419
PSC + AIH	31	3 (9.7%)	3 (23%)	0 (0%)	0.064
Small-duct PSC	31	1 (3.2%)	0 (0%)	1 (5.6%)	> 0.999
Diagnostic testing					
MRCP realized	32	23 (72%)	10 (77%)	13 (68%)	0.704
MRCP	32	20 (62%)	10 (77%)	10 (53%)	0.163
ERPC	32	12 (38%)	3 (23%)	9 (47%)	0.163
Biopsy	31	20 (65%)	7 (54%)	13 (72%)	0.449
Comorbidities					
Diabetes	32	7 (22%)	2 (15%)	5 (26%)	0.671
Hypertension	32	6 (19%)	4 (31%)	2 (11%)	0.194
Dyslipidemia	32	4 (12%)	2 (15%)	2 (11%)	> 0.999
Obesity	32	1 (3.1%)	0 (0%)	1 (5.3%)	> 0.999
Smoking	32	4 (12%)	2 (15%)	2 (11%)	> 0.999
Drinking	32	6 (19%)	2 (15%)	4 (21%)	> 0.999
Others	31	8 (26%)	2 (17%)	6 (32%)	0.433
IBD	18				> 0.999
Ulcerative rectocolitis		15 (83%)	7 (88%)	8 (80%)	
Crohn's disease		3 (17%)	1 (12%)	2 (20%)	
Ankylosing spondylitis	32	0 (0%)	0 (0%)	0 (0%)	
Seronegative arthritis	32	0 (0%)	0 (0%)	0 (0%)	
Gallbladder calculus	32	7 (22%)	3 (23%)	4 (21%)	> 0.999
Gallbladder polyps	31	0 (0%)	0 (0%)	0 (0%)	
Neoplasia	31	4 (13%)	2 (15%)	2 (11%)	> 0.999
Dyslipidemia	32	4 (12%)	2 (15%)	2 (11%)	> 0.999
Obesity	32	1 (3.1%)	0 (0%)	1 (5.3%)	> 0.999
Smoking	32	4 (12%)	2 (15%)	2 (11%)	> 0.999

Drinking	32	6 (19%)	2 (15%)	4 (21%)	> 0.999
Other	31	8 (26%)	2 (17%)	6 (32%)	0.433
Treatment					
Ursodeoxycholic acid	29	27 (93%)	11 (92%)	16 (94%)	0.665
Prednisone	27	14 (52%)	9 (75%)	5 (33%)	0.031
Endoscopic treatment	16	8 (50%)	1 (12%)	7 (88%)	0.010
Indication for LTx					> 0.999
Untreatable pruritus		4 (12%)	2 (15%)	2 (11%)	
Decompensated cirrhosis		27 (84%)	11 (85%)	16 (84%)	
Hepatocellular carcinoma		1 (3.1%)	0 (0%)	1 (5.3%)	
Dominant stenosis	26	4 (15%)	1 (9.1%)	3 (20%)	0.614

[1]Range; mean ± SD (median); *n* (%).
[2]Wilcoxon rank sum exact test; Wilcoxon rank sum test; Fisher's exact test; Pearson's χ^2 test.
AIH: Autoimmune hepatitis; ERCP: Endoscopic retrograde cholangiopancreatography; IBD: Inflammatory bowel disease; LTx: Liver transplantation; PSC: Primary sclerosing cholangitis; MRCP: Magnetic resonance cholangiopancreatography.

Table 2 Baseline and pretransplantation laboratory findings of patients with primary sclerosing cholangitis

Variables	*n*	Total[1]	Females (*n* = 14[1])	Males (*n* = 19[1])	*P* value[2]
ALP/RV1c	19	3.72 ± 3.02 (2.86)	2.23 ± 1.58 (1.89)	4.60 ± 3.37 (3.35)	0.045
GGT/RV1c	19	10 ± 9 (5)	5 ± 4 (4)	12 ± 9 (10)	0.210
AST/RV1c	23	5.86 ± 11.29 (3.00)	10.63 ± 18.86 (3.30)	3.31 ± 1.53 (2.86)	0.591
ALT/RV1c	23	3.28 ± 3.19 (2.46)	3.80 ± 5.18 (1.93)	3.01 ± 1.53 (2.75)	0.302
DB	24	7.4 ± 5.3 (5.9)	8.9 ± 5.2 (7.3)	6.8 ± 5.4 (5.9)	0.383
Antibody testing					
ANA	20	3 (15%)	1 (17%)	2 (14%)	> 0.999
AASM	19	2 (11%)	1 (17%)	1 (7.7%)	> 0.999
AMA	19	0 (0%)	0 (0%)	0 (0%)	
ANTI-SLA	11	0 (0%)	0 (0%)	0 (0%)	
pANCA	12	4 (33%)	1 (33%)	3 (33%)	> 0.999
Pre-LTx lab results					
ALT/RV	22	5.5 ± 8.8 (3.0)	6.2 ± 12.6 (2.1)	4.9 ± 4.1 (4.0)	0.138
AST/RV	23	16 ± 44 (5)	24 ± 66	9 ± 14 (5)	0.107
ALP/RV	22	2.79 ± 2.24 (1.84)	2.39 ± 2.37 (1.64)	3.06 ± 2.20 (2.54)	0.393
TB	22	15 ± 10 (11)	13 ± 10 (10)	17 ± 10 (12)	0.324
INR	27	2.08 ± 2.34 (1.51)	1.40 ± 0.32 (1.37)	2.54 ± 2.98 (1.71)	0.025
Creatinine	28	0.89 ± 0.80 (0.75)	0.94 ± 1.19 (0.52)	0.86 ± 0.34 (0.83)	0.143
MELD	27	22.4 ± 6.5 (22.0)	19.9 ± 8.1 (19.0)	24.1 ± 4.7 (23.5)	0.011

[1]Range; mean ± SD (median); *n* (%).
[2]Wilcoxon rank sum exact test; Wilcoxon rank sum test; Fisher's exact test; Pearson's chi-squared test.
AASM: Anti-smooth muscle antibodies; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AMA: Anti-mitochondrial antibody; ANA: Anti-nuclear antibodies; Anti-SLA: Anti-soluble liver antigen; AST: Aspartate transferase; DB: Direct bilirubin; GGT: γ-Glutamyl transferase; INR: International normalized ratio; MELD: Model for end-stage liver disease; pANCA: Perinuclear anti-neutrophil cytoplasmic antibody; RV: Reference value; TB: Total bilirubin; LTx: Liver transplantation.

Table 3 Clinical variables of patients with primary sclerosing cholangitis according to the presence/absence of inflammatory bowel disease

	Total[1]	IBD[1] yes	IBD[1] no	*P* value[2]
Total	26	18 (69.2%)	9 (30.8%)	0.440
Sex				0.692
Male	16 (59%)	6 (67%)	10 (56%)	
Female	11 (41%)	3 (33%)	8 (44%)	
Age	40 ± 13 (36)	38 ± 15 (34)	35 ± 14 (32)	
Ulcerative colitis	15 (83%)			
Crohn's disease	3 (17%)			
AST/RV1c	6.1 ± 12.1 (2.9)	12.2 ± 19.8 (3.7)	2.8 ± 1.6 (2.6)	0.014
DM	5 (19%)	4 (44%)	1 (5.6%)	0.030
Esophageal varices				0.027
No	9 (36%)	0 (0%)	9 (50%)	
Yes	16 (64%)	7 (100%)	9 (50%)	
MELD	21.3 ± 5.5 (22.0)	24.6 ± 5.4 (23.0)	19.3 ± 4.7 (19.0)	0.033
Anastomosis				
Roux-en-Y	8 (31%)	0 (0%)	8 (47%)	0.023
End-to-end	18 (69%)	9 (100%)	9 (53%)	

[1]Range; mean ± SD (median); *n* (%).
[2]Wilcoxon rank sum exact test; Wilcoxon rank sum test; Fisher's exact test; Pearson's χ^2 test.
AST: Aspartate transferase; DM: Diabetes mellitus; MELD: Model for end-stage liver disease; RV: reference value.

and 2 mo) and at 12 years and 6 mo (survival: 18 years).

The overall median post-LTx survival was 66 mo (range: 0–234 mo), with no significant difference between the sexes ($P = 0.282$). Ten deaths occurred, most of which in the first year (infection, $n = 2$; primary liver graft dysfunction, $n = 3$; unknown cause, $n = 1$). Three patients died with coronavirus disease 2019 after 4, 6 and 10 years, respectively, and one patient died of infection 1 year and 7 mo after LTx.

The 1-year and 5-year survival rates of our cohort were 82.3% and 70.6%, respectively. This is compatible with the average overall survival rates of LTx patients at our institution (87.1% and 69.43% respectively) ($P = 0.83$) (Figure 1).

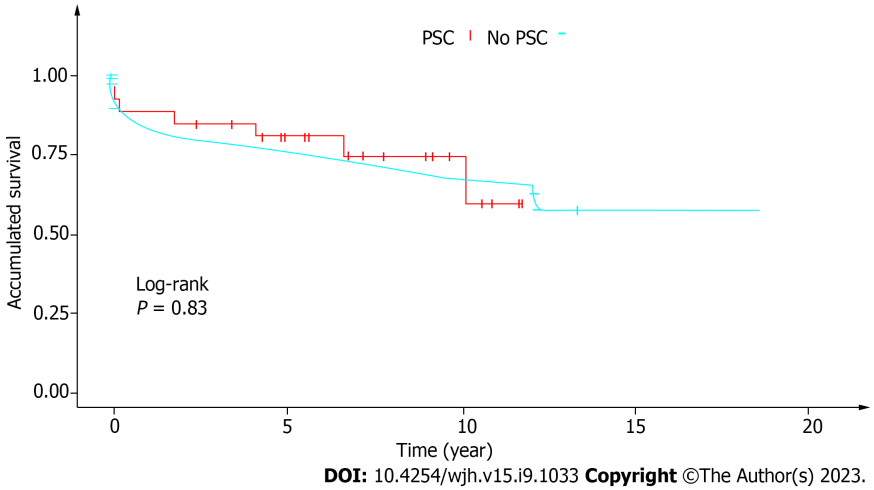
DISCUSSION

PSC represented only 1.6% of all LTx patients in our study, compared with, for example, 15.3% in Nordic countries[6]. The balanced sex distribution in our cohort also differed from that in the international literature, which shows a male predominance (up to 2:1)[7], while matching the proportion observed in a Brazilian multicenter study, in which 45% of the patients were female[8].

The prevalence of classic PSC in our Brazilian cohort matched that of studies from Europe, North America and Australia[9]. The average age of our patients at diagnosis (33 years; range 11–61 years) was similar to that of a Latin American study (29 years; range 19–40 years), but lower than that of a British study (54 years; range 6–93 years)[3,8]. The mean time from the onset of symptoms to diagnosis of PSC was almost twice as long as that in a Swedish study (16 mo) [10].

Elevated serum alkaline phosphatase and γ-glutamyl transferase levels are typical fin PSC patients, but we also observed aspartate aminotransferase and alanine aminotransferase levels on average five and three times above the normal range at the time of diagnosis[11]. According to Williamson and Chapman[12], serum bilirubin levels tend to be normal at disease onset and occasionally fluctuate during the course of the disease. In our cohort, the median bilirubin level was 8.72 mg/dL.

PSC is often associated with IBD[7,13]. PSC may manifest before, concomitantly with, or after the diagnosis of IBD[11]. IBD was observed in 76% of our patients; 67% of whom had concomitant PSC and IDB. The proportion of patients diagnosed with IBD before PSC was similar to that of other studies, as was the predominance of ulcerative rectocolitis[3]. In our cohort, biochemical changes were more pronounced in patients without IBD than in patients with IBD, as was liver disease severity, the occurrence of esophageal varices, and the prevalence of DM, possibly due to the concomitant use of corticoids to treat IBD.



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Figure 1 Kaplan–Meier survival curve of the general population and patients with primary sclerosing cholangitis. PSC: Primary sclerosing cholangitis.

Current evidence suggests PSC–IBD may be a condition altogether different from PSC alone, and some have argued that PSC may have a protective effect on the course of IBD[12,14], considering the invariably benign course of IBD, with mild or no clinical symptoms and possibly even normal endoscopic appearance observed in PSC patients with a subdiagnosis of IBD. However, concomitant ulcerative rectocolitis increases the risk of colorectal cancer[15].

The presence of a range of autoantibodies in the serum of PSC patients suggests autoimmunity plays a role in pathogenesis, but diagnostic testing for autoantibodies is of limited use due to low sensitivity and specificity[16]. A review on PSC found a high prevalence of p-anti-neutrophil cytoplasmic antibody (50%–80%), anti-nuclear antibody (7%–77%) and anti-mitochondrial autoantibodies (13%–20%)[17], but in our cohort, few patients were tested for antibodies and the prevalence was low.

Most of our patients (93%) were treated with UDCA at least until the time of LTx. UDCA is hepatoprotective in chronic cholestatic liver disease, but its efficacy in PSC has been questioned[18]. In a European study on treatment for PSC[19], 50% of physicians routinely prescribed UDCA for all patients, while 12% never prescribed it. The American Association for the Study of Liver Diseases and the British Society of Gastroenterology do not encourage the use of UDCA in PSC patients[20,21]. The 2009 guidelines of the European Association for the Study of the Liver state that "UDCA (15-20 mg/d) improves serum liver tests and surrogate markers of prognosis (I/B1), but does not reveal a proven benefit on survival (III/C2)"[22].

Lindor *et al*[23] (2009) conducted a double-blind randomized controlled trial on 150 adult PSC patients to evaluate prolonged use of high doses of UDCA (28–30 mg/kg/d). Liver tests did improve, but patients taking UDCA were at higher risk of severe adverse events and clinical outcomes such as cirrhosis, LTx, esophageal varices, CCA and death, when compared with patients receiving placebo. The drug is believed to modify the composition of the bile acids.

Wunsch *et al*[18] (2014) prospectively evaluated the withdrawal of UDCA over 3 mo in 26 PSC patients and found a significant increase in biochemical parameters, nonsignificant deterioration of quality of life in certain domains, and improvement of well-being in the social functioning domain and the mental component summary in SF-36.

Just over half the patients (52%) used prednisone. Immunosuppressants are rarely prescribed for PSC patients and are only indicated in cases of overlap[24].

According to Carey *et al*[4] (2015), up to one fourth of PSC patients submitted to LTx may experience recurrence. In this study, the only patient (3%) with recurrence had concomitant IBD. The association between PSC and IBD is well documented and may affect two thirds of PSC patients, especially when IBD is combined with ulcerative pancolitis[25].

According to Lopens *et al*[24] (2020), patients with concomitant PSC and IBD are at increased risk of liver disease, and the absence of IBD tends to improve the prognosis of PSC and lessen the risk of complications. In contrast, in our study, patients without IBD were not only significantly more severe at the time of LTx but also displayed greater biochemical changes in the early stages of the disease, when compared with patients with concomitant PSC and IBD.

In a large study from the Netherlands involving 3020 PSC patients, the mean time between diagnosis of PSC and indication for LTx was 27 years, compared to 9.7 years in our study[26].

A wide-ranging review by Song *et al*[27] has shown that the risk of CCA is 10 to 1000 times higher in patients with PSC than in the general population. The early diagnosis of CCA in two of our patients agrees with the literature, according to which CCA develops one year after LTx in 50% of cases[27].

In an epidemiological populational study evaluating the risk and malignancy of PSC in 590 patients, the time between diagnosis of PSC and the diagnosis of CCA was on average 6 years, and only 12% were diagnosed with PSC and CCA at the initial presentation. CCA was diagnosed in the first year in 15%, between the first and the tenth year in 37%, and > 10 years later in 37%. The cumulative risk of CCA after 10, 20 and 30 years was 6%, 14% and 20%, respectively[26].

CCA is a formal contraindication for LTx in Brazil. In our cohort, the rate of survival after early recurrence (2 patients) was better than the mean rate given in the literature, according to which the overall survival rate of intrahepatic CCA is 40.8% (39.8%–41.9%) at 1 year, and 9.8% (9%–10.5%) at 5 years[28]. Our 5-year post-LTx survival rate was higher than that

of a British study (75%)[3].

Some caveats apply to this retrospective study: (1) The medical records displayed differences in completeness; (2) PSC and IBD may have been under-reported; and (3) some laboratory findings were inadequately recorded in the database. To obtain the most reliable data possible, primary information was collected from the initial physical, laboratory and image records through active search, while incomplete information and doubts arising from the medical records were addressed by directly contacting the patients by phone.

CONCLUSION

PSC is a rare cause of LTx in our service. In our cohort, the proportion of women was larger than expected. Survival at 1 and 5 years was satisfactory and similar to other LTx indications. CCA findings in explants with good survival rates raise the hypothesis that CCA may be an acceptable indication for LTx in selected cases.

ARTICLE HIGHLIGHTS

Research background

Primary sclerosing cholangitis (PSC) is a rare indication for liver transplantation (LTx). Male sex is predominant in European studies. The ideal moment for LTx can be difficult to determine. PSC is often associated with inflammatory bowel disease (IBD) and may recur after LTx.

Research motivation

A Brazilian multicenter study on PSC showed that LTx patient data are limited and little explored in research. Our LTx service is the largest in North/Northeastern Brazil, with an average of 150 procedures a year, indicating a potential for research. The diagnosis of IBD in PSC patients before and after LTx is often inadequate and requires more attention on part of LTx teams. The finding of associated cholangiocarcinoma (CCA) in explants, associated with good survival, was an additional motivating factor.

Research objectives

To evaluate the clinical profile, complications and survival rates of PSC patients submitted to LTx at a Brazilian referral center.

Research methods

Retrospective study of medical records supplemented by telephone interviews with patients. The study contributed to setting up a database of PSC patients submitted to LTx at our service.

Research results

PSC was observed in 1.6% of LTx patients. Male sex was predominant, but the proportion of women was considerably higher than in the literature. Women were diagnosed later than men, but PSC was more severe in men, including CCA in explants. The prevalence of IBD was 73%. PSC was diagnosed later in IBD patients. The median time from the diagnosis of IBD to the diagnosis of PSC was 9.8 years. Diabetes was significantly more common in patients without IBD. Aspartate transferase was 1.6 times higher in PSC patients with IBD. Esophageal varices were more frequent in non-IBD patients. The most prevalent treatment before LTx was ursodeoxycholic acid. Most men (88%) were treated endoscopically for dominant stenosis prior to LTx. CCA was an incidental finding in two patients with satisfactory survival. The survival of our PSC patients was better than that of LTx patients with other indications at our service. Survival was 81.9% (1 year) and 78.8% (5 years). PSC recurred in 5.88%.

Research conclusions

In our cohort of 34 PSC patients submitted to LTx (2002-2023), the proportion of women was unusually high. CCA patients had satisfactory survival, despite the recurrence of PSC. In patients with both PSC and IBD, the disease was less severe.

Research perspectives

Our study raises the hypothesis that early-stage CCA may be an acceptable indication for LTx. The observed differences in severity in the male sex and the high proportion of women in the cohort require further investigations into the genetic profile of this population.

FOOTNOTES

Author contributions: Freitas LTS and Hyppolito EB designed the study; Freitas LTS, Hyppolito EB, Barreto VL, Júnior LHJC, Jorge BCM,

Háteras FCTSB and Marzola MB reviewed the literature, collected data and drafted the original manuscript; Hyppolito EB performed statistical analyses and reviewed the manuscript; Hyppolito EB, Coelho GR, Garcia JHP, Lima CA and Celedonio RM reviewed the intellectual content of the manuscript; all authors read and approved the final version of the manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Baseline metabolites could predict responders with hepatitis B virus-related liver fibrosis for entecavir or combined with FuzhengHuayu tablet

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Abstract

BACKGROUND

After receiving entecavir or combined with FuzhengHuayu tablet (FZHY) treatment, some sufferers with hepatitis B virus (HBV)-related liver fibrosis could achieve a histological improvement while the others may fail to improve even worsen. Serum metabolomics at baseline in these patients who were effective in treatment remain unclear.

AIM

To explore baseline serum metabolites characteristics in responders.

METHODS

A total of 132 patients with HBV-related liver fibrosis and 18 volunteers as healthy controls were recruited. First, all subjects were divided into training set and validation set. Second, the included patients were subdivided into entecavir responders (E-R), entecavir no-responders (E-N), FZHY + entecavir responders (F-R), and FZHY + entecavir no-responders (F-N) following the pathological histological changes after 48 wk' treatments. Then, Serum samples of all subjects before treatment were tested by high performance liquid chromatography-tandem mass spectrometry (LC-MS) high-performance LC-MS. Data processing was conducted using multivariate principal component analysis and orthogonal

partial least squares discriminant analysis. Diagnostic tests of selected differential metabolites were used for Boruta analyses and logistic regression.

RESULTS

As for the intersection about differential metabolic pathways between the groups E-R *vs* E-N and F-R *vs* F-N, results showed that 4 pathways including linoleic acid metabolism, aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, alanine, aspartate and glutamate metabolism were screened out. As for the differential metabolites, these 7 intersected metabolites including hydroxypropionic acid, tyrosine, citric acid, taurochenodeoxycholic acid, benzoic acid, 2-Furoic acid, and propionic acid were selected.

CONCLUSION

Our findings showed that 4 metabolic pathways and 7 differential metabolites had potential usefulness in clinical prediction of the response of entecavir or combined with FZHY on HBV fibrotic liver.

Key Words: Serum metabolomics; Differential metabolites; Therapeutic responders; Entecavir; FuzhengHuayu tablet; Hepatitis B virus-related liver fibrosis

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Core Tip: This study will use high-performance liquid chromatography-tandem mass spectrometry and multivariate statistical modelings to predict serum metabolites of the treatment (entecavir or entecavir + FuzhengHuayu tablet) that effectively reversed hepatitis B virus-related liver fibrosis. It is of great theoretical and practical significance to prevent the transformation of liver fibrosis to cirrhosis or even hepatocellular carcinoma and reduce the social burden.

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INTRODUCTION

Liver fibrosis, characterized by the progressive and reversible accumulation of fibrillar extracellular matrix components in the liver, poses a significant threat to the physiological architecture of the liver and accounts for nearly half of all-cause mortality associated with various liver diseases worldwide[1-2]. Among the numerous causes of acute and chronic liver diseases, hepatitis B virus (HBV) infection stands out as a prevalent culprit and a leading instigator of liver fibrosis[3]. Epidemiological studies have revealed that more than 240 million individuals are afflicted by HBV infection[4]. Given the insidious nature of chronic hepatitis B (CHB), it can swiftly advance to fibrosis, cirrhosis, or even hepatocellular carcinoma (HCC) if left unchecked[5]. Hence, it is imperative to consider the use of antiviral agents in the treatment of HBV, with entecavir serving as a prominent representative.

In recent years, the study of liver fibrosis has consistently been a focal point of medical research[6]. Serving as a reversible lesion, liver fibrosis acts as the intermediary stage between the development of chronic liver diseases and the progression to cirrhosis[7]. Presently, effective treatments for cirrhosis remain limited, underscoring the significance of anti-liver fibrosis as a crucial therapeutic strategy. FuzhengHuayu tablet (FZHY), a novel traditional Chinese medicine (TCM) remedy, has gained widespread usage in clinical practice for the treatment of liver fibrosis and cirrhosis[8]. Furthermore, our prior multi-center clinical investigation has substantiated that entecavir + FZHY therapy significantly enhances the histological reversal rate of CHB fibrosis[9]. Nonetheless, approximately one-third of patients fail to exhibit a substantial histological response[10]. Consequently, elucidating the biological characteristics of individuals who respond to entecavir or entecavir + FZHY will undoubtedly contribute to the enhancement of precision therapy's therapeutic efficacy.

To date, no single biomarker or scoring system has achieved the ideal balance of sensitivity and specificity for the detection and characterization of liver fibrosis[11]. While liver biopsy remains the gold standard for staging liver fibrosis, it is burdened by limitations such as invasiveness, sampling errors, and the potential for complications[12]. Furthermore, this method lacks convenience in tracking the dynamic progression of liver fibrosis and assessing therapeutic outcomes. Fortunately, non-invasive diagnostic techniques for liver fibrosis, including transient elastography (Fibroscan), elastography, and diffusion-weighted magnetic resonance imaging, have made significant advancements and gained widespread clinical utility. However, these approaches are susceptible to interference from factors such as a patient's body mass index (BMI), liver inflammation, or hepatocyte degeneration[13].

Metabolomics, an emerging field following in the footsteps of genomics, transcriptomics, and proteomics, represents a novel approach to systematically study changes in small-molecule metabolites produced by the body's metabolism[14]. Often referred to as the "end point" of the genome and proteome, metabolomics allows for the comprehensive analysis of

various metabolites and their metabolic pathways in a population, offering high-throughput and modeling capabilities. Furthermore, metabolomics can unveil downstream products of gene and protein expression within an organism, providing insight into all physiological processes within the body. Due to its close proximity to disease phenotypes, metabolomics is particularly well-suited for disease classification and biomarker discovery. In this study, we intend to employ high-performance liquid chromatography-mass spectrometry (HPLC-MS) and advanced multivariate statistical modeling to predict serum metabolite profiles associated with the effective reversal of HBV-related liver fibrosis induced by treatment with entecavir or entecavir + FZHY. This research holds profound theoretical and practical significance in preventing the progression of liver fibrosis to cirrhosis or HCC, thereby reducing the societal burden associated with these conditions.

MATERIALS AND METHODS

Patient selection

This is a cross-sectional study that encompasses multi-center randomized controlled clinical trials. We enrolled a total of 132 patients with HBV-related liver fibrosis, along with 18 healthy volunteers as controls, during the period from September 9, 2014, to October 25, 2018. The study comprised two distinct sets: A training set and a validation set. All participants were recruited from 20 hospitals across China and provided voluntary informed consent. The research protocol received ethical approval from the Ethics Committee of Shuguang Hospital Affiliated with Shanghai University of TCM (ethical approval number: 2014-331-27-01). The diagnostic criteria for HBV-related liver fibrosis were in accordance with the guidelines for the prevention and treatment of CHB (2019)[15]. The primary focus of this study was on the progression of liver fibrosis, assessed primarily through liver histopathology using the Ishak scoring system as the indicator for therapeutic evaluation. The primary outcome measured was the proportion of patients demonstrating a 1-point improvement in liver fibrosis stage, as per the Ishak score, from baseline to 48 wk. Liver biopsies were performed both before and 48 wk after the initiation of combination TCM treatment, and the histopathological evaluation was independently conducted by three pathologists. Fibrosis regression was defined as a decrease in the Ishak score of 1 or greater[16]. The final fibrotic scores were established based on consensus among two or more pathologists; any disagreements were resolved by a central pathologist. However, a detailed assessment of inflammation levels was not performed. For the noninvasive diagnosis and staging of liver fibrosis, aminotransferase-to-platelet ratio index (APRI) and fibrosis index based on the 4 factor (FIB-4) were primarily employed as adjunct diagnostic tools to assess the severity of liver fibrosis. Consequently, the two treatment groups were further subdivided into four subgroups: Entecavir responders (E-R), entecavir non-responders (E-N), FZHY + entecavir responders (F-R), and FZHY + entecavir non-responders (F-N). Inclusion criteria for this study encompassed individuals aged 18 years or older who met the aforementioned diagnostic criteria. Exclusion criteria included the following: individuals with liver fibrosis not associated with HBV infection; those with cardio-cerebrovascular or infectious diseases or other digestive system disorders; pregnant or lactating women; and patients with poor compliance.

Sample collection

All subjects were asked to have normal regular diets and schedules on the day before blood collection, and venous blood was collected on an empty stomach the next morning. 500 μL serum was centrifuged at 4 ℃ at 4000 r/min and stored in a -80 ℃ for later use.

Sample processing

The cryopreserved serum was thawed on ice-bath in case of degradation. 25 μL of serum was added to a 96-well plate for the transferring to the Biomek 4000 workstation (Biomek 4000, Beckman Coulter, Inc., Brea, CA, United States). 120 μL of methanol was automatically added to each serum and vortexed for 5 min. The plate was centrifuged at 4000 g for half an hour and it was returned back to the workstation. 30 μL of supernatant fluid was transferred to a clean 96-well plate, where each well was filled with 20 μL of freshly prepared derivative reagents. Then the plate was sealed for derivatization at 30 ℃ for an hour and the sample was diluted by 330 μL of ice-cold 50% methanol solution. Next, the plate was left at -20 ℃ for 20 min and centrifuged at 4 ℃ for half an hour. Finally, 135 μL of supernatant fluid was taken to a new 96-well plate, which was sealed for liquid chromatography-tandem mass spectrometry (LC-MS) analysis.

Quality control analysis

All samples were mixed into one quality control sample for quality control. The quality control samples were analyzed 6 times and randomly respectively tested 2 times before, during and after analysis. The total ion flow chromatograms of the quality control samples were overlapped and the total principal component analysis (PCA) was performed. It would show good repeatability if the results of the quality control samples were close to each other.

Materials and reagents

Formic acid (Optima grade) was obtained from Sigma-Aldrich (St. Louis, MO, United States). Methanol (Optima LC-MS) and acetonitrile (Optima LC-MS) were purchased from Thermo-Fisher Scientific (FairLawn, NJ, United States). The experimental water was distilled water.

Instrument analysis platform

We used a ultra-performance liquid chromatography coupled to tandem mass spectrometry system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, United States) in order to quantitate all targeted metabolites in this study. A briefly description of the optimized instrument settings can be shown in Supplementary Table 1. Meanwhile, the instrument performance optimization and routine maintenance were conducted every week.

LC-MS analysis

Extraction of ion flow chromatograms based on HPLC-MS. (1) Chromatographic elution gradient: The initial gradients were 5% solution B (acetonitrile + 0.1% formic acid) and 95% solution A (distilled water + 0.1% formic acid), whose elution time lasted 2-10 min. Meanwhile, solution B increased linearly to 95% for 5 min and then dropped back to 5%. The injection volume was 4 uL and the automatic sampler temperature was 4 ℃; and (2) mass spectrometry scanning mode: Positive and negative ions were used for detection by mass spectrometry. The ion scanning time was 0.03 s, the time interval was 0.02 s, and the data collection range was 50-100 m/z.

Screening and identification of potential metabolites

The data of group A and group B were analyzed by total PCA, then partial least squares discriminant analysis (PLS-DA) was used, and finally the supervised orthogonal PLS-DA (OPLS-DA) was used for modeling analysis. Variable importance in the projection (VIP) values (threshold > 1) based on the OPLS-DA model, combined with P value ($P < 0.05$) of t test, were used to find metabolites which were differentially expressed. Potential metabolites were identified by searching online database (http://metlin.scripps.edu/) to compare the mass charge ratio or molecular mass of mass spectrometry.

Potential metabolite enrichment analysis and metabolic pathway analysis

Metabo-Analyst online analysis software (https://www.metaboanalyst.ca) and Kyoto Encyclopedia of Genes and Genomes databases (https://www.kegg.jp/) were used for metabolic pathway analysis and enrichment analysis of the identified potential metabolites so as to determine the metabolic pathways involved in the potential metabolites, and to evaluate the diagnostic performance of the potential metabolites enriched in pathways.

Diagnostic tests

In order to validate the applicability and stability of the selected differential metabolites, random forest (RF), Support vector machine (SVM) and Boruta analyses were conducted for each selected metabolite in sequence. Boruta analysis, the maximum number of runs with 1000, was an RF-based feature selection method that it selects key features with more significant distinguishing ability than random lag features. When provisional features were included, a secondary selection was made to determine whether certain metabolites with large fluctuations should be included in the selected features.

These differential metabolites used for subsequent model construction were modeled and predicted using logistic regression. After modeling, sensitivity and specificity values were calculated to evaluate the model effects through drawing the receiver operating characteristic curve. Meanwhile, the closer the area under the curve (AUC) value is to 1, the better the sensitivity, specificity and diagnostic abilities. The conventional AUC of metabolites with the value ≥ 0.75 indicated relatively good sensitivity and specificity.

Statistical analysis

Statistical analysis software packages in R studio (http://cran.r-project.org/) were performed for the statistical algorithms. All the included data were calculate with mean ± SD or median-interquartile range. The Mann-Whitney U test or t test was used for the statistical differences in pairwise comparison. Multivariate statistical modelings including PCA, PLS-DA, and OPLS-DA were used for the multi-class classification and identification of differently altered metabolites. Among these modelings, each spatial dot in the K-dimensional space represented an individual sample with the samples color-coded based on grouping information. R^2X and R^2Y respectively represented the fraction of the variance of X matrix and Y matrix, while Q^2Y represented the predictive accuracy of the model. Cumulative values of R^2X and R^2Y approaching 1.0, along with Q^2Y greater than 0.2 (permutation test), indicated a model with a satisfactory predictive ability. Those variables with VIP greater than 1.0 are considered significantly different between classes. If multidimensional statistics cannot establish a robust discriminant model (such as uneven distribution of inter-group sample categories or large intra-group deviation), differential metabolites between the two groups would be acquired with the aid of univariate analysis.

RESULTS

Baseline clinical characteristics of participants

In the training set, there were 23 sufferers in each subgroup and 13 normal volunteers as control. In the validation set, there were 10 patients in each subgroup and 5 volunteers as control. Details of the baseline clinical characteristics of the two datasets can be found in Table 1. Specifically, there were no significant differences in the gender, age, BMI, alanine aminotransferase, aspartate aminotransferase, albumin (ALB), total bilirubin (TBIL), creatinine, prothrombin time, platelet count, alpha fetoprotein, FIB-4, aspartate APRI, Ishak score in the training set ($P > 0.05$). However, in the

Table 1 Demographic and clinical data of patients and volunteers in training set and validation set

	Training set (*n* = 105)					Validation set (*n* = 45)				
	F-R (*n* = 23)	F-N (*n* = 23)	E-R (*n* = 23)	E-N (*n* = 23)	NOR (*n* = 13)	F-R (*n* = 10)	F-N (*n* = 10)	E-R (*n* = 10)	E-N (*n* = 10)	NOR (*n* = 5)
Male/Female	15/8	15/8	16/7	17/6	9/4	7/3	10/0	6/4	8/2	4/1
Age (yr)	44.17 ± 6.25	42.43 ± 8.36	40.65 ± 7.73	42.22 ± 7.97	36.92 ± 6.18	42.80 ± 5.01	38.10 ± 11.95	45.00 ± 7.93	47.40 ± 10.44	37.80 ± 8.79
BMI (kg/m^2)	23.60 ± 2.56	23.64 ± 3.17	23.54 ± 2.06	23.92 ± 2.73	24.20 ± 1.34	23.22 ± 3.30	24.76 ± 1.65	24.61 ± 2.29	23.16 ± 3.86	22.60 ± 1.71
ALT (IU/L)	42.52 ± 29.59	41.03 ± 20.48	68.91 ± 89.81	47.34 ± 27.83	/	49.80 ± 50.83	48.80 ± 33.14	57.08 ± 46.97	58.33 ± 73.02	/
AST (IU/L)	40.50 ± 21.35	41.80 ± 19.41	53.47 ± 51.20	50.53 ± 27.59	/	38.71 ± 16.39	47.75 ± 28.32	77.64 ± 120.70	48.73 ± 35.54	/
ALB (IU/L)	43.84 ± 5.50	41.43 ± 6.07	43.51 ± 5.75	42.22 ± 4.61	/	42.40 ± 5.08	35.70 ± 6.67	39.68 ± 5.95	41.53 ± 4.64	/
TBIL (μmol/L)	16.15 ± 10.88	13.27 ± 6.36	13.36 ± 9.25	14.16 ± 6.51	/	11.98 ± 4.90	24.49 ± 16.83	22.08 ± 13.61	12.18 ± 5.79	/
Cr (μmol/L)	64.43 ± 17.03	66.39 ± 11.99	69.57 ± 16.59	64.57 ± 14.67	/	72.80 ± 18.27	71.10 ± 9.71	65.80 ± 13.70	83.60 ± 25.07	/
PT (S)	13.22 ± 1.48	13.23 ± 1.41	13.31 ± 1.46	13.78 ± 1.52	/	13.21 ± 1.32	14.27 ± 2.90	14.10 ± 1.33	13.88 ± 1.83	/
PLT (× 10 ×^{9}/L)	119.02 ± 49.99	113.47 ± 61.33	131.70 ± 49.26	104.65 ± 41.65	/	145.10 ± 64.42	112.00 ± 36.18	98.88 ± 45.13	106.40 ± 37.97	/
AFP (ng/ml)	23.70 ± 59.55	12.46 ± 13.22	18.63 ± 42.03	14.75 ± 15.56	/	16.73 ± 31.93	54.81 ± 93.15	25.25 ± 41.79	14.86 ± 15.91	/
FIB-4	2.99 ± 2.06	3.31 ± 2.38	2.54 ± 1.62	3.46 ± 1.75	/	2.29 ± 1.95	2.72 ± 1.51	5.63 ± 8.14	3.55 ± 1.89	/
APRI	1.10 ± 0.96	1.23 ± 0.95	1.40 ± 1.60	1.31 ± 0.73	/	0.81 ± 0.54	1.18 ± 0.84	3.17 ± 6.23	1.28 ± 0.92	/
Ishak score	5.48 ± 0.51	5.43 ± 0.51	5.35 ± 0.49	5.39 ± 0.50	/	5.40 ± 0.52	5.40 ± 0.52	5.50 ± 0.53	5.10 ± 0.32	/

BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; TBIL: Total bilirubin; Cr: Creatinine; PT: Prothrombin time; PLT: Platelet count; AFP: Alpha fetoprotein; FIB-4: Fibrosis index based on the 4 factor; APRI: Aspartate aminotransferase-to-platelet ratio index; E-R: Entecavir responders; E-N: Entecavir no-responders; F-R: FuzhengHuayu tablet + entecavir responders; F-N: FuzhengHuayu tablet + entecavir no-responders; NOR: Normal.

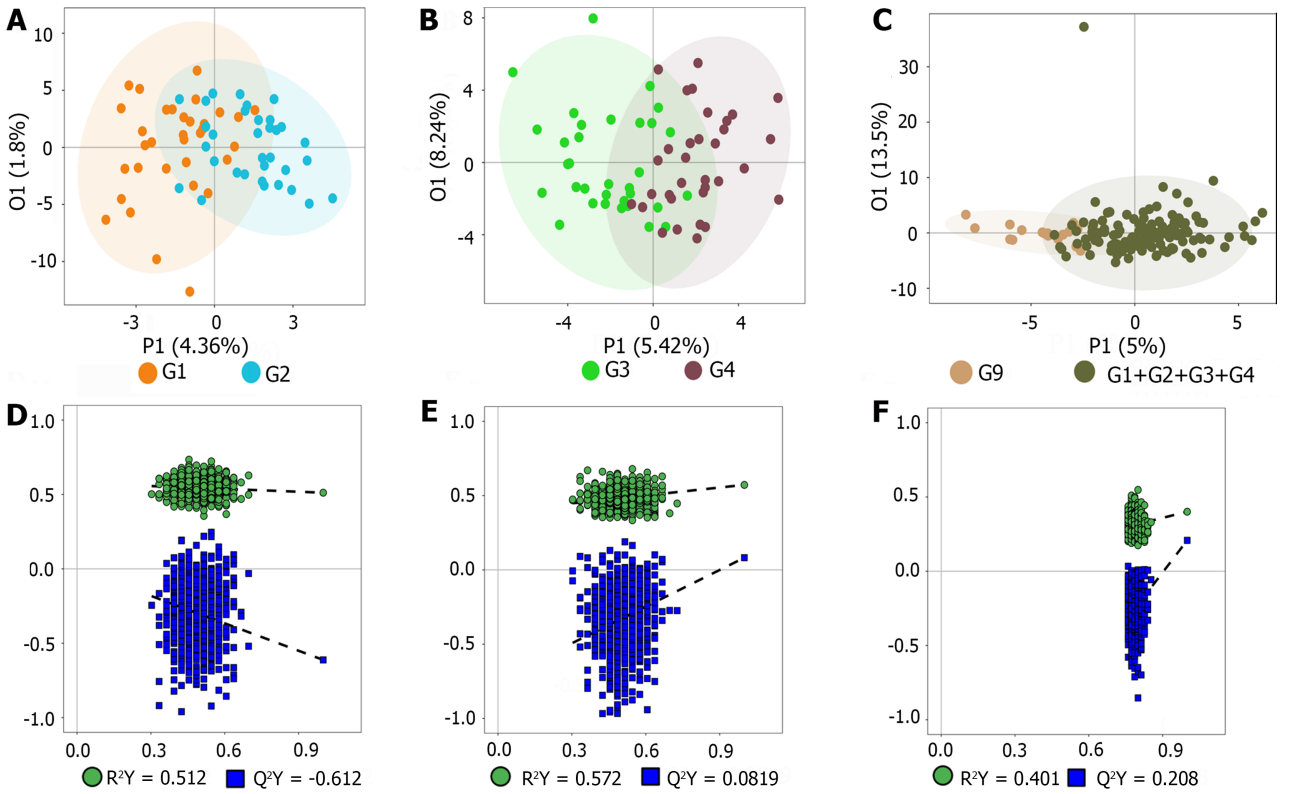
validation set, the serum ALB and TBIL levels significantly differed between the F-R and F-N patients ($P < 0.05$), but the other indexes were not statistically significant ($P > 0.05$).

The pathological histological results of the liver biopsy

The obtained tissues *via* liver biopsy were fixed in 10% formalin and embedded in paraffin. Sections of each liver tissue were cut and stained using hematoxylin-eosin (HE) staining for histopathological analysis. Based on the HE staining results and Ishak score, staging of liver fibrosis was determined as F1 to F6[17]. Briefly, F1: Some portal areas have fibrosis but no fibrous septum; F2: Many portal areas have fibrosis along with one fibrous septum; F3: Many portal areas have fibrosis along with two or three fibrous septa; F4: Portal areas have obvious portal-junction bridge fibrosis along with more than four fibrous septa; F5: Portal areas have obvious portal-junction bridge fibrosis or portal-central bridge fibrosis along with one to three pseudolobuli and F6: More than three pseudolobuli. Details of relevant figures can be found in Supplementary Figure 1.

Overall metabolomics analysis of serum samples

Representative nuclear magnetic resonance spectra with targeted metabolites are exhibited in Supplementary Figure 2. The serum spectra included high-intensity signals from Maleic acid, Glycine (G1 *vs* G2), dihomo-gamma-linolenic acid, arachidonic acid, hydroxypropionic acid, (G3 *vs* G4), 2-Furoic acid, 2-Phenylpropionate, arachidonic acid, benzoic acid, butyric acid, aconitic acid, citric acid, dimethylglycine, glycochenodeoxycholic acid (GCDCA), homovanillic acid, hydrocinnamic acid, hydroxyphenyllactic acid, isocitric acid, tyrosine, phenyllactic acid, propionic acid, taurochenodeoxycholic acid (TCDCA), tricarboxylic acid (TCA) (G9 *vs* G1-G4). Because all patients were suffered from HBV-related



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Figure 1 Orthogonal partial least squares discriminant analysis of all the metabolites. A: Score of entecavir responders (E-R) *vs* entecavir no-responders (E-N); B: Score of FuzhengHuayu tablet (FZHY) + entecavir responders (F-R) *vs* FZHY + entecavir no-responders (F-N); C: Score of patients vs volunteers; D: R^2X and R^2Y of E-R vs E-N; E: R^2X and R^2Y of F-R vs F-N; F: R^2X and R^2Y of patients vs volunteers.

liver fibrosis in this study, statistical assessment by PCA indicated not clear separation in each group (E-R *vs* E-N; F-R *vs* F-N; patients vs volunteers) (Supplementary Figure 3). Besides, in order to exclude the possible confounding factors irrelevant to the group differences and to assess the statistical meaning of those signals, OPLS-DA was conducted and the result showed that the discrimination model could differentiate the two groups despite within a small overlap in one orthogonal component (Figure 1A-C). Moreover, as shown in Figure 1D-F, the models with $R^2(Y)$ of 0.512 (E-R *vs* E-N), 0.572 (F-R *vs* F-N), 0.401 (patients *vs* volunteers) suggested relatively good predictability and no potential over-fit. However, the models with $Q^2(Y)$ of -0.612 (E-R *vs* E-N), 0.0819 (F-R *vs* F-N), and 0.208 (patients *vs* volunteers) indicated the potential risk of over-fit.

Serum metabolites relevant to responders and HBV-related liver fibrosis

Due to the possibility of potential risk of the over-fit in these models, differential metabolites between the two groups were acquired with the aid of univariate analysis instead of analysis together with the VIP values from the above OPLS-DA model. Furthermore, in order to explore the applicability and stability of the distinctive models, serum samples from all the included patients and volunteers were collected and analyzed using the training set and validation set for the subsequent analyses.

In order to find out potential metabolites involving in responders and HBV-related liver fibrosis among the thousands of variables, a pairwise comparison in each group was conducted. According to the threshold value ($P < 0.05$ and $|\log2FC| \geq 0$, FC: Fold change), a total of 2 (E-R *vs* E-N), 16 (F-R *vs* F-N) and 35 (patients *vs* volunteers) potential metabolites in the training set (Figure 2A-C) were obtained while a total of 8 (E-R *vs* E-N), 7 (F-R *vs* F-N) and 23 (patients *vs* volunteers) potential metabolites in the validation set (Figure 2D-F) were acquired.

Selection of potential metabolites in different sets

By taking intersection and union set in terms of the aforementioned obtained unidimensional and multidimensional potential metabolites, these metabolites that may have biological significance can be selected on the basis of OPLS-DA (VIP > 1) and univariate ($P < 0.05$ and $|\log2FC| \geq 0$) analyses. A total of 53 potential metabolites in the training set and 38 potential metabolites in the validation set were obtained. Detailed information of these selected potential metabolites were shown in Table 2. The distribution of data for all the metabolites in each group can be found in Supplementary Figure 4. Furthermore, a heat map, together with Z-score, was used for analysis of these selected metabolites and the results suggested that the pairwise comparisons between the two groups could be separated no matter which data set was (Figure 3).

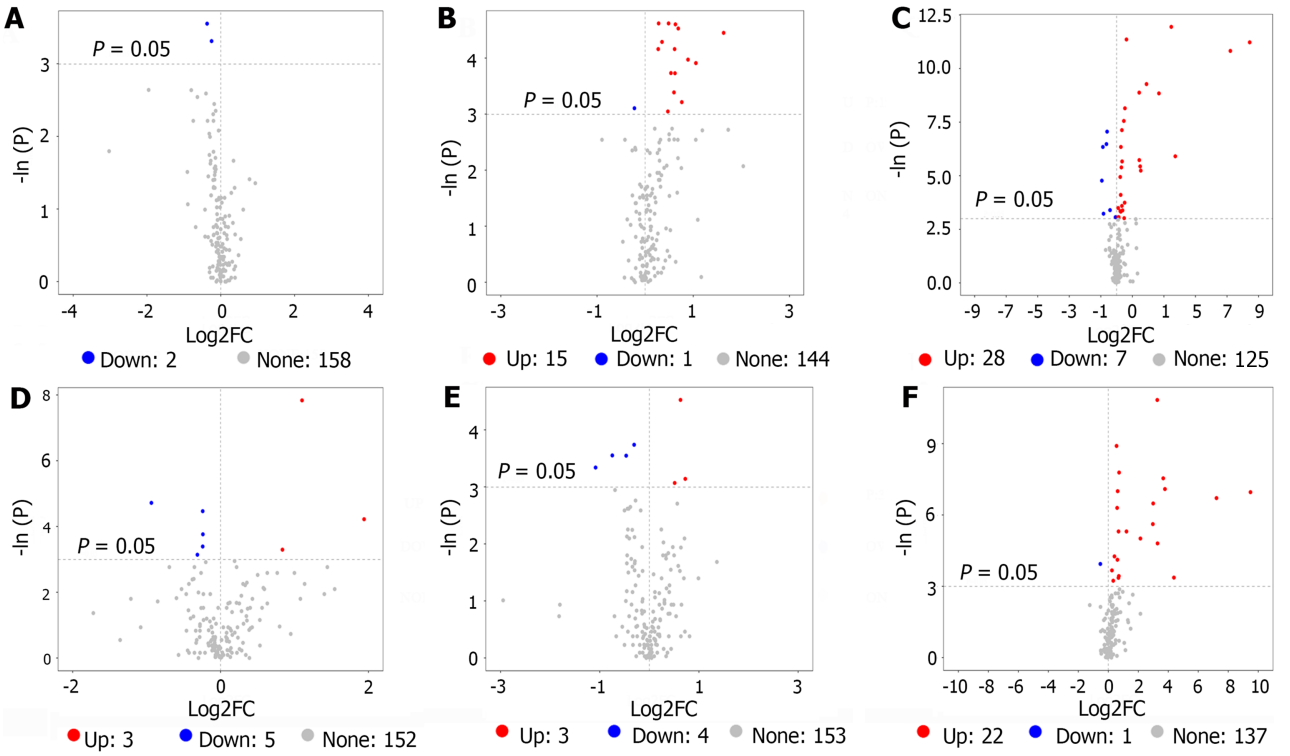
Table 2 The selected potential metabolites in training set and validation set

Class	HMDB	KEGG	Metabolite	Uni_P	Uni_FDR	FC	log2FC	OPLSDA_VIP
Training set								
Fatty acids	HMDB0000448	C06104	Adipic acid	0.03	1.00	0.77	-0.37	1.65
Organic acids	HMDB0000176	C01384	Maleic acid	0.04	1.00	0.84	-0.25	1.73
Fatty acids	HMDB0060038	NA	10Z-Heptadecenoic acid	0.01	0.31	3.10	1.63	1.90
	HMDB0002925	C03242	Dihomo-gamma-linolenic acid	0.02	0.32	2.08	1.05	1.60
	HMDB0001043	C00219	Arachidonic acid	0.04	0.44	1.70	0.76	1.79
	HMDB0002183	C06429	DHA	0.02	0.32	1.85	0.89	2.03
	HMDB0006528	C16513	DPA	0.01	0.31	1.55	0.63	1.99
	HMDB0001999	C06428	EPA	0.02	0.32	1.54	0.62	1.70
Organic acids	HMDB0000700	C01013	Hydroxypropionic acid	0.01	0.31	1.27	0.35	1.21
Fatty acids	HMDB0000673	C01595	Linoleic acid	0.05	0.44	1.39	0.47	1.54
Carnitines	HMDB0006469	NA	Linoleylcarnitine	0.01	0.31	1.40	0.49	2.16
Fatty acids	HMDB0000806	C06424	Myristic acid	0.01	0.31	1.61	0.69	1.76
	HMDB0000207	C00712	Oleic acid	0.02	0.32	1.45	0.54	1.86
Carnitines	HMDB0005065	NA	Oleylcarnitine	0.01	0.31	1.21	0.28	2.32
Fatty acids	HMDB0003229	C08362	Palmitoleic acid	0.02	0.31	1.53	0.61	1.62
	HMDB0000826	C16537	Pentadecanoic acid	0.03	0.42	1.51	0.60	1.51
Benzenoids	HMDB0000205	C00166	Phenylpyruvic acid	0.04	0.44	0.86	-0.22	0.85
Carnitines	HMDB0013128	NA	Valerylcarnitine	0.02	0.31	1.21	0.27	1.47
Organic acids	HMDB0000617	C01546	2-Furoic acid	0.00	0.00	148.12	7.21	2.10
Phenylpropanoic acids	HMDB0011743	NA	2-Phenylpropionate	0.01	0.04	2.89	1.53	1.26
Organic acids	HMDB0000357	C01089	3-Hydroxybutyric acid	0.04	0.20	0.56	-0.83	0.59
Fatty acids	HMDB0000555	NA	3-Methyladipic acid	0.05	0.22	0.95	-0.07	2.09
	HMDB0001043	C00219	Arachidonic acid	0.00	0.01	0.66	-0.60	2.11
	HMDB0000784	C08261	Azelaic acid	0.05	0.22	1.07	0.10	0.86
Organic acids	HMDB0001870	C00180	Benzoic acid	0.00	0.00	346.31	8.44	2.42
Bile acids	HMDB0000686	C17662	bUDCA	0.00	0.02	0.64	-0.64	1.37
SCFAs	HMDB0000039	C00246	Butyric acid	0.00	0.00	3.72	1.89	2.54
Carnitines	HMDB0002013	C02862	Butyrylcarnitine	0.00	0.02	0.55	-0.87	1.80
Bile acids	HMDB0000619	C00695	CA	0.02	0.16	1.42	0.51	1.27
Organic acids	HMDB0000072	C02341	Aconitic acid	0.00	0.01	1.37	0.46	0.76
	HMDB0000094	C00158	Citric acid	0.00	0.02	1.21	0.27	1.31
Carbohydrates	HMDB0000122	C00221	Glucose	0.05	0.22	1.10	0.14	0.75
Carnitines	HMDB0000651	NA	Decanoylcarnitine	0.01	0.06	0.52	-0.94	0.55
Amino acids	HMDB0000092	C01026	Dimethylglycine	0.00	0.03	1.27	0.35	0.90
Amino acids	HMDB0000112	C00334	GABA	0.01	0.05	1.17	0.23	0.46
	HMDB0000123	C00037	Glycine	0.04	0.19	1.18	0.23	1.07
Bile acids	HMDB0000637	C05466	GCDCA	0.00	0.00	6.42	2.68	2.30
Organic acids	HMDB0000115	C00160	Glycolic acid	0.03	0.18	1.26	0.33	1.03
Fatty acids	HMDB0000666	C17714	Heptanoic acid	0.03	0.19	1.30	0.38	1.07

Phenols	HMDB0000118	C05582	Homovanillic acid	0.00	0.01	1.26	0.33	2.40
Phenylpropanoic acids	HMDB0000764	C05629	Hydrocinnamic acid	0.00	0.03	2.71	1.44	1.23
	HMDB0000755	C03672	Hydroxyphenyllactic acid	0.00	0.01	1.44	0.53	2.20
Organic acids	HMDB0000193	C00311	Isocitric acid	0.05	0.22	1.39	0.48	0.65
	HMDB0000168	C00152	Asparagine	0.03	0.19	1.07	0.10	1.85
	HMDB0000719	C00263	Homoserine	0.04	0.19	1.20	0.26	1.41
	HMDB0000696	C00073	Methionine	0.00	0.04	1.23	0.30	1.59
	HMDB0000716	C00408	Pipecolic acid	0.02	0.11	1.20	0.26	0.98
	HMDB0000158	C00082	Tyrosine	0.00	0.00	1.54	0.62	2.51
Carnitines	HMDB0000791	C02838	Octanoylcarnitine	0.03	0.19	0.75	-0.42	0.57
Phenylpropanoic acids	HMDB0000779	NA	Phenyllactic acid	0.00	0.04	2.81	1.49	1.44
SCFAs	HMDB0000237	C00163	Propionic acid	0.00	0.00	2.70	1.43	3.00
Bile Acids	HMDB0000951	C05465	TCDCA	0.00	0.00	11.04	3.47	1.80
	HMDB0000036	C05122	TCA	0.00	0.03	13.19	3.72	1.55
Validation set								
Carnitines	HMDB0000062	C00318	Carnitine	0.04	0.65	0.80	-0.31	1.73
	HMDB0001976	NA	DPAn-6	0.04	0.65	1.79	0.84	2.00
	HMDB0003073	C06426	gamma-Linolenic acid	0.01	0.59	3.84	1.94	2.54
Amino acids	HMDB0000123	C00037	Glycine	0.03	0.65	0.85	-0.24	1.60
Peptides	HMDB0000721	NA	Glycylproline	0.01	0.59	0.85	-0.24	1.59
Amino acids	HMDB0000168	C00152	Asparagine	0.02	0.65	0.85	-0.24	1.41
	HMDB0000162	C00148	Proline	0.01	0.59	0.52	-0.94	1.53
	HMDB0006270	NA	Linoelaidic acid	0.00	0.06	2.15	1.10	2.62
	HMDB0002925	C03242	Dihomo-gamma-linolenic acid	0.04	0.80	1.65	0.72	1.39
	HMDB0001043	C00219	Arachidonic acid	0.05	0.80	1.42	0.51	1.59
Organic acids	HMDB0000700	C01013	Hydroxypropionic acid	0.04	0.80	0.47	-1.08	2.07
Amino acids	HMDB0000168	C00152	Asparagine	0.02	0.80	0.81	-0.31	2.66
	HMDB0000191	C00049	Aspartic acid	0.01	0.80	1.55	0.63	2.29
	HMDB0000158	C00082	Tyrosine	0.03	0.80	0.72	-0.47	2.51
Phenylpropanoic acids	HMDB0000779	NA	Phenyllactic acid	0.03	0.80	0.60	-0.75	2.14
Organic acids	HMDB0000617	C01546	2-Furoic acid	0.00	0.02	148.81	7.22	1.66
Phenylpropanoic acids	HMDB0011743	NA	2-Phenylpropionate	0.00	0.02	13.73	3.78	1.90
Fatty acids	HMDB0001043	C00219	Arachidonic acid	0.02	0.17	0.69	-0.53	1.88
Organic acids	HMDB0001870	C00180	Benzoic acid	0.00	0.02	715.57	9.48	1.71
SCFAs	HMDB0000039	C00246	Butyric acid	0.00	0.05	7.84	2.97	1.80
	HMDB0000072	C02341	Aconitic acid	0.00	0.06	1.60	0.68	1.90
	HMDB0000094	C00158	Citric acid	0.00	0.02	1.54	0.62	2.32
Amino acids	HMDB0000092	C01026	Dimethylglycine	0.00	0.02	1.65	0.72	0.97
Bile acids	HMDB0000637	C05466	GCDCA	0.00	0.02	12.69	3.67	1.53
Phenols	HMDB0000118	C05582	Homovanillic acid	0.00	0.03	1.51	0.59	2.32
	HMDB0000764	C05629	Hydrocinnamic acid	0.00	0.03	7.97	2.99	1.88

	HMDB0000755	C03672	Hydroxyphenyllactic acid	0.03	0.25	1.63	0.70	2.10
Organic acids	HMDB0000193	C00311	Isocitric acid	0.00	0.06	2.32	1.21	1.29
	HMDB0000641	C00064	Glutamine	0.04	0.27	1.26	0.33	1.85
	HMDB0000684	C00328	Kynurenine	0.02	0.15	1.53	0.61	0.95
	HMDB0000158	C00082	Tyrosine	0.00	0.01	1.46	0.55	2.23
	HMDB0002931	NA	N-acetylserine	0.03	0.21	1.18	0.24	0.83
Fatty acids	HMDB0003229	C08362	Palmitoleic acid	0.03	0.25	1.60	0.68	1.07
Phenylpropanoic acids	HMDB0000779	NA	Phenyllactic acid	0.03	0.25	20.79	4.38	1.84
Benzoic acids	HMDB0002107	C01606	Phthalic acid	0.01	0.14	1.33	0.41	2.21
SCFAs	HMDB0000237	C00163	Propionic acid	0.01	0.08	4.41	2.14	2.19
Bile acids	HMDB0000951	C05465	TCDCA	0.00	0.00	9.63	3.27	1.75
	HMDB0000036	C05122	TCA	0.01	0.09	9.75	3.28	1.54

GCDCA: Glycochenodeoxycholic acid; TCA: Tricarboxylic acid; TCDCA: Taurochenodeoxycholic acid; NA: Not Applicable; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid; UDCA: Ursodeoxycholic acid; CA: Citric acid; GABA: Gamma-amino butyric acid.

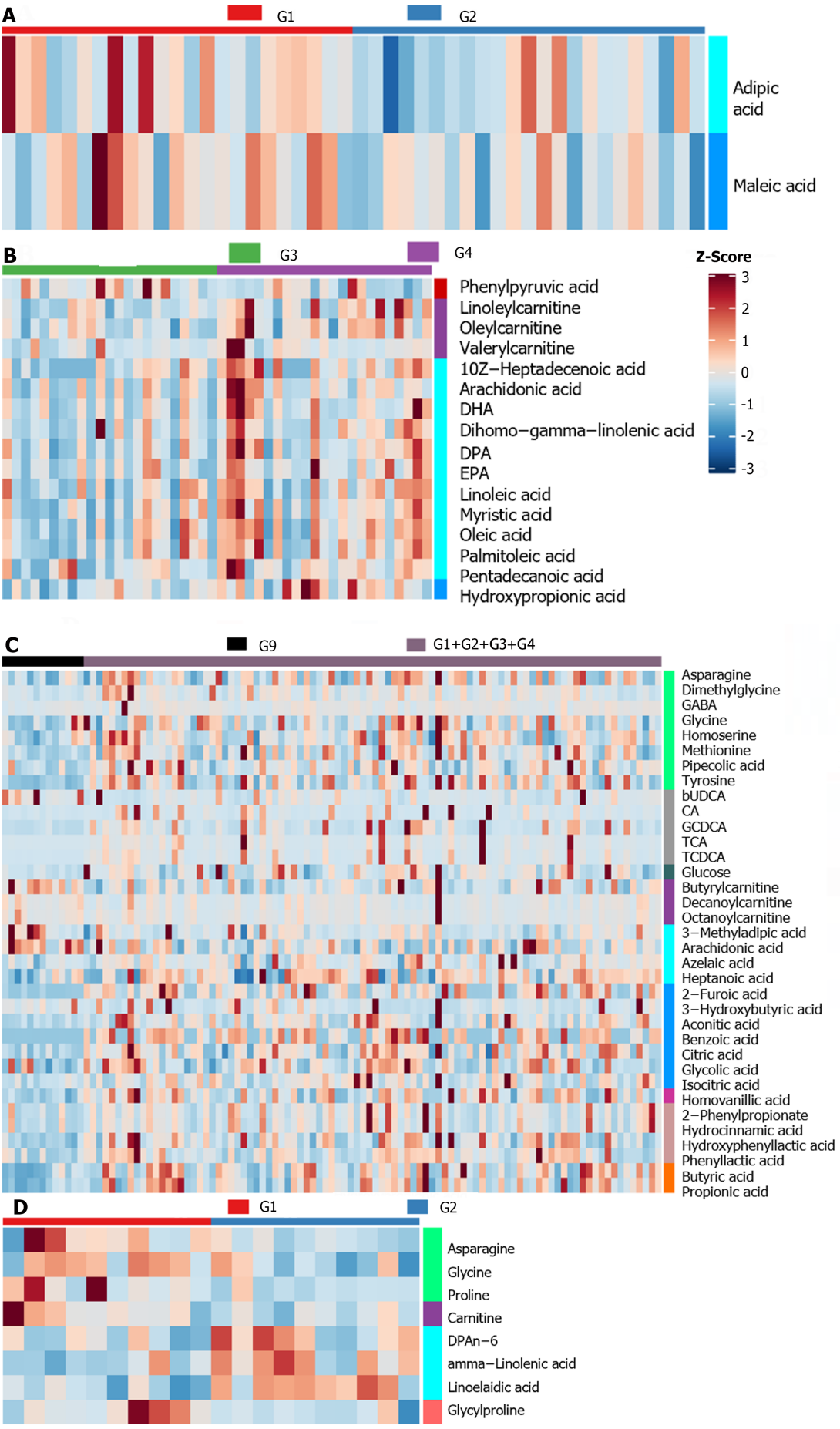


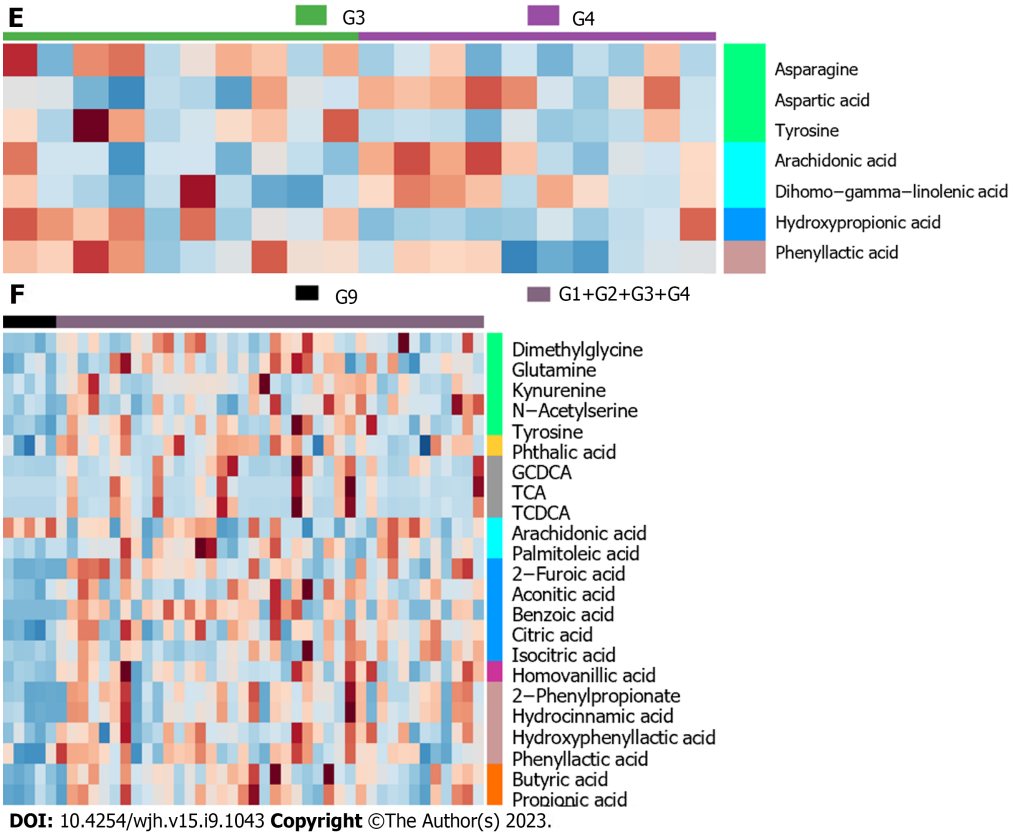
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Figure 2 Volcano plot of serum metabolites. A: Volcano plot of entecavir responders (E-R) *vs* entecavir no-responders (E-N) (training set); B: Volcano plot of FuzhengHuayu tablet (FZHY) + entecavir responders (F-R) *vs* FZHY + entecavir no-responders (F-N) (training set); C: Volcano plot of patients *vs* volunteers (training set); D: Volcano plot of E-R *vs* E-N (validation set); E: Volcano plot of F-R *vs* F-N (validation set); F: Volcano plot of patients *vs* volunteers (validation set).

Metabolic pathways related to the selected metabolites in different sets

Both topological centrality (impact value > 0) and enrichment significance [-ln(p) > 2.99, namely $P < 0.05$] were used to evaluate the analyses of enrichment and metabolic pathways for the selected potential metabolites. As shown in Figure 4, there were 2 pathways (butanoate metabolism, nicotinate and nicotinamide metabolism) (E-R *vs* E-N), 1 pathway (fatty acid biosynthesis) (F-R *vs* F-N), and 11 pathways (primary bile acid biosynthesis, nitrogen metabolism, butanoate metabolism, aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, phenylalanine metabolism, glycine, serine and threonine metabolism, glyoxylate and dicarboxylate metabolism, citrate cycle (TCA cycle), thiamine metabolism, alanine, aspartate and glutamate metabolism) (patients *vs* volunteers) in the training set (Figure 4A-C); and there were 6 pathways (aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, nitrogen metabolism, linoleic acid metabolism, thiamine


A
G1
G2
Adipic acid
Maleic acid
B
G3
G4
Z-Score
3
2
1
0
-1
-2
-3
Phenylpyruvic acid
Linoleylcarnitine
Oleylcarnitine
Valerylcarnitine
10Z−Heptadecenoic acid
Arachidonic acid
DHA
Dihomo−gamma−linolenic acid
DPA
EPA
Linoleic acid
Myristic acid
Oleic acid
Palmitoleic acid
Pentadecanoic acid
Hydroxypropionic acid
C
G9
G1+G2+G3+G4
Asparagine
Dimethylglycine
GABA
Glycine
Homoserine
Methionine
Pipecolic acid
Tyrosine
bUDCA
CA
GCDCA
TCA
TCDCA
Glucose
Butyrylcarnitine
Decanoylcarnitine
Octanoylcarnitine
3−Methyladipic acid
Arachidonic acid
Azelaic acid
Heptanoic acid
2−Furoic acid
3−Hydroxybutyric acid
Aconitic acid
Benzoic acid
Citric acid
Glycolic acid
Isocitric acid
Homovanillic acid
2−Phenylpropionate
Hydrocinnamic acid
Hydroxyphenyllactic acid
Phenyllactic acid
Butyric acid
Propionic acid
D
G1
G2
Asparagine
Glycine
Proline
Carnitine
DPAn−6
amma−Linolenic acid
Linoelaidic acid
Glycylproline



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Figure 3 Heatmap of all the selected potential metabolites. A: Heatmap of entecavir responders (E-R) *vs* entecavir no-responders (E-N) (training set); B: Heatmap of FuzhengHuayu tablet (FZHY) + entecavir responders (F-R) *vs* FZHY + entecavir no-responders (F-N) (training set); C: Heatmap of patients *vs* volunteers (training set); D: Heatmap of E-R *vs* E-N (validation set); E: Heatmap of F-R *vs* F-N (validation set); F: Heatmap of patients *vs* volunteers (validation set). CA: Citric acid; GCDCA: Glycochenodeoxycholic acid; TCA: Tricarboxylic acid; TCDCA: Aurochenodeoxycholic acid; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid.

metabolism, alanine, aspartate and glutamate metabolism) (E-R *vs* E-N), 5 pathways (nitrogen metabolism, aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, alanine, aspartate and glutamate metabolism, beta-alanine metabolism) (F-R *vs* F-N), and 6 pathways (phenylalanine metabolism, primary bile acid biosynthesis, TCA cycle, tyrosine metabolism, ubiquinone and other terpenoid-quinone biosynthesis, nitrogen metabolism) (patients *vs* volunteers) in the validation set (Figure 4D-F).

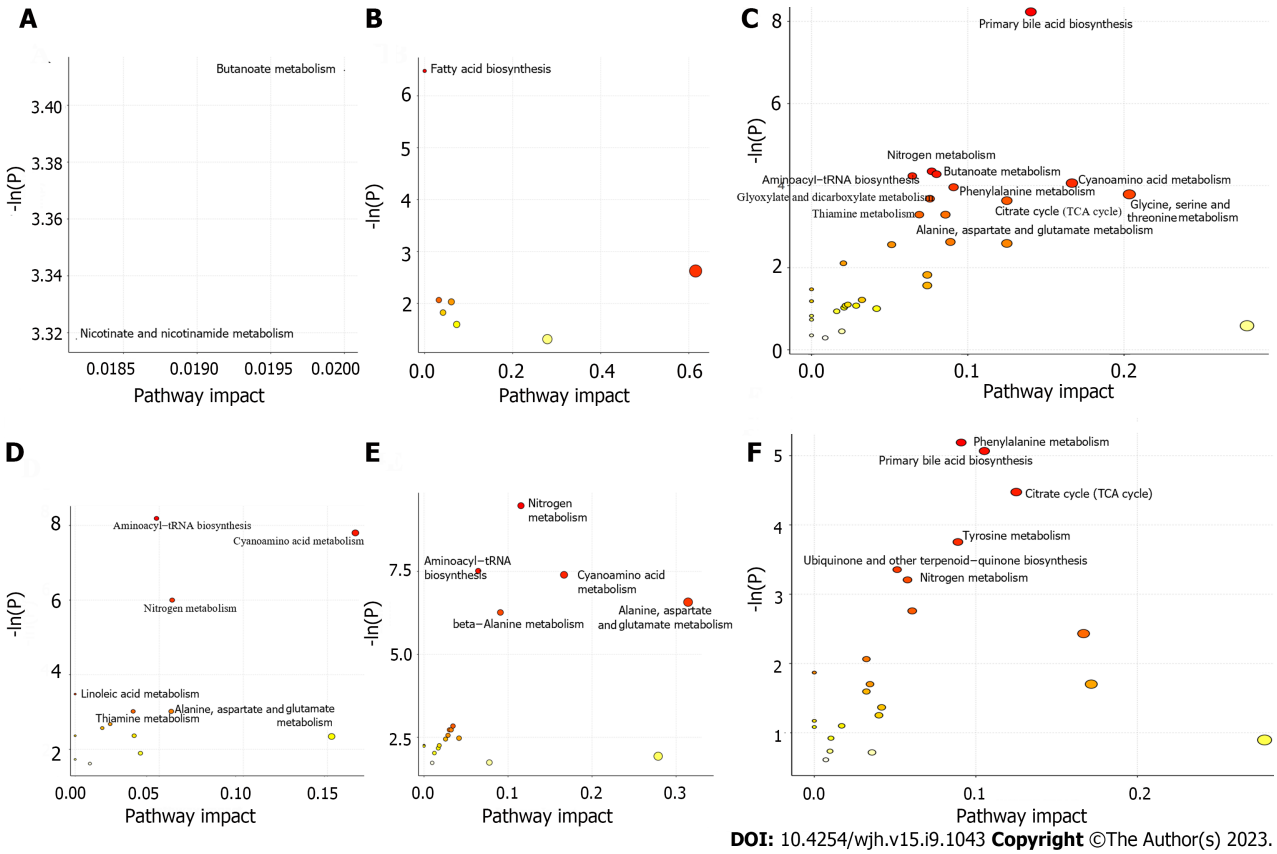
Selection of differential metabolites in different sets

In order to find differential metabolites from these selected potential metabolites, RF, SVM and Boruta analyses were conducted for each selected metabolite in sequence. And intersection of these potential metabolites in the three analyses can be found in Supplementary Figure 5. Specifically, there were Maleic acid and Adipic acid (E-R *vs* E-N), Hydroxypropionic acid, 10Z-heptadecenoic acid, and linoleylcarnitine (F-R *vs* F-N), tyrosine, benzoic acid, 2-Furoic acid, aconitic acid, and butyrylcarnitine (patients *vs* volunteers) in the training set while there were linoelaidic acid, gamma-linolenic acid, glycylproline, proline, asparagine, and carnitine (E-R *vs* E-N), hydroxypropionic acid, aspartic acid, dihomo-gamma-linolenic acid, and tyrosine (F-R *vs* F-N), dimethylglycine, citric acid, GCDCA, and 2-phenylpropionate (patients *vs* volunteers) in the validation set.

In the results of Boruta analysis (Figure 5), the metabolites marked as confirmed are the differential metabolites obtained by the final screening for subsequent model construction. As shown in Figure 5A-C, in addition to the above intersection metabolites, there were arachidonic acid, oleylcarnitine, and docosahexaenoic acid (F-R *vs* F-N), butyric acid, TCDCA, arachidonic acid, citric acid, and propionic acid (patients *vs* volunteers) confirmed in the training set. As shown in Figure 5D-F, in addition to the above intersection metabolites, there were TCDCA, benzoic acid, tyrosine, 2-Furoic acid, butyric acid, TCA, isocitric acid, hydrocinnamic acid, and propionic acid (patients *vs* volunteers) confirmed in the validation set.

Evaluation of model effects in different sets

In the training set, there were good sensitivity and specificity with the AUC value of 0.851 (F-R *vs* F-N) and 0.985 (patients *vs* volunteers) except for 0.733 (E-R *vs* E-N) (Figure 6A-C). In the validation set, there were good sensitivity and specificity with the AUC value of 1 (E-R *vs* E-N, patients *vs* volunteers) and 0.94 (F-R *vs* F-N) (Figure 6D-F). On the whole, the above AUC values of the two sets indicated good diagnostic capability in this study.



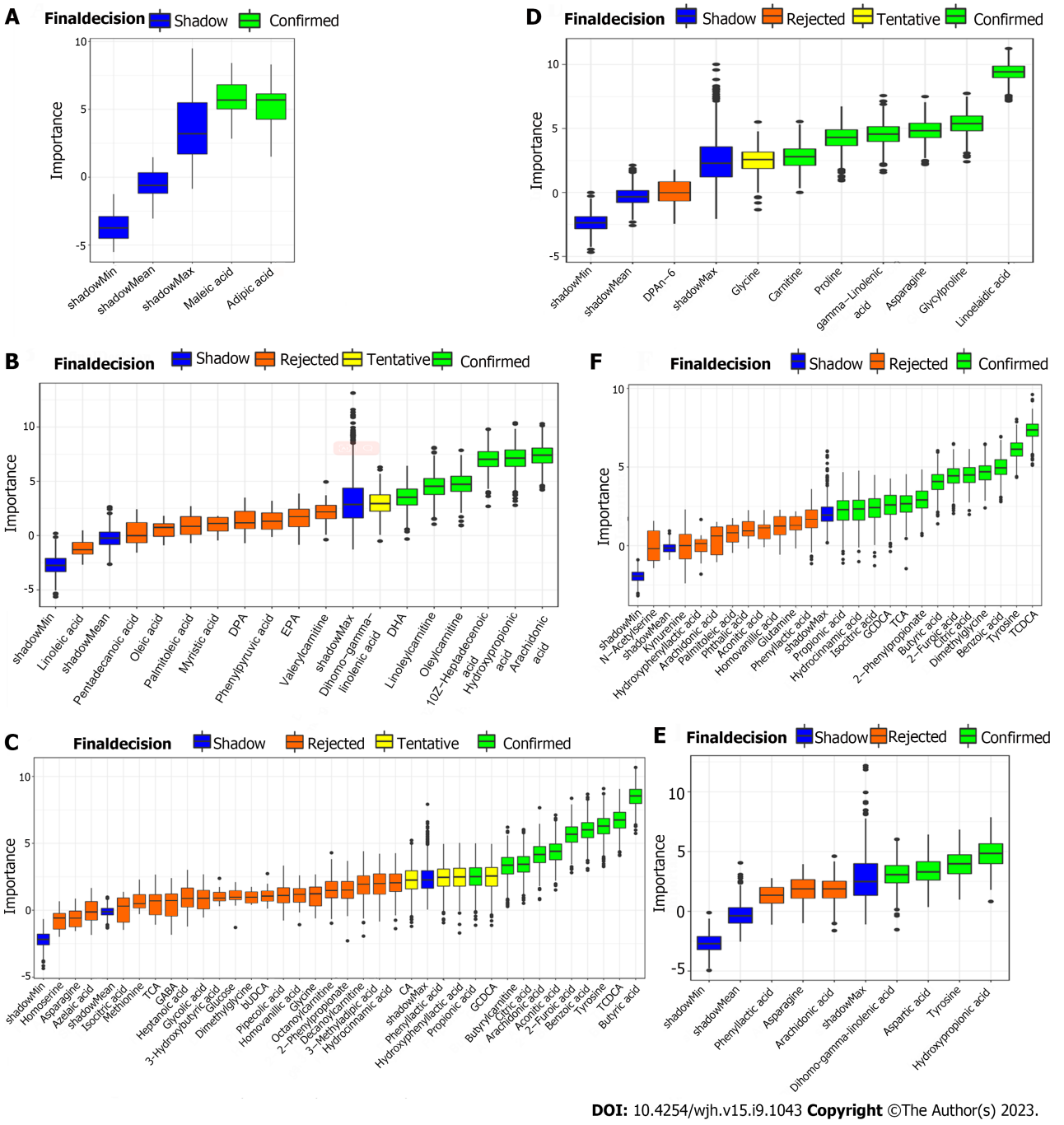
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Figure 4 Bubbleplot of the selected metabolites pathways. A: Bubbleplot of entecavir responders (E-R) *vs* entecavir no-responders (E-N) (training set); B: Bubbleplot of FuzhengHuayu tablet (FZHY) + entecavir responders (F-R) *vs* FZHY + entecavir no-responders (F-N) (training set); C: Bubbleplot of patients *vs* volunteers (training set); D: Bubbleplot of E-R *vs* E-N (validation set); E: Bubbleplot of F-R *vs* F-N (validation set); F: Bubbleplot of patients *vs* volunteers (validation set). TCA: Tricarboxylic acid.

DISCUSSION

With the global prevalence of HBV-related liver fibrosis on the rise, precise targeting of the population that responds to entecavir or entecavir + FZHY is of paramount importance for improving clinical efficacy through precision treatment. Metabolomics serves as a valuable tool for biomarker discovery[18]. In this study, we employed HPLC-MS and advanced multivariate statistical modeling to predict the serum differential metabolites associated with interventions effectively reversing HBV-related liver fibrosis. Our findings revealed the involvement of 7 metabolic pathways (E-R *vs* E-N), including linoleic acid metabolism, aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, alanine, aspartate, and glutamate metabolism, nitrogen metabolism, butanoate metabolism, and nicotinate and nicotinamide metabolism. Similarly, 7 metabolic pathways (F-R *vs* F-N) were identified, encompassing linoleic acid metabolism, aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, alanine, aspartate, and glutamate metabolism, nitrogen metabolism, beta-alanine metabolism, and fatty acid biosynthesis. Furthermore, 3 metabolic pathways (patients *vs*. volunteers) were noted, which included nitrogen metabolism, primary bile acid biosynthesis, and the TCA cycle. Regarding the intersection of differential metabolic pathways between the E-R *vs* E-N and F-R *vs* F-N groups, our study highlighted 4 common pathways: Linoleic acid metabolism, aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, and alanine, aspartate, and glutamate metabolism.

Regarding linoleic acid metabolism, a study suggested an inverse association between dietary linoleic acid intake and the risk of significant liver fibrosis, particularly emphasizing the ratio of unsaturated to saturated fatty acids[19]. Another clinical investigation demonstrated that specific alterations in linoleic acid metabolites could differentiate individuals with moderate alcohol-associated hepatitis from those with mild alcohol-associated liver disease among heavy drinkers. It is noteworthy that alcohol-associated liver diseases share common characteristics, spanning from steatosis to steatohepatitis, fibrosis, and cirrhosis[20]. Concerning aminoacyl-tRNA biosynthesis, an animal experiment revealed that Ganfule capsules could mitigate liver injury and liver fibrosis induced by bile duct ligation in mice. These effects were associated with the regulation and control of metabolic pathways, including glutamine metabolism, valine, leucine, and isoleucine biosynthesis, as well as aminoacyl-tRNA biosynthesis[21]. Furthermore, findings from a nonalcoholic fatty liver disease rat model indicated that metabolic disturbances primarily revolved around aminoacyl-tRNA biosynthesis, nitrogen metabolism, lipid metabolism, glyoxylate and dicarboxylate metabolism, and amino metabolism[22]. As for alanine, aspartate, and glutamate metabolism, a study aimed at investigating the role of the Wnt/β-catenin signaling pathway and the enzyme l-glutaminase in liver fibrosis pathogenesis and the potential benefits of niclosamide in treating liver fibrosis. It was observed that the group of rats treated with niclosamide and CC cytokine ligand-4 exhibited significant reductions

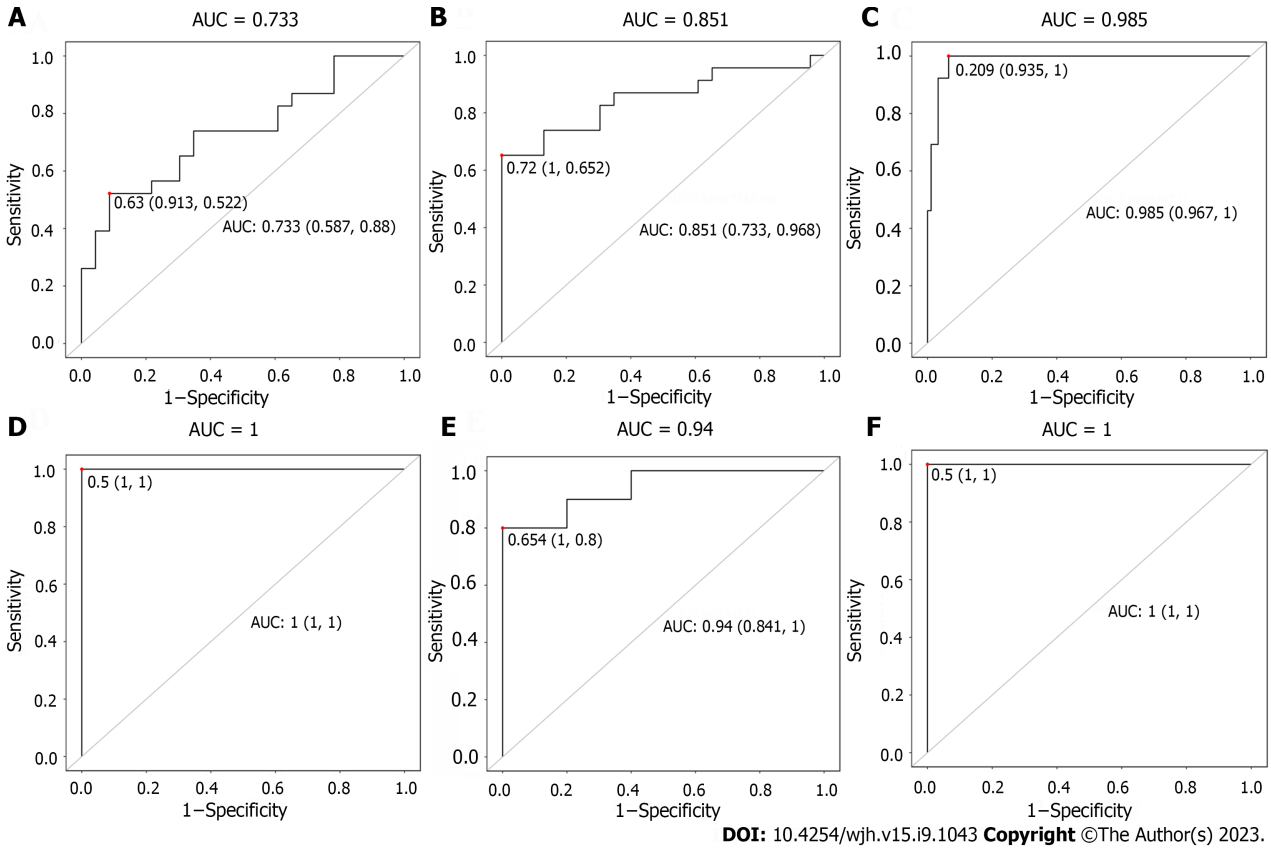


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Figure 5 Boxplot of all the differential metabolites. A: Boxplot of entecavir responders (E-R) *vs* entecavir no-responders (E-N) (training set); B: Boxplot of FuzhengHuayu tablet (FZHY) + entecavir responders (F-R) *vs* FZHY + entecavir no-responders (F-N) (training set); C: Boxplot of patients *vs* volunteers (training set); D: Boxplot of E-R *vs* E-N (validation set); E: Boxplot of F-R *vs* F-N (validation set); F: Boxplot of patients *vs* volunteers (validation set).

in TBIL, alanine transaminase, aspartate transaminase, β-catenin, l-hydroxyproline, and l-glutaminase activity. These findings led to the conclusion that Niclosamide protected rats against liver fibrosis by inhibiting the Wnt/β-catenin pathway and glutaminolysis[23]. In summary, the metabolic pathways identified in this study are intricately linked to the initiation and progression of liver fibrosis.

The investigation into baseline differential metabolites for predicting the response to entecavir or entecavir + FZHY in HBV-related fibrotic livers has unveiled crucial insights with the potential to enhance tailored treatments for individuals. Notably, our findings indicated that specific differential metabolites, as mentioned earlier, were closely associated with the response to entecavir and entecavir + FZHY in HBV-related fibrotic livers. Furthermore, this study proposed that these baseline differential metabolites could be effectively combined with clinical parameters to enhance the precision of personalized treatment for patients grappling with HBV-related liver fibrosis. This approach holds the key to reducing the incidence of treatment failures stemming from inappropriate therapeutic interventions. Moreover, the insights gleaned from this research bear significant implications for the advancement of biomarker-guided precision medicine. These differential metabolites can potentially be employed to predict disease progression, select the most suitable treatment modalities, and monitor treatment outcomes among HBV patients. Additionally, this study provides a



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Figure 6 Receiver operating characteristic curve of all the differential metabolites. A: Receiver operating characteristic (ROC) curve of entecavir responders (E-R) *vs* entecavir no-responders (E-N) (training set); B: ROC curve of FuzhengHuayu tablet (FZHY) + entecavir responders (F-R) *vs* FZHY + entecavir no-responders (F-N) (training set); C: ROC curve of patients *vs* volunteers (training set); D: ROC curve of E-R *vs* E-N (validation set); E: ROC curve of F-R *vs* F-N (validation set); F: ROC curve of patients *vs* volunteers (validation set). AUC: Area under the curve.

foundation for the exploration of novel metabolites or biomarkers that might serve as superior predictors of the response to entecavir or entecavir + FZHY in HBV-related fibrotic livers. Ultimately, these findings contribute to an enhanced understanding of the molecular mechanisms underpinning HBV-related liver fibrosis and may offer opportunities to more accurately evaluate the efficacy of individualized treatments. By comprehending the intricate association between these differential metabolites and the response to entecavir and entecavir + FZHY, healthcare practitioners can fine-tune treatment options for each patient, thereby optimizing the effectiveness of HBV-related liver fibrosis therapy. Furthermore, the outcomes of this study can serve as a valuable resource for the development of future pharmacological treatments that target different pathways more effectively in combatting HBV-related liver fibrosis.

There are several noteworthy limitations in our study. Firstly, all of our sample sources were confined to China. This geographically limited distribution could potentially restrict the broader applicability of our therapeutic regimen. Secondly, there was no dedicated FZHY monotherapy group. Given that all participants included in our study were CHB patients, and the development of liver fibrosis in these individuals was directly or indirectly attributed to HBV infection, antiviral therapy was considered the foundational treatment. Administering FZHY as the sole treatment to HBV-related liver fibrosis patients would be ethically inconsistent with clinical standards. Consequently, we lacked an observation of the therapeutic efficacy of FZHY in isolation. In regard to the FZHY monotherapy group, for future research endeavors, it may be considered to further validate the identified differential metabolites and metabolic pathways by selecting alternative etiologies of liver fibrosis for validation or by investigating the distinctions between monotherapy and combination therapy in animal experiments. Thirdly, our study exclusively focused on patients with hepatitis B, and whether our conclusions can be extrapolated to the treatment of liver fibrosis arising from other causes necessitates further exploration. Lastly, due to the cross-sectional nature of our study, external reproducibility should be further evaluated through prospective studies.

CONCLUSION

In summary, through metabolomis analysis, we have identified 4 metabolic pathways and 7 differential metabolites from serum that accurately differentiated responders from no-responders in the treatment of HBV-related liver fibrosis. If validated in future studies, these metabolic pathways and differential metabolites will be useful in improving the curative effect of entecavir + FZHY and promoting the development of precision medicine.

ARTICLE HIGHLIGHTS

Research background

After receiving entecavir or combined with FuzhengHuayu tablet (FZHY) treatment, some sufferers with hepatitis B virus (HBV)-related liver fibrosis could achieve a histological improvement while the others may fail to improve even worsen. Serum metabolomics at baseline in these patients who were effective in treatment remain unclear.

Research motivation

The key significance of this cross-sectional study is to predict the serum metabolites of the treatment (entecavir or entecavir + FZHY) that effectively reversed HBV-related liver fibrosis.

Research objectives

We are about to explore serum differential metabolites and metabolic pathways at baseline in HBV-related liver fibrosis patients who are response to the treatments.

Research methods

A total of 132 patients with HBV-related liver fibrosis and 18 volunteers as healthy controls were recruited. First, all subjects were divided into training set and validation set. Second, the included patients were subdivided into entecavir responders (E-R), entecavir no-responders (E-N), FZHY + entecavir responders (F-R), and FZHY + entecavir no-responders (F-N) following the pathological histological changes after 48 wk' treatments. Then, serum samples of all subjects before treatment were tested by high-performance liquid chromatography-tandem mass spectrometry. Data processing was conducted using multivariate principal component analysis and orthogonal partial least squares discriminant analysis. Diagnostic tests of selected differential metabolites were used for Boruta analyses and logistic regression.

Research results

As for the intersection about differential metabolic pathways between the groups E-R *vs* E-N and F-R *vs* F-N, results showed that 4 pathways including Linoleic acid metabolism, aminoacyl-tRNA biosynthesis, cyanoamino acid metabolism, alanine, aspartate and glutamate metabolism were screened out. As for the differential metabolites, these 7 intersected metabolites including hydroxypropionic acid, tyrosine, citric acid, taurochenodeoxycholic acid, benzoic acid, 2-furoic acid, and propionic acid were selected.

Research conclusions

Our findings showed that 4 metabolic pathways and 7 differential metabolites have potential usefulness in clinical prediction of the response of entecavir or combined with FZHY on HBV fibrotic liver.

Research perspectives

It is of great theoretical and practical significance to prevent the transformation of liver fibrosis to cirrhosis or even hepatocellular carcinoma and reduce the social burden.

FOOTNOTES

Author contributions: Liu CH and Zhao ZM conceived and designed the study; Dai YK, Fan HN, Huang K and Sun X performed the experiment; Dai YK, Fan HN, Huang K and Sun X analyzed the data; Dai YK wrote the paper; Liu CH and Zhao ZM contributed to supervision; All authors approved the final manuscript as submitted.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine.

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: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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META-ANALYSIS

Corrected QT interval in cirrhosis: A systematic review and meta-analysis

Vasileios Periklis Papadopoulos, Konstantinos Mimidis

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Abstract

BACKGROUND

Corrected QT (QTc) interval is prolonged in patients with liver cirrhosis and has been proposed to correlate with the severity of the disease. However, the effects of sex, age, severity, and etiology of cirrhosis on QTc have not been elucidated. At the same time, the role of treatment, acute illness, and liver transplantation (Tx) remains largely unknown.

AIM

To determine the mean QTc in patients with cirrhosis, assess whether QTc is prolonged in patients with cirrhosis, and investigate whether QTc is affected by factors such as sex, age, severity, etiology, treatment, acute illness, and liver Tx.

METHODS

In the present systematic review and meta-analysis, the searching protocol "{[QTc] OR [QT interval] OR [QT-interval] OR [Q-T syndrome]} AND {[cirrhosis] OR [Child-Pugh] OR [MELD]}" was applied in PubMed, EMBASE, and Google Scholar databases to identify studies that reported QTc in patients with cirrhosis and published after 1998. Seventy-three studies were considered eligible. Data concerning first author, year of publication, type of study, method used, sample size, mean age, female ratio, alcoholic etiology of cirrhosis ratio, Child-Pugh A/B/C ratio, mean model for end-stage liver disease (MELD) score, treatment with β-blockers, episode of acute gastrointestinal bleeding, formula for QT correction, mean pulse rate, QTc in patients with cirrhosis and controls, and QTc according to etiology of cirrhosis, sex, Child-Pugh stage, MELD score, and liver Tx status (pre-Tx/post-Tx) were retrieved. The Newcastle-Ottawa quality assessment scale appraised the quality of the eligible studies. Effect estimates, expressed as proportions or standardized mean differences, were combined using the random-effects, generic inverse variance method of DerSimonian and Laird. Subgroup, sensitivity analysis, and meta-regressions were applied to assess heterogeneity.

The study has been registered in the PROSPERO database (CRD42023416595).

RESULTS

QTc combined mean in patients with cirrhosis was 444.8 ms [95% confidence interval (CI): 440.4-449.2; $P < 0.001$ when compared with the upper normal limit of 440 ms], presenting high heterogeneity ($I2 = 97.5\%$; 95%CI: 97.2%-97.8%); both Egger's and Begg's tests showed non-significance. QTc was elongated in patients with cirrhosis compared with controls ($P < 0.001$). QTc was longer in patients with Child-Pugh C cirrhosis when compared with Child-Pugh B and A ($P < 0.001$); Child-Pugh B patients presented longer QTc when compared with Child-Pugh A patients ($P = 0.003$). The MELD score was higher in patients with cirrhosis with QTc > 440 ms when compared with QTc ≤ 440 ms ($P < 0.001$). No correlation of QTc with age ($P = 0.693$), sex ($P = 0.753$), or etiology ($P = 0.418$) was detected. β-blockers shortened QTc ($P< 0.001$). QTc was prolonged during acute gastrointestinal bleeding ($P = 0.020$). Tx tended to improve QTc ($P < 0.001$). No other sources of QTc heterogeneity were revealed.

CONCLUSION

QTc is prolonged in cirrhosis independently of sex, age, and etiology but is correlated with severity and affected by β-blockers and acute gastrointestinal bleeding. QTc is improved after liver Tx.

Key Words: Liver cirrhosis; Corrected QT interval; Child-Pugh stage; Model for end-stage liver disease score; Liver transplantation; Meta-analysis

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Core Tip: Corrected QT (QTc) interval is prolonged in patients with liver cirrhosis and has been proposed to correlate with the severity of the disease. The QTc upper normal limit in cirrhosis is widely debated. Moreover, the effects of sex, age, Child-Pugh stage, model for end-stage liver disease score, and etiology of cirrhosis have not been elucidated, while the role of liver transplantation has been largely unknown. The present study is the first systematic review and meta-analysis focusing on the topics mentioned above, thus aiming to determine whether QTc interval is a useful, easy, and inexpensive tool in the assessment of liver cirrhosis by clinicians.

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INTRODUCTION

The prolongation of ventricular repolarization, as reflected in rate-corrected QT (QTc) electrocardiogram interval, was first reported in 44% of patients with cirrhosis of alcoholic etiology[1,2]. QTc prolongation was soon recognized as a frequent electrocardiographic abnormality in patients with cirrhosis, regardless of the subsequent etiology[3,4]. QTc prolongation has been historically attributed to a broad spectrum of pathophysiological mechanisms involving electrolyte imbalance, sympathetic nervous system hyperactivity, portal hypertension, elevated bile salt plasma concentrations, direct alcohol toxicity, regimens such as β-blockers and diuretics, and stressful events such as acute gastrointestinal bleeding[1,3,5-10]. However, QTc prolongation is currently considered to reflect delayed ventricular repolarization in the presence of cirrhotic cardiomyopathy, an entity characterized mainly by ventricular diastolic dysfunction associated with liver cirrhosis in the absence of other known cardiac disease[11-19]. Of note, some contradictory evidence support that QTc prolongation is independent of the structural and functional abnormalities that characterize cirrhotic cardiomyopathy[20]. Moreover, it is debatable whether taming the gonadal hormone metabolism in cirrhosis might blur QTc sex-dependence observed in patients without cirrhosis[21]. Interestingly, liver transplantation (Tx) has been demonstrated to at least partly restore prolonged QTc[4,21-25].

QTc prolongation > 440 ms has been correlated with shortened overall survival in cirrhosis[3,26,27]; however, there is contradictory evidence obscuring this proposal[28]. More commonly, QTc length has been considered to reflect the severity of the disease in terms of either Child-Pugh stage[26,29-37] or model for end-stage liver disease (MELD) score[27,37-40]. On the contrary, several studies support dissimilar conclusions[4,41-45]. Additionally, the direct (due to alcoholic cardiomyopathy) and indirect (due to the aggravated course of the disease) role of alcohol in QTc cirrhosis-linked prolongation is still debatable[1]. Whether QTc abnormalities are more pronounced in alcoholic cirrhosis has not been elucidated yet, as there is contradictory evidence either for[33,46] or against[24,25,47] that possibility. Finally, QTc sex dependence might be less evident or even absent in patients with cirrhosis[3,21,23,25,30,38-41].

The present systematic review and meta-analysis was conducted to provide further evidence regarding a potential correlation between QTc length in patients with cirrhosis and age, sex, etiology of cirrhosis, severity of the disease in terms of Child-Pugh stage, and MELD score, treatment with β-blockers, episode of acute gastrointestinal bleeding, as well

as liver Tx by identifying all relevant studies and summarizing their results.

MATERIALS AND METHODS

Literature search

The study was conducted following Preferred Reporting in Systematic and Meta-Analysis (PRISMA) guidelines[48]. We used the PubMed and EMBASE databases to identify studies that reported QTc in patients with cirrhosis and were published between January 1998 and April 2023. We also utilized the Google Scholar database to retrieve any additional published or unpublished data, such as conference proceedings and other grey literature. We performed an iterative search until we could trace no additional publications. Moreover, a search for unpublished dissertations as well as other unpublished work was completed. The literature search was performed by both authors (Papadopoulos VP and Mimidis K). The study has been registered in the PROSPERO database (CRD42023416595); PROSPERO data were revised on August 8, 2023[49].

Study selection

The present systematic review was conducted following a search strategy that included the terms {[QTc] OR [QT interval] OR [QT-interval] OR [Q-T syndrome]} AND {[cirrhosis] OR [Child-Pugh] OR [MELD]}. Pre-specified eligibility criteria used the PICO strategy [P: Populations/people/patient/problem: Patients who have cirrhosis and healthy individuals (controls), I: Intervention(s): Liver Tx, C: Comparison: QTc in (1) Patients with cirrhosis *vs* upper normal limit; (2) Patients with cirrhosis *vs* controls; (3) Males with cirrhosis *vs* females with cirrhosis; (4) Patients with cirrhosis of Child-Pugh stage A *vs* B *vs* C; (5) Patients with cirrhosis of alcoholic etiology *vs* viral etiology; (6) Relation with age; (7) Relation with MELD score; (8) Patients with cirrhosis before *vs* after liver Tx; (9) Relation with β-blockers; (10) Relation with episode of acute gastrointestinal bleeding; and (11) Relation with age, sex, and etiology of cirrhosis in transplanted patients, O: Outcome: Combined mean, percentage; standardized mean difference (SMD)][50]. Exclusion criteria were: (1) Review articles, case reports, and letters; (2) Duplicated or overlapping studies (if that was the case, only the most recent or the highest level of study or the most informative study was included); and (3) Studies published only as abstracts. The process was performed independently by both authors. Mimidis K was responsible for resolving any discordance. The Cohen kappa statistic was preferred to assess the level of agreement between the two investigators. No software was used for study retrieval. Sources of financial support were traced where possible.

Data extraction

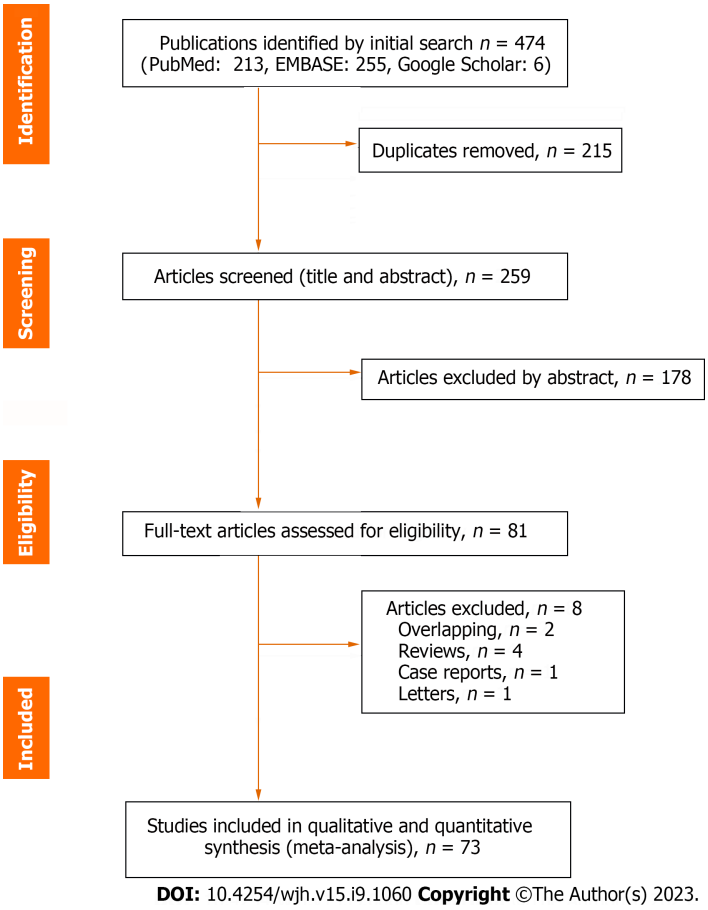
Data concerning first author, year of publication, type of study, method used, sample size, mean age, female ratio, alcoholic etiology of cirrhosis ratio, Child-Pugh A/B/C ratio, mean MELD score, use of β-blockers, formula for QT correction, mean pulse rate, QTc in patients with cirrhosis and controls, and QTc according to etiology of cirrhosis, sex, Child-Pugh stage, and Tx status (pre-Tx/post-Tx) were retrieved independently by both authors. Mimidis K supervised the process and resolved any potential discordance.

Risk of bias

Funnel plots assessed the risk of publication bias. Trim-and-fill analysis was used to impute missing studies in cases of significant publication bias. The Newcastle-Ottawa Scale (NOS) evaluated the risk of bias assessment of the eligible studies; scores ≥ 7-9, 4-6, and < 4 were considered to reflect low, intermediate, and high risk, respectively[51]. Furthermore, the GRADE assessment was used to evaluate evidence certainty rating risk of bias, imprecision, inconsistency, indirectness, publication bias, and effect size for every endpoint[52]. When comparing hazard ratios (HRs) between two groups, small, medium, and large effect sizes were considered to be approximately 1.3, 1.9, and 2.8, respectively[53].

Statistical analysis

Data were synthesized using MedCalc Statistical Software version 20.218 (MedCalc Software bv, Ostend, Belgium; https://www.medcalc.org; 2023). Effect estimates, expressed as QTc/upper normal limit percentage or SMD, were extracted from every study possible and combined using the random-effects, generic inverse variance method of DerSimonian and Laird[54], which assigned the weight of each study in the pooled analysis inversely to its variance. Combination of means and standard deviations (SDs) were performed using the freely available online tool located at https://www.statstodo.com/CombineMeansSDs.php. Means and estimates based on sample size, median, range, and interquartile range were calculated using the freely available online tool located at https://www.math.hkbu.edu.hk/~tongt/papers/median2mean.html[55-57]. SD estimates were computed from the mean, confidence interval (CI), and sample size using the freely available online tool https://www.omnicalculator.com/statistics/confidence-interval. Effect size Cohen's *d* was calculated from 2^2 contingency data using the freely available online tool https://www.psychometrica.de/effect_size.html. The correlation coefficient was calculated for paired data using the formula $(SD_{baseline}^2 + SD_{final}^2 - SD_{change}^2)/(2 \times SD_{baseline} \times SD_{final})$. A subgroup analysis was performed to investigate the potential effect of hospitalization, comorbidities, and treatments affecting QT. Sensitivity analysis assessed the correlation coefficient *r* concerning pre-Tx and post-Tx status. HR was calculated from time-to-event data and log-rank *P* value as described elsewhere[58]. The NORMSINV function, freely available from MedCalc software, was used for that purpose. The formula $QT_{Bazett} = QT_{Fridericia} \times RR^{-1/6}$ was used to convert $QT_{Fridericia}$ (QTc corrected with the use of Fridericia formula) to QT_{Bazett} (QTc corrected with the use of



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Figure 1 Study selection.

Bazett formula) given that the mean heart rate was 60-100 beats/min. Heterogeneity was approached using the Q test and I^2 statistic; Q test P value < 0.10 was indicative of a statistically significant result. Furthermore, a value of $I^2 \leq 25\%$ was indicative of insignificant heterogeneity, 26%-50% of low heterogeneity, 51%-75% of moderate heterogeneity, and > 75% of high heterogeneity[59,60]. Heterogeneity was analyzed through meta-regressions and derived standardized coefficients beta (bSD) focusing separately on study characteristics and quality assessment. Multivariate analysis was omitted in cases where the available studies numbered less than 10. Meta-regressions were performed using SPSS 26.0 software (IBM Corp., Armonk, NY, United States). Synthesis of effect sizes was performed using the MedCalc® Statistical Software version 20.218 and Meta-Essentials Excel-based software[61].

RESULTS

Study selection

Four hundred and sixty-eight potentially relevant publications (PubMed: 213, EMBASE: 255, Google Scholar: 6) were identified. No unpublished data of interest were detected. The authors removed duplicates and critically appraised the title, the abstract, and the full text of the remaining publications (Figure 1). Finally, 73 studies, including 14495 patients, were eligible for qualitative and quantitative meta-analyses (Table 1)[3,4,6,7,10-15,17,18,21,23-47,62-96].

QTc interval in patients with cirrhosis

QTc was elongated in patients with cirrhosis when compared with controls (SMD = 1.187; 95%CI: 0.804-1.570; P < 0.001). The I^2 was 88.8% (95%CI: 81.0%-93.4%; P < 0.001) (Figure 2A). QTc combined mean in patients with cirrhosis (n = 7715) was 444.8 ms (95%CI: 440.4-449.2; P < 0.001 when compared with the upper normal limit of 440 ms), presenting high heterogeneity (I^2: 97.5%; 95%CI: 97.2%-97.8%; P < 0.001) (Figure 2B).

A subgroup analysis was performed to investigate the potential effect of hospitalization, comorbidities, and treatments affecting QT. Thus, when non-hospitalized patients with cirrhosis without any other comorbid condition or treatment with known effect of QT were considered (n = 1448), the QTc combined mean was 444.0 ms (95%CI: 437.8-450.1) with an I^2 of 92.4% (95%CI: 89.6%-94.5%; P < 0.001). When patients with cirrhosis who either might have been hospitalized or presented other comorbidities or were treated with regimens affecting QT were considered (n = 6267), the QTc combined mean was 445.3 ms (95%CI: 439.6-450.6) with an I^2 of 98.1% (95%CI: 97.9%-98.4%; P < 0.001). These two groups yielded comparable results (P = 0.823) (Supplementary Figures 1 and 2).

Table 1 Characteristics of eligible studies

Ref.	Type of study	Device	Formula	Patients, *n*	Controls, *n*	Female ratio	Alcoholic etiology ratio	Child-Pugh			MELD score	Age in yr	QTc									
								A, *n*	B, *n*	C, *n*				Prolongation ratio	Controls	Females	Viral etiology	Acohol etiology	Child-Pugh A	Child-Pugh B	Child-Pugh C	Tx pre	Tx post
Wang *et al*[62], 2023	R	E	B	1022		0.095	1					52.6 ± 11.6		0.107									
Bilous *et al*[10], 2023	P	H	B	33		0.394		8	14	11		48.0 ± 12.0	393.7 ± 35.0										
Barutcu *et al*[37], 2023	R	E	B	100	100	0.440		32	34	34	16 ± 8	60.0 ± 42.2	446.3 ± 49.3		439 ± 33				410 ± 23	455 ± 39	473 ± 53		
Lu *et al*[46], 2022	R	E	B	3529		0.233	0.182					55.0 ± 11.0		0.158									
Wang *et al*[63], 2022	R	E	B	189		0.783		102	56	31		59.4 ± 11.8	435.9 ± 46.1	0.243					433 ± 45	439 ± 47	439 ± 47		
Li *et al*[27], 2021	P	E	F	274		0.456		108	122	44	12 ± 4	61.8 ± 12.8		0.328									
Ou *et al*[40], 2021	R	E	B	167		0.281	0.168	70	81	16	11 ± 4	52.9 ± 10.8		0.665									
Ko *et al*[25], 2021	R	E	B	408		0.236	0.093					57.1 ± 12.0	452.0 ± 31.0	0.650								452 ± 31	430 ± 32
Héla *et al*[36], 2020	P	H	B	42		0.429	0.095	12	15	15		60.0 ± 13.2	435.9 ± 21.8	0.476					423 ± 19	429 ± 17	453 ± 17		
Abrahamovych *et al*[64], 2020	R	H	B	87		0.276	1					44.5 ± 4.3	443.8 ± 34.4										
Ibrahim *et al*[65], 2020	P	E	B	50		0.580	0	38	12	0		52.0 ± 12.0	415.8 ± 24.4										
Hussain *et al*[66],	P	E	B	87	87	0.460	0.316					47.0	470.0	0.218	400 ± 50								

2020											± 13.3	± 50.0										
Kim *et al*[35], 2020	R	E	B	310		0.274	0.274	105	94	111	46.0 ± 17.0	450.0 ± 43.0					460 ± 44	417 ± 29	452 ± 34	480 ± 38		
Toma *et al*[67], 2020	P	E	B	63		0.508	0	18	20	25	56.2 ± 13.5	452.5 ± 27.7	0.460			453 ± 28		445 ± 27	451 ± 23	459 ± 31		
Bhardwaj *et al*[68], 2020	P	E	B	100	100	0.150	0.530	4	35	61	49.8 ± 13.6	458.5 ± 27.0		424 ± 28								
Gaafar *et al*[69], 2019	P	E	B	112			0					424.4 ± 36.6										
Kazankov *et al*[28], 2019	R	E	B	915								415.0 ± 30.0										
Moaref *et al*[70], 2019	P	E	B	30		0.367				16 ± 5	41.0 ± 6.6											
Santeusanio *et al*[71], 2019	R	E	B	258		0.337	0.097			18 ± 10	59.5 ± 9.7	454.4 ± 27.9	0.403									
Biselli *et al*[72], 2019	R	E	F	474		0.352	0.236			13 ± 5	61.7 ± 12.6	438.0 ± 42.0										
Tieranu *et al*[45], 2018	P	E	B	60		0.433		6	28	26	59.4 ± 7.3	457.8 ± 23.9						456 ± 27	452 ± 26	462 ± 20		
Lee *et al*[39], 2018	R	E	B	283		0.247	0.113			17 ± 11	55.1 ± 7.7	449.9 ± 31.6	0.636								450 ± 32	435 ± 32
Hajiaghamohammadi *et al*[73], 2018	P	E	B	37		0.432		12	12	13	58.8 ± 11.5	418.5 ± 41.9										
Główczyńska *et al* [44], 2018	R	E	B	151		0.371	0.179	50	73	28	12 ± 5	49.0 ± 12.3	426.3 ± 41.6	0.338		426 ± 41	432 ± 45	423 ± 38	424 ± 46	438 ± 34		
Tahata *et al*[74], 2018	P	E	B	104		0.654		104	0	0		71.1 ± 8.4	415.9 ± 30.6					416 ± 31				

Tsiompanidis *et al* [75], 2018	P	E	B	51		0.373	0.333	22	18	11	28 ± 19	55.2 ± 14.2	428.1 ± 31.0	0.431				437 ± 31	419 ± 30	419 ± 30	435 ± 30		
Yap *et al*[43], 2018	R	E	B	148		0.527	0.155	17	57	9		72.4 ± 14.0	440.3 ± 45.6						464 ± 63	432 ± 33	448 ± 63		
Kim *et al*[76], 2017	R	E	B	406		0.404	0.389				18 ± 9	56.4 ± 9.0	454.5 ± 27.8	0.510									
Rimbaş *et al*[18], 2018	P	E	B	46	46	0.348	0.522	23	16	7	13 ± 5	57.0 ± 9.0	436.0 ± 30.0	0.413	404 ± 21			438 ± 35					
Salgado *et al*[42], 2016	P	E	B	67		0.478	0.239	25	26	16		54.0 ± 12.9	418.7 ± 26.6	0.224					415 ± 34	418 ± 21	426 ± 22		
Naqvi *et al*[34], 2016	P	E	B	89		0.438	0	17	29	43		51.5 ± 12.4	475.1 ± 73.3	0.461					420 ± 36	459 ± 56	508 ± 78		
Zhao *et al*[38], 2016	R	E	B	1268		0.347	0.253	497	528	140	6 ± 7	56.0 ± 12.1		0.382									
Sonny *et al*[77], 2016	R	E	B	106		0.280	0.179				17 ± 8	55.0 ± 9.0	453.0 ± 28.0									453 ± 28	442 ± 29
Barbosa *et al*[17], 2016	C	E	B	26		0.154	0.769	17	8	1	9 ± 5	54.6 ± 10.4	460.0 ± 23.0	0.769									
Carvalheiro *et al*[78], 2016	R	E	F	106		0.198	0.651	23	24	59	17 ± 8	54.8 ± 8.5		0.189									
Pourafkari *et al*[79], 2016	R	E	B	69		0.348	0.217	14	28	27	17 ± 7	56.8 ± 16.0	452.2 ± 46.0	0.507					453 ± 52	455 ± 40	449 ± 50		
Barakat *et al*[80], 2015	P	E	B	74		0.324	0				19 ± 26		473.1 ± 25.1										
Voiosu *et al*[81], 2015	P	E	F	74		0.378	0.378	43	12	19	13 ± 5	58.0 ± 11.0	418.3 ± 26.8										
Cichoż-Lach *et al*[82], 2015	R	E	B	122	32	0.344	0.664	28	40	54		42.1 ±	447.5 ±		394 ± 23		465 ± 50	443 ± 7	438 ± 35	434 ± 40	476 ± 40		

											12.7	29.9										
Peter *et al*[83], 2014	P	E	B	78	0.154	0.538				15 ± 5	51.2 ± 11.9	424.0 ± 10.2										
Moaref *et al*[15], 2014	P	E	B	80	0.375		0	0	80		41.0 ± 14.0	490.0 ± 51.0									490 ± 51	
Josefsson *et al*[84], 2014	R	E	B	202	0.307		22	91	89	17 ± 6	52.0 ± 11.0		0.262									
Karagiannakis *et al*[14], 2014	P	E	B	45	0.267	0.444	26	15	3	12 ± 4	57.2 ± 12.4	428.0 ± 27.5										
Yataco *et al*[41], 2014	R	E	B	17	0.471	0.235	0	1	16	29 ± 7	57.9 ± 7.9	461.4 ± 37.4	0.529		461 ± 38	459 ± 31	479 ± 42					
Bhatti *et al*[85], 2014	C	E	B	166	0.422	0	22	69	75		57.1 ± 12.0	429.9 ± 45.1	0.247									
Møller *et al*[86], 2012	P	E	B	10	0.500						55.6 ± 10.0	450.0 ± 20.0										
Trevisani *et al*[87], 2012	R	E	F	70	0.329	0.186				16 ± 1	61.7 ± 12.5	415.9 ± 36.0	0.243									
Zamirian *et al*[24], 2012	P	E	B	243	0.380	0.049						446.9 ± 12.5	0.432			483 ± 20	478 ± 25				447 ± 13	383 ± 49
Kim *et al*[88], 2011	P	E	B	100	0.140		18	48	34	14 ± 8	52.3 ± 8.3											
Shin *et al*[89], 2011	P	E	B	50	0.200		14	19	17		52.0 ± 7.0	433.2 ± 32.7	0.440									
Mozos *et al*[33], 2011	P	E	B	38	0.500	0.395	16	13	9		58.0 ± 12.0	493.0 ± 46.0	0.711			481 ± 51	527 ± 50	462 ± 25	493 ± 62	520 ± 45		
Vuppalanchi *et al*[90], 2011	P	E	B	14	0.286	0.500				11 ± 2	53.9 ± 9.6											

Møller *et al*[91], 2010	P	E	B	43		0.326	1	17	18	8	11 ± 3	57.4 ± 9.1	453.0 ± 31.0									
Zurick *et al*[23], 2010	R	E	B	269		0.372					21	50.9	448.3 ± 41.3	0.502		449 ± 11					449 ± 41	417 ± 41
Lossnitzer *et al*[13], 2010	P	E	B	20		0.450	0.600				15 ± 6	52.4 ± 9.4		0.250								
Genovesi *et al*[32], 2009	P	H	B	40		0.000	0.450	26	15	7		56.1 ± 11.2	439.3 ± 30.2	0.525					425 ± 24	452 ± 30	465 ± 24	
Zambruni *et al*[31], 2008	P	E	B	30		0.167	0.233	15	10	5	13 ± 1	58.8 ± 11.0	455.4 ± 26.8	0.367			459 ± 38	450 ± 38	440 ± 16	466 ± 26	486 ± 20	
Henriksen *et al*[92], 2007	P	E	B	48		0.354	0.917	15	19	14		52.1 ± 11.1	450.0 ± 33.9	0.563					441 ± 53	441 ± 39	470 ± 76	
Kosar *et al*[93], 2007	R	E	B	33		0.455		9	15	9		53.0 ± 11.0	412.5 ± 89.6						405 ± 51	411 ± 46	427 ± 64	
Hansen *et al*[12], 2007	P	H	B	23		0.261	0.609	6	10	7		55.0 ± 8.6	441.0 ± 31.7	0.478					420 ± 53	438 ± 48	461 ± 45	
Zuberi *et al*[94], 2007	C	E	B	78		0.269		6	19	53		34.4	438.0 ± 15.0	0.192								
Zambruni *et al*[47], 2007	P	E	B	100	53	0.370	0.210	34	39	27		57.2 ± 7.6	465.2 ± 33.3	0.520	416 ± 24							
Ytting *et al*[7], 2005	P	E	B	80		0.263	0.488	33	33	14		51.0 ± 10.4	447.0 ± 42.3	0.513								
Adigun *et al*[21], 2005	P	E	B	183		0.366	0.164	7	143	33		47.7 ± 7.4	419.6 ± 31.8	0.279		419 ± 35					436 ± 21	421 ± 21
Henriksen *et al*[95], 2004	P	E	B	24		0.125	1	3	12	9		58.3 ± 11.0	462.0 ± 40.3	0.583				462 ± 40				
Bal and Thuluvath [26], 2003	R	E	B	409		0.440	0.196	0	273	136		49.0 ±	439.4 ±	0.396				456 ± 42		434 ± 31	451 ± 43	450 ±	429 ± 29

											14.2	36.4										39	
Trevisani *et al*[6], 2003	P	E	B	19		0.474					61.2 ± 6.5	465.0 ± 26.2	0.842										
Mimidis *et al*[30], 2005	R	E	B	52		0.250	0.558	23	19	10	61.3 ± 11.1	466.6 ± 37.8	0.596		468 ± 15	466 ± 43	468 ± 15	446 ± 27	480 ± 39	489 ± 36		
Henriksen *et al*[11], 2002	P	E	B	24	17	0.125	1	3	12	9	58.3 ± 11.0	462.0 ± 40.3	0.375	410 ± 41			462 ± 40					
Puthumana *et al*[29], 2001	R	E	B	130		0.469		42	53	35	52.0 ± 10.0	442.7 ± 29.3	0.446					432 ± 23	443 ± 29	455 ± 33		
Quera *et al*[96], 2000	R	E	B	47								460.0 ± 30.0	0.723									
Bernardi *et al*[3], 1998	P	E	B	94	37	0.277	0.074	24	45	25	53.1 ± 13.6	440.3 ± 31.0	0.468	394 ± 36	444 ± 25		453 ± 25					
Finucci *et al*[4], 1998	P	E	B	75	24	0.360	0.453	23	37	15	57.0 ± 11.0	452.0 ± 33.0	0.600	414 ± 28			463 ± 31				449 ± 31	415 ± 26

C: Case-control; NOS: Newcastle-Ottawa scale; P: Prospective; R: Retrospective; E: Electrocardiography; B: Bazett's formula; H: Holter; F: Fridericia's formula; QTc: Corrected QT; MELD: Model for end-stage liver disease; Tx: Transplantation.

The pre-specified upper normal limit for QTc was higher for females (median: 440 ms; range: 440-470 ms) when compared with males (median: 440 ms; range: 420-462 ms). The related samples Wilcoxon signed rank test showed statistical significance ($P < 0.001$) (Figure 3).

Effect of sex and age on QTc interval

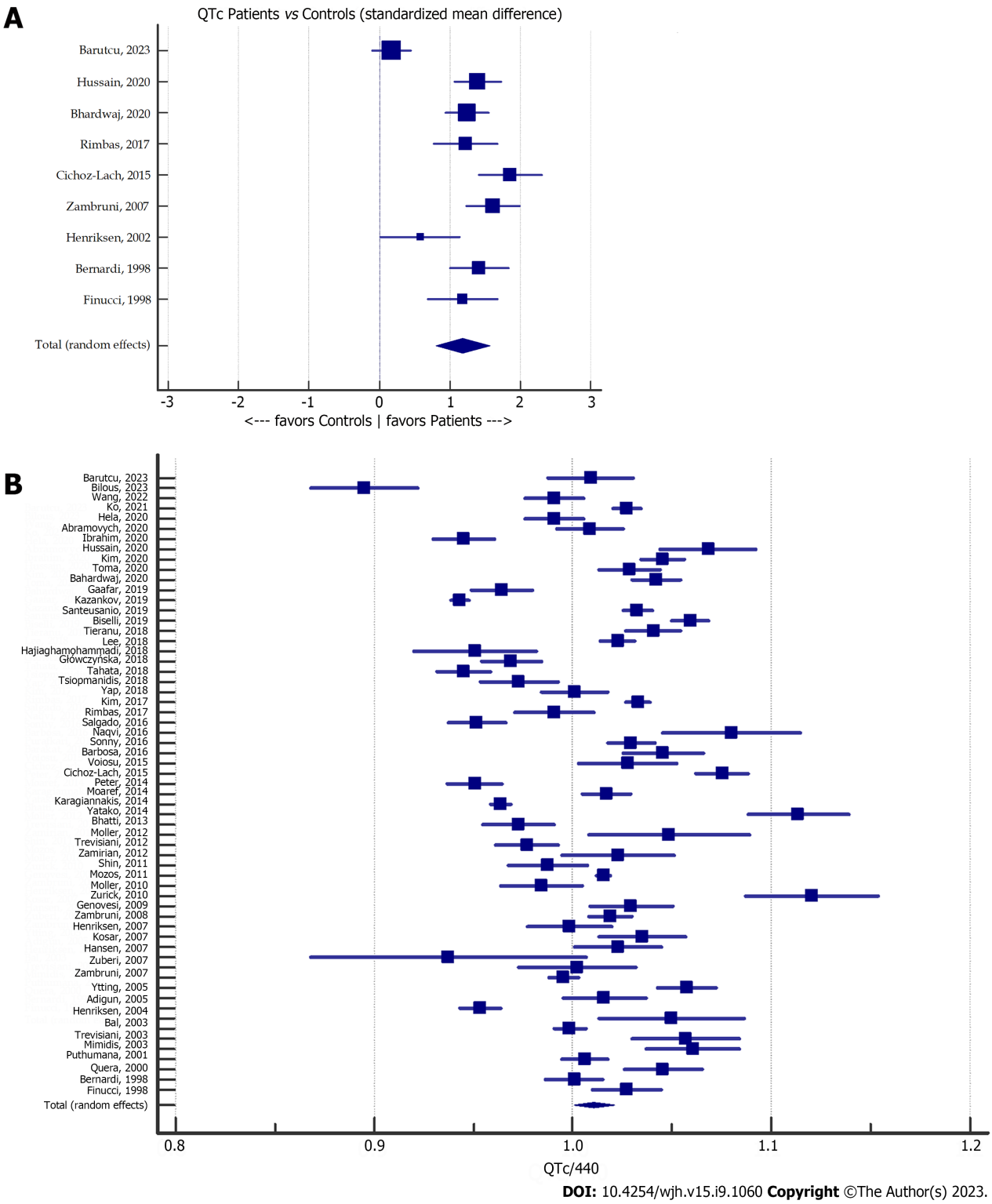
QTc was comparable between male and female patients (SMD = -0.032; 95%CI: -0.229 to 0.165; $P = 0.753$); I^2 was 78.5% (95%CI: 62.0%-87.8%; $P < 0.001$) (Figure 4A). Moreover, no correlation of QTc with age ($P = 0.974$) was detected, even after adjustment for alcoholic etiology rate and MELD score ($P_{adj} = 0.160$).

Effect of etiology of cirrhosis on QTc interval

Patients with cirrhosis of alcoholic etiology exhibited comparable QTc with those of viral etiology (SMD = 0.095; 95%CI: -0.109 to 0.264; $P = 0.418$). Heterogeneity was moderate (I^2: 47.8%; 95%CI: 0.0%-74.8%; $P = 0.045$) (Figure 4B).

Effect of Child-Pugh stage and MELD score on QTc interval

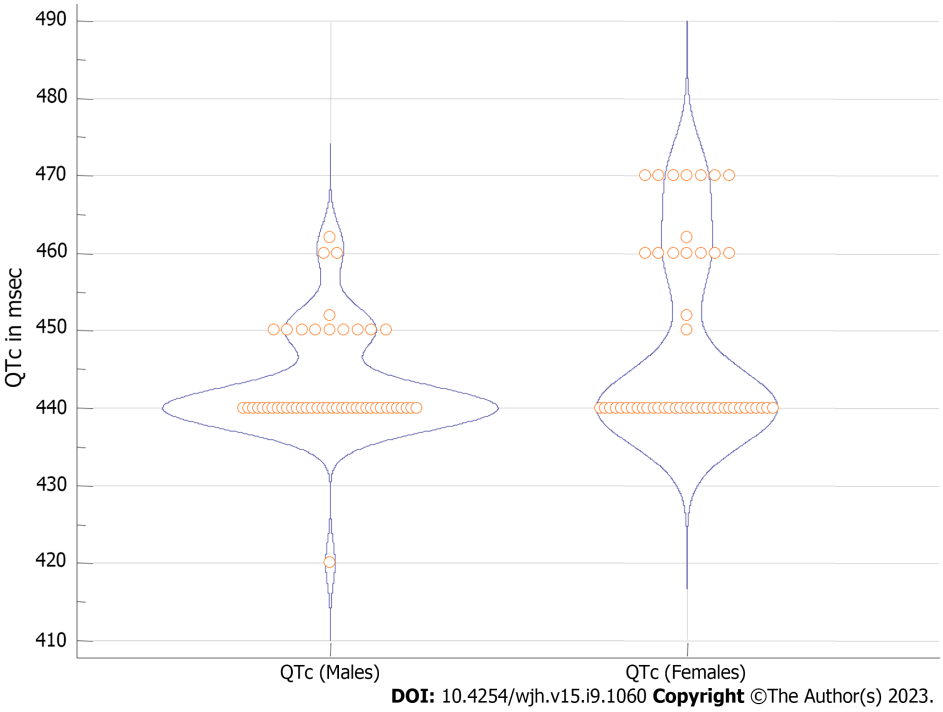
QTc was longer in patients with Child-Pugh C cirrhosis when compared with Child-Pugh A (SMD = 0.860; 95%CI: 0.547-



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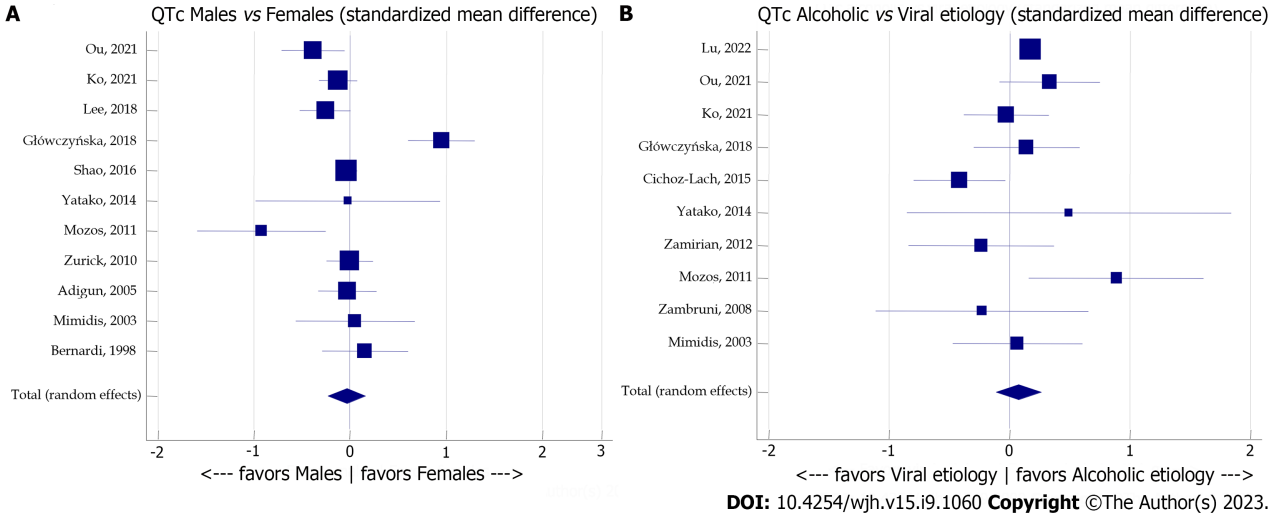
Figure 2 Meta-analysis forest plot. A: Corrected QT (QTc) in patients with cirrhosis *vs* controls; B: QTc compared with the upper normal limit (440 ms) ratio in patients with cirrhosis. QTc: Corrected QT.

1.173; $P < 0.001$) and B (SMD = 0.474; 95%CI: 0.344-0.6003; $P < 0.001$); I^2 was 80.8% (95%CI: 71.5%-87.1%; $P < 0.001$) and 24.9% (95%CI: 0.0%-55.4%; $P = 0.647$), respectively (Figures 5A and B). Moreover, Child-Pugh B patients with cirrhosis were characterized by longer QTc when compared with Child-Pugh A patients (SMD = 0.372; 95%CI: 0.126-0.619; $P = 0.003$); I^2 was 76.0% (95%CI: 63.5%-84.2%; $P < 0.001$) (Figure 5C). Considering the effect of the Child-Pugh score on QTc, a significant dose-response gradient was observed using Spearman's non-parametric correlation coefficient (rho = 0.526, $P < 0.001$). The MELD score was higher in patients with cirrhosis with QTc > 440 ms when compared with patients with QTc ≤ 440 ms (SMD = 0.509; 95%CI: 0.249-0.769; $P < 0.001$); I^2 was 78.1% (95%CI: 47.4%-90.9%; $P = 0.001$) (Figure 6A).



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Figure 3 Violi plot for corrected QT upper normal limit used in the included studies. QTc: Corrected QT.



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Figure 4 Meta-analysis forest plot concerning the effect of sex and etiology of cirrhosis on corrected QT. A: The effect of sex on corrected QT (QTc) in patients with cirrhosis; B: The effect of etiology of cirrhosis on QTc. QTc: Corrected QT.

Role of liver Tx regarding QTc interval

Liver Tx tended to improve QTc (pre-Tx *vs* post-Tx QTc SMD = 0.808; 95%CI: 0.488-1.129; $P < 0.001$). I^2 was 93.9% (95%CI: 90.1%-96.2%; $P < 0.001$) (Figure 6B). Since pre-Tx and post-Tx QTc values were correlated, the correlation coefficient r was 0.7, using two separate approaches: (1) Sensitivity analysis for $r = 0.1$ to $r = 0.9$ (step 0.1), which suggested that the combined Hedges'g (0.714; 95%CI: 0.645-0.783) with I^2: 0.00% ($P = 0.988$) corresponded to $0.7 < r < 0.8$; and (2) Direct calculation from Finucci *et al*[4], which resulted in $r = 0.642$ (Figure 7). QTc improvement after Tx remained unaffected by age ($P = 0.417$) and was negatively correlated with female ratio ($P = 0.002$), alcoholic etiology of cirrhosis ratio ($P < 0.001$), and age of the study ($P = 0.019$) (Figures 8A-D).

Pharmacological effects on QTc: The paradigm of β-blockers

The effect of β-blockers on QTc was investigated using data from three relevant studies. Patients with cirrhosis who were treated with β-blockers presented shorter QTc than those who were not (SMD = -0.540; 95%CI: -0.836 to -0.243; $P < 0.001$); I^2 was 0.0% (95%CI: 0.0%-92.1%; $P = 0.653$) (Supplementary Figure 3).

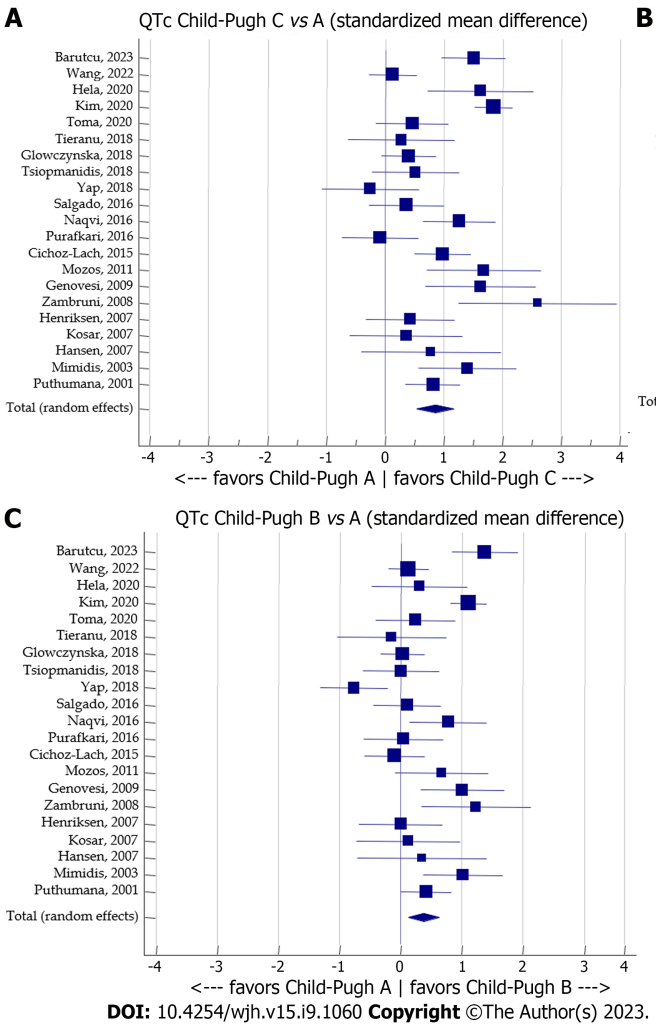


Figure 5 Meta-analysis forest plots concerning the effect of the Child-Pugh stage on corrected QT. A: Child-Pugh stage C *vs* A; B: Child-Pugh stage C *vs* B; C: Child-Pugh stage B *vs* A. QTc: Corrected QT.

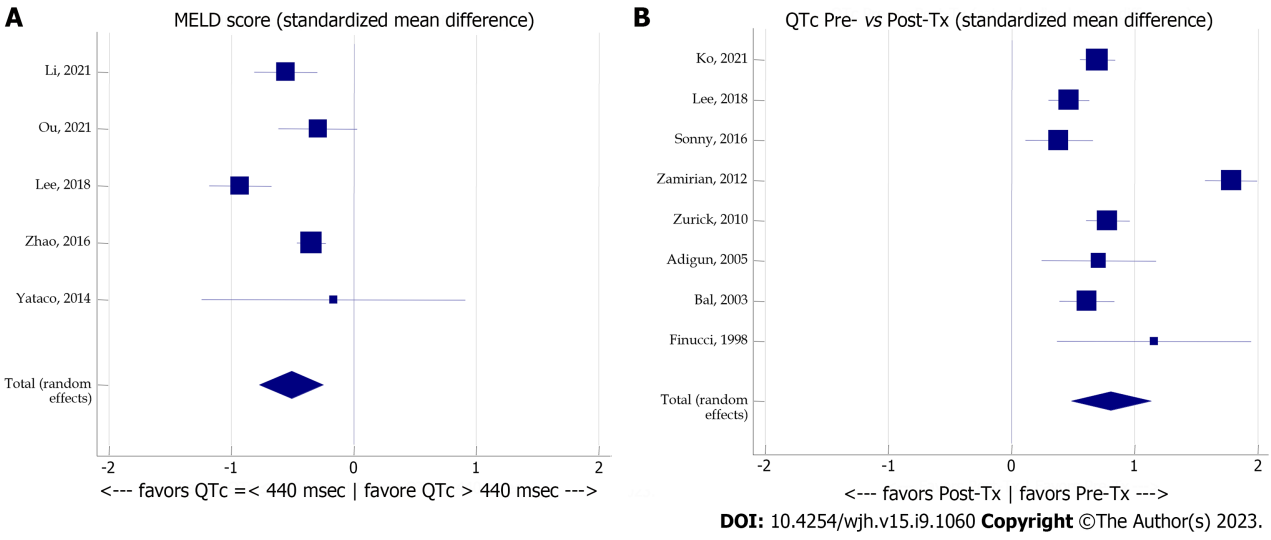


Figure 6 Meta-analysis forest plot concerning the effect of the model for end-stage liver disease score and liver transplantation on corrected QT. A: The effect of the model for end-stage liver disease score on corrected QT (QTc); B: The effect of liver transplantation on QTc. Tx: Transplantation; MELD: Model for end-stage liver disease; QTc: Corrected QT.

Acute gastrointestinal bleeding

QTc was prolonged during acute gastrointestinal bleeding, as deduced from two studies providing paired data (SMD = 1.800; 95%CI: 0.287-3.313; P = 0.020); I^2 was 96.7% (95%CI: 91.1%-98.8%; P < 0.001) (Supplementary Figure 4). Moreover, QTc was restored among survivors of an episode of gastrointestinal bleeding (SMD = 0.183; 95%CI: -0.051 to 0.417; P = 0.124); I^2 was 0.0% (95%CI: 0.0%-0.0%; P = 0.770) (Supplementary Figure 5).

Other potential sources of QTc heterogeneity

Meta-regression over 54 studies providing complete data revealed no independent correlation of QTc with study type (prospective *vs* others; bSD = -0.089; P = 0.517), device used (electrocardiograph *vs* Holter; bSD = 0.164; P = 0.237), or year of publication (bSD = 0.218; P = 0.118).

Overall survival according to QTc

Patients with cirrhosis with QTc ≤ 440 ms (n = 46) when compared with those with QTc > 440 ms (n = 40) had a survival HR of 2.666 [95%CI: 1.131-6.284; P = 0.025; standard error (SE) = 0.4375][3]. Similarly, patients with cirrhosis with QTc ≤ 440 ms (n = 247) when compared with those with QTc > 440 ms (n = 162) had a survival HR of 1.727 (95%CI: 1.054-2.828; P = 0.030; SE = 0.2518)[26]. Lastly, patients with cirrhosis with QTc ≤ 440 ms (n = 55) when compared with those with QTc > 440 ms (n = 55) had a survival HR of 2.464 (95%CI: 1.407-4.313; P = 0.0016; SE = 0.2858)[27]. These data demonstrated that patients with cirrhosis with QTc ≤ 440 ms when compared with those with QTc > 440 ms had a survival HR of 2.228 (95%CI: 1.640-2.815; P < 0.001) with an I^2 of 63.1% (95%CI: 0.0%-89.5%; P = 0.067) (Figure 9A).

Risk of bias assessment

The funnel plot referring to QTc/440 ratio combined mean was symmetric (Figure 9B). Moreover, both Egger's and Begg's tests showed non-significance (P = 0.151 and P = 0.985, respectively). The risk of bias assessment with the aid of the NOS and evaluation of evidence certainty derived from GRADE assessment are provided in Tables 2 and 3, respectively. Of note, no correlation of QTc with NOS low, intermediate, and high risk (P = 0.772) was detected, even after adjustment for alcoholic etiology rate and MELD score (P_{adj} = 0.651).

DISCUSSION

The present work represents the first systematic review and meta-analysis of QTc interval in cirrhosis. We demonstrated that QTc is prolonged in patients with cirrhosis compared with the most commonly used upper normal limit for QT interval (440 ms). Moreover, we showed that QTc prolongation in cirrhosis is linked with overall survival and is more evident in severe forms of the disease, as described by Child-Pugh stage, as well as in cases where alcohol as the etiology factor prevails when compared with viral hepatitis B or C. Interestingly, the fact that QTc prolongation in cirrhosis is a potentially reversible electrocardiographic abnormality is reflected by the fact that is at least partly restored after liver Tx.

Evidence of high quality indicates that liver Tx exerts a large beneficial effect in QTc. In contrast with Adigun *et al*[21], this amelioration has been shown to be negatively associated with age, male sex, and alcohol as the etiology of cirrhosis. This phenomenon could be partly attributed to the redefinition of QTc-affecting drugs, such as β-blockers and diuretics [77]. Moreover, both restoration of hepatocellular function and remission of portal hypertension might be considered helpful[6,32,92]. However, as portal decompression following transjugular intrahepatic portosystemic shunt increases QTc, the beneficial effect of liver Tx reflects only the amelioration of liver function[3,6,90]. Therefore, this compensatory mechanism might be compromised in patients with cirrhosis of alcohol etiology in cases that alcohol consumption persists. Moreover, diastolic dysfunction reflecting cirrhotic cardiomyopathy persists after liver Tx[77]. Patients with persistent QTc prolongation after liver Tx exhibit a worse prognosis[39].

High certainty of evidence has been also demonstrated that QTc prolongation in cirrhosis is more pronounced in severe forms of the disease, revealing a dose-response gradient effect of Child-Pugh score on QTc. It has been shown that patients with cirrhosis with QTc > 440 ms had higher MELD scores when compared with patients with QTc ≤ 440 ms. The correlation between the severity of cirrhosis and QTc prolongation might reflect the key role that aggravating hyperdynamic circulation leading to cirrhotic cardiomyopathy plays in the pathophysiology of the disease as well as the electrolyte imbalance superimposed by diuretic administration[80].

We have also concluded that alcohol, compared to the viral etiology of cirrhosis, leads to comparable QTc prolongation. This finding is in contrast with the fact that patients with cirrhosis related to alcoholic liver disease have been reported to present a worse outcome than those with cirrhosis related to chronic hepatitis C virus infection or non-alcoholic fatty liver disease[97]. Moreover, considering that alcohol causes cardiomyopathy *per se*, it could be argued that alcohol might well contribute to an inextricably intertwined entity involving alcoholic and cirrhotic cardiomyopathy in cases that it constitutes the unique or dominant cause of cirrhosis[62,64]. However, our result suggests that the contribution of alcohol in the pathophysiology of cirrhotic cardiomyopathy might be limited, if any.

Of note, other factors such as β-blockers, electrolyte imbalance due to diuretic treatment, and a recent episode of gastrointestinal bleeding might affect QTc. Similar to previous studies, we have demonstrated that β-blockers exert a negative effect on QTc[31,88,95]. Moreover, we showed that QTc is prolonged during acute gastrointestinal bleeding and is restored among survivors. This finding is also similar to recent studies[40,72]. However, the overall effect of treatments affecting QT, hospitalization for acute illness, and comorbidities on QTc prolongation in patients with cirrhosis is debatable if not negligible as suggested by the relevant sensitivity analysis carried out in the present study.

Table 2 Newcastle-Ottawa risk of bias assessment tool for all eligible studies

Ref.	Type	NOS selection	NOS comparability	NOS exposure[1] or outcome of interest[2]	Risk of bias
Wang *et al*[62], 2023	Retrospective study	**	*	***	Intermediate
Bilous *et al*[10], 2023	Prospective study	**	*	***	Intermediate
Barutcu *et al*[37], 2023	Retrospective study	***	**	***	Low
Lu *et al*[46], 2022	Retrospective study	***	*	***	Low
Wang *et al*[63], 2022	Retrospective study	**	**	***	Low
Li *et al*[27], 2021	Prospective study	****	**	***	Low
Ou *et al*[40], 2021	Retrospective study	****	*	***	Low
Ko *et al*[25], 2021	Retrospective study	****	**	***	Low
Héla *et al*[36], 2020	Prospective study	**	**	***	Low
Abrahamovych *et al*[64], 2020	Retrospective study	***	*	***	Low
Ibrahim *et al*[65], 2020	Prospective study	****	*	***	Low
Hussain *et al*[66], 2020	Prospective study	***	*	***	Low
Kim *et al*[35], 2020	Retrospective study	***	**	***	Low
Toma *et al*[67], 2020	Prospective study	***	**	***	Low
Bhardwaj *et al*[68], 2020	Prospective study	***	*	***	Low
Gaafar *et al*[69], 2019	Prospective study	***	*	***	Low
Kazankov *et al*[28], 2019	Retrospective study	****	*	***	Low
Moaref *et al*[70], 2019	Prospective study	***	*	***	Low
Santeusanio *et al*[71], 2019	Retrospective study	****	*	***	Low
Biselli *et al*[72], 2019	Retrospective study	****	*	**	Low
Tieranu *et al*[45], 2018	Prospective study	***	**	***	Low
Lee *et al*[39], 2018	Retrospective study	****	**	***	Low
Hajiaghamohammadi *et al*[73], 2018	Prospective study	***	*	**	Intermediate
Główczyńska *et al*[44], 2018	Retrospective study	***	**	***	Low
Tahata *et al*[74], 2018	Prospective study	****	*	***	Low
Tsiompanidis *et al*[75], 2018	Prospective study	***	**	***	Low
Yap *et al*[43], 2018	Retrospective study	**	**	***	Low
Kim *et al*[76], 2017	Retrospective study	***	*	**	Intermediate
Rimbaş *et al*[18], 2018	Prospective study	****	*	***	Low
Salgado *et al*[42], 2016	Prospective study	****	**	***	Low
Naqvi *et al*[34], 2016	Prospective study	***	**	***	Low

Zhao *et al*[38], 2016	Retrospective study	***	*	***	Low
Sonny *et al*[77], 2016	Retrospective study	***	**	***	Low
Barbosa *et al*[17], 2016	Case-control study	***	*	***	Low
Carvalheiro *et al*[78], 2016	Retrospective study	***	*	***	Low
Pourafkari *et al*[79], 2016	Retrospective study	***	**	***	Low
Barakat *et al*[80], 2015	Prospective study	**	*	***	Intermediate
Voiosu *et al*[81], 2015	Prospective study	***	*	***	Low
Cichoż-Lach *et al*[82], 2015	Retrospective study	**	**	***	Low
Peter *et al*[83], 2014	Prospective study	**	*	***	Intermediate
Moaref *et al*[15], 2014	Prospective study	**	*	***	Intermediate
Josefsson *et al*[84], 2014	Retrospective study	***	*	***	Low
Karagiannakis *et al*[14], 2014	Prospective study	***	*	***	Low
Yataco *et al*[41], 2014	Retrospective study	***	**	***	Low
Bhatti *et al*[85], 2014	Case-control study	**	*	***	Intermediate
Møller *et al*[86], 2012	Prospective study	***	*	***	Low
Trevisani *et al*[87], 2012	Retrospective study	****	*	***	Low
Zamirian *et al*[24], 2012	Prospective study	**	*	***	Intermediate
Kim *et al*[88], 2011	Prospective study	****	*	***	Low
Shin *et al*[89], 2011	Prospective study	***	*	***	Low
Mozos *et al*[33], 2011	Prospective study	***	**	***	Low
Vuppalanchi *et al*[90], 2011	Prospective study	**	*	***	Intermediate
Møller *et al*[91], 2010	Prospective study	***	*	***	Low
Zurick *et al*[23], 2010	Retrospective study	****	**	***	Low
Lossnitzer *et al*[13], 2010	Prospective study	***	*	***	Low
Genovesi *et al*[32], 2009	Prospective study	****	**	***	Low
Zambruni *et al*[31], 2008	Prospective study	****	**	**	Low
Henriksen *et al*[92], 2007	Prospective study	****	**	***	Low
Kosar *et al*[93], 2007	Retrospective study	****	**	***	Low
Hansen *et al*[12], 2007	Prospective study	**	**	***	Low
Zuberi *et al*[94], 2007	Case-control study	***	*	**	Intermediate
Zambruni *et al*[47], 2007	Prospective study	****	*	***	Low
Ytting *et al*[7], 2005	Prospective study	***	*	***	Low
Adigun *et al*[21], 2005	Prospective study	***	**	***	Low
Henriksen *et al*[95], 2004	Prospective study	***	*	***	Low
Bal and Thuluvath[26], 2003	Retrospective study	****	**	***	Low
Trevisani *et al*[6], 2003	Prospective study	***	*	***	Low
Mimidis *et al*[30], 2005	Retrospective	***	**	***	Low

	study				
Henriksen *et al*[11], 2002	Prospective study	***	*	***	Low
Puthumana *et al*[29], 2001	Retrospective study	***	**	***	Low
Quera *et al*[96], 2000	Retrospective study	***	*	**	Intermediate
Bernardi *et al*[3], 1998	Prospective study	****	**	***	Low
Finucci *et al*[4], 1998	Prospective study	****	**	***	Low

[1]For case-control studies.
[2]For cross-sectional studies.
NOS: Newcastle-Ottawa scale.

Table 3 GRADE assessment of evidence certainty (quality) for every endpoint

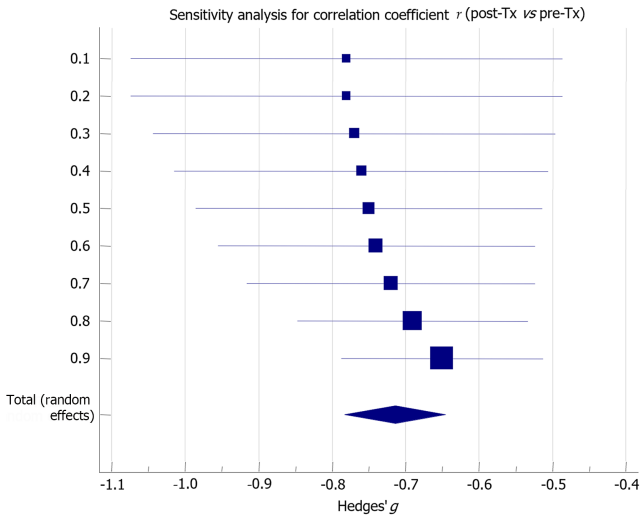
Endpoint	Risk of bias	Imprecision	Inconsistency	Indirectness	Publication bias	Effect size	Quality
Cirrhosis effect (patients *vs* controls) on QTc	No important risk of bias	No important imprecision	No important inconsistency	No important indirectness	No important publication bias	Very large	Very high
QTc prolongation in cirrhosis (QTc *vs* 440 ms)	No important risk of bias	No important imprecision	No important inconsistency	No important indirectness	No important publication bias	Very large	Very high
Sex effect on QTc in cirrhosis	No important risk of bias	No important imprecision	Serious inconsistency	No important indirectness	No important publication bias	Trivial	Very low
Etiology of cirrhosis (alcohol *vs* viral) effect on QTc	No important risk of bias	No important imprecision	Serious inconsistency	No important indirectness	No important publication bias	Trivial	Very low
Child-Pugh stage (C *vs* B *vs* A) effect on QTc	No important risk of bias	No important imprecision	No important inconsistency	No important indirectness	No important publication bias	Medium with dose-response gradient	High
MELD score effect on QTc prolongation	No important risk of bias	No important imprecision	No important inconsistency	No important indirectness	No important publication bias	Medium	Low
β-blockers effect on QTc	No important risk of bias	No important imprecision	No important inconsistency	No important indirectness	No important publication bias	Medium	Low
Acute gastrointestinal bleeding effect on QTc	No important risk of bias	No important imprecision	Serious inconsistency	No important indirectness	No important publication bias	Very large	High
Liver transplantation effect on QTc	No important risk of bias	No important imprecision	No important inconsistency	No important indirectness	No important publication bias	Large	High
QTc prolongation effect on overall survival in cirrhosis	No important risk of bias	No important imprecision	No important inconsistency	No important indirectness	No important publication bias	Medium	Low

MELD: Model for end-stage liver disease; QTc: Corrected QT.

In line with Adigun *et al*[21], we found no essential effect of sex or age on QTc of patients with cirrhosis. However, sex-dependent QTc prespecified upper normal limits are often adopted in the overall relevant literature, as demonstrated in the present meta-analysis and Figure 3. The QTc prolongation ratio recorded in studies that do not share a common QTc upper normal limit for both males and females might be erroneous. According to our findings, using sex-specific or age-specific QTc upper normal values in this group of patients is not justified.

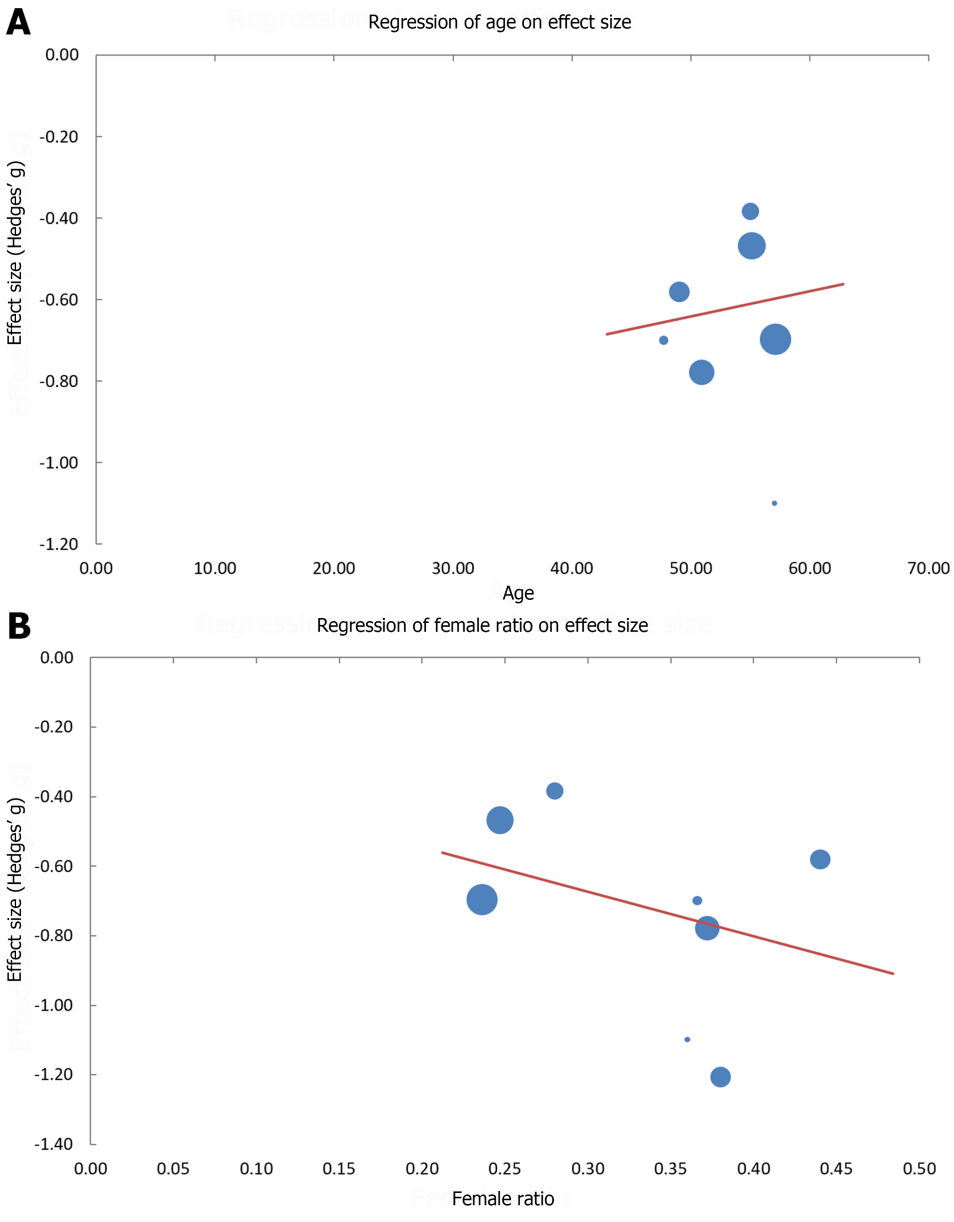
It is widely debated which upper normal limit should be used for QTc in patients with cirrhosis. In contrast with what is considered as QTc upper normal limit for patients without cirrhosis, namely < 430 ms for males and < 450 ms for females, 440 ms was adopted as the upper normal limit for QTc for both male and female patients with cirrhosis by the majority of the studies included (38/60; 63.3%; Figure 3)[98,99]. This choice was further supported by our result that patients with cirrhosis with QTc ≤ 440 ms, when compared with those with QTc > 440 ms, have at least twice the probability of surviving, thus conveying a clear-cut clinical meaning. The evidence above suggests that QTc ≤ 440 ms can be introduced as a surrogate prognostic marker for prolonged overall survival in cirrhosis.

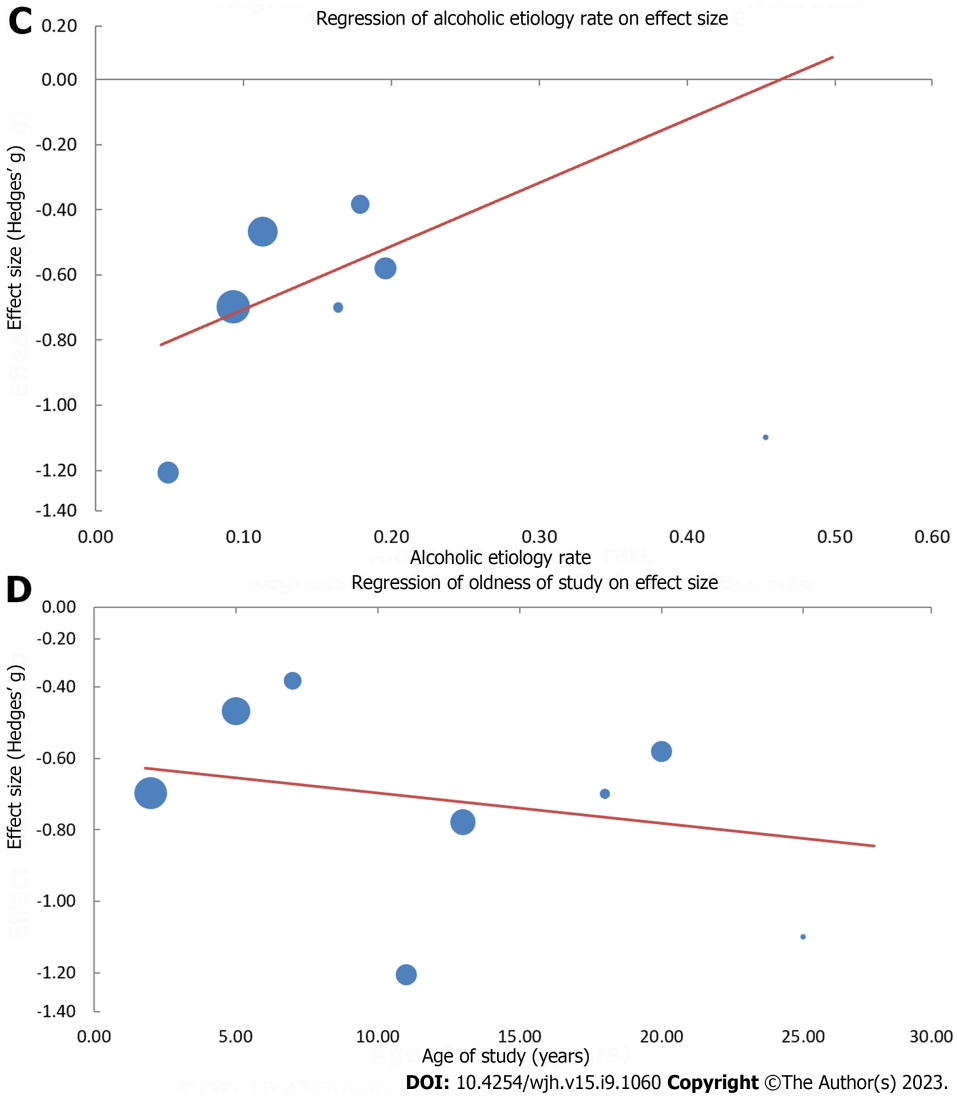
Most studies adopted the Bazett formula ($QT_{Bazett} = QT/RR^{1/2}$), while the second most common formula was Fridericia ($QT_{Fridericia} = QT/RR^{1/3}$). In cases where the heart rate was 60-100 beats/min, $QT_{Fridericia}$ can be safely converted to QT_{Bazett} using the formula $QT_{Bazett} = QT_{Fridericia}/RR^{-1/6}$, given that QT_{Bazett} and $QT_{Fridericia}$ produce comparable QT corrections under these circumstances[100]. There is still much debate regarding the procedure that should be selected for the correction of



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Figure 7 Sensitivity analysis forest plot concerning the estimation of the correlation coefficient between post-transplantation and pre-transplantation corrected QT values. Tx: Transplantation.





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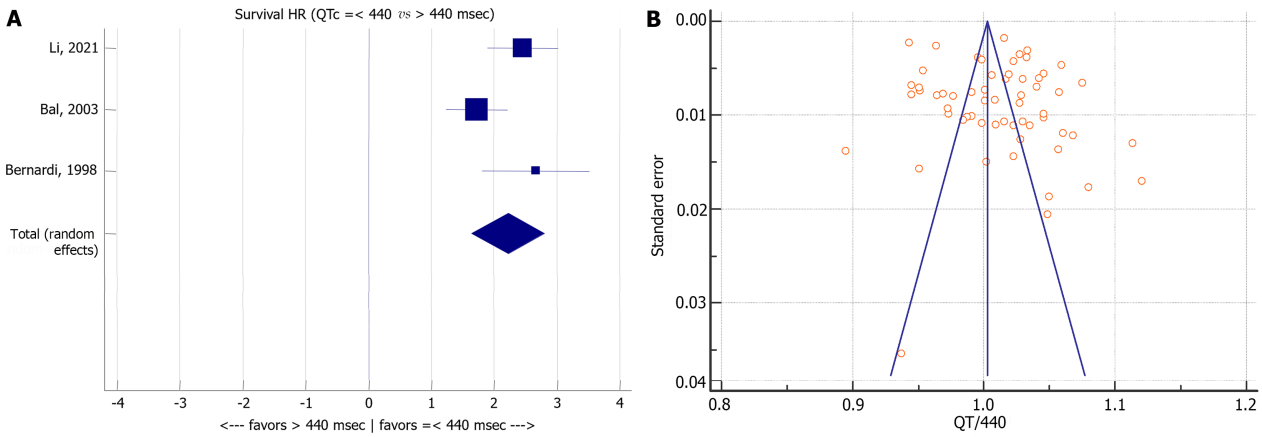
Figure 8 Plot points and regression lines on effect size, namely Hedges' *g*, reflecting correlations of pre-transplantation *vs* post-transplantation corrected QT. A: Age [H_g = -0.95 + 0.01 (yr); *P* = 0.417]; B: Female ratio [H_g = -0.29 - 1.28 (female ratio); *P* < 0.001]; C: Alcoholic etiology rate [H_g = -0.90 - 1.95 (alcoholic etiology rate); *P* < 0.001]; D: Age of study [H_g = -0.61 - 0.01 (age of study); *P* = 0.019]. H_g refers to Hedges'g.

QT in patients with cirrhosis, as there is evidence that they may lead to different clinical conclusions[31]. However, QT_{Bazett} was selected as the formula of choice in most of the included studies (68/73; 93.2%). Therefore, all current evidence derived from combining relevant effect sizes and summarized in Table 3 was based on QT_{Bazett}. Hence, authors should consider using the QT_{Bazett} as an at least additional formula to correct QT.

Interestingly, QTc length was not correlated with study type, year of publication, or even device used (electrocardiograph or Holter). This finding underlies that since no confounding parameters have been detected, the quality of evidence concerning QTc length remains very high, having been upgraded by two levels due to the very high relevant effect size.

Limitations

Apart from the apparent strengths regarding the quantitative and qualitative assessment of endpoints, the present study also had some limitations. First, the literature review was conducted by only two authors; while no different coauthor was available to resolve any discrepancies, the most experienced author (Mimidis K) undertook the latter task. Second, high heterogeneity was detected, which was not attributed to any specified potential confounder, such as publication bias, NOS scoring, study type, device used, year of publication, hospitalization for acute illness, comorbidities, and treatments affecting QT except β-blockers. Third, the effect of drugs on QTc could not be explicitly determined as detailed information concerning the use of medications, other than β-blockers, affecting QTc (including diuretics, anti-rejection regimens such as tacrolimus, antibiotics, antipsychotics, antidepressants, antiemetics, analgesics, antihistamines, and the direct antiviral agents lepidasvir and sofosbuvir) are lacking. Last, it might be claimed that performing meta-analysis with very few studies, as in the cases of the effect of β-blockers on QTc, acute gastrointestinal bleeding effect on QTc, and QTc prolongation effect on overall survival in cirrhosis, might be a limitation. However, when the results are not inconclusive, a quantitative meta-analysis is an acceptable approach[101].



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Figure 9 Meta-analysis forest plot concerning overall survival of patients with cirrhosis relating to corrected QT and corrected QT to upper normal limit (440 ms) ratio in patients with cirrhosis. A: Overall survival of patients with cirrhosis relating to corrected QT (QTc); B: QTc to upper normal limit (440 ms) ratio in patients with cirrhosis. QTc: Corrected QT; HR: Hazard ratio.

CONCLUSION

QTc is prolonged in cirrhosis independent of sex, age, and etiology. QTc is correlated with severity and is affected by β-blockers and acute gastrointestinal bleeding. QTc is improved after liver Tx.

ARTICLE HIGHLIGHTS

Research background

The effects of sex, age, severity, and etiology, as well as the role of treatment, acute illness, and liver transplantation (Tx) are largely unknown regarding corrected QT (QTc) in cirrhosis.

Research motivation

It is unknown whether QTc is prolonged in patients with cirrhosis and whether QTc is affected by factors such as sex, age, severity, etiology, regimens, acute illness, and liver Tx.

Research objectives

To investigate QTc clinical usefulness in cirrhosis.

Research methods

Seventy-three studies were considered eligible, as identified by application of the search protocol "{[QTc] OR [QT interval] OR [QT-interval] OR [Q-T syndrome]} AND {[cirrhosis] OR [Child-Pugh] OR [MELD]}" in PubMed, EMBASE, and Google Scholar databases.

Research results

QTc was prolonged in patients with cirrhosis independent of sex and age (444.8 ± 4.4 ms). QTc correlated with Child-Pugh stage and model for end-stage liver disease score. QTc improved after liver Tx.

Research conclusions

QT prolongation in cirrhosis is independent of sex and age, is aggravated in severe cases, and benefited by liver Tx.

Research perspectives

QTc interval could be further evaluated as a tool in the assessment of liver cirrhosis by clinicians.

FOOTNOTES

Author contributions: Papadopoulos VP contributed to design of the study; Papadopoulos VP and Mimidis K contributed to conception of the study, acquisition, analysis and interpretation of the data, and drafting of the manuscript; and all authors gave final approval of the manuscript.

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OPINION REVIEW

Dietary salt in liver cirrhosis: With a pinch of salt!

Ramesh Kumar, Sudheer Marrapu

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Abstract

Patients with liver cirrhosis are advised to limit their sodium consumption to control excessive fluid accumulation. Salt is the most common form in which sodium is consumed daily. Consequently, various recommendations urge patients to limit salt intake. However, there is a lack of consistency regarding salt restriction across the guidelines. Moreover, there is conflicting evidence regarding the efficacy of salt restriction in the treatment of ascites. Numerous studies have shown that there is no difference in ascites control between patients with restriction of salt intake and those without restriction. Moreover, patients with cirrhosis may have several negative effects from consuming too little salt, although there are no recommendations on the lower limit of salt intake. Sodium is necessary to maintain the extracellular fluid volume; hence, excessive salt restriction can result in volume contraction, which could negatively impact kidney function in a cirrhotic patient. Salt restriction in cirrhotic patients can also compromise nutrient intake, which can have a negative impact on the overall outcome. There is insufficient evidence to recommend restricted salt intake for all patients with cirrhosis, including those with severe hyponatremia. The existing guidelines on salt restriction do not consider the salt sensitivity of patients; their nutritional state, volume status and sodium storage sites; and the risk of hypochloremia. This opinion article aims to critically analyze the existing literature with regard to salt recommendations for patients with liver cirrhosis and identify potential knowledge gaps that call for further research.

Key Words: Salt; Cirrhosis; Sodium; Hyponatremia; Ascites; Malnutrition

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Core Tip: There are still many inconsistencies in the guidelines regarding salt recommendations for patients with liver cirrhosis. Although controlling ascites is the core tenet of salt restriction, there is insufficient data to back up this assertion. Moreover, the guidelines have no recommendations for minimum salt intake, even though too little salt consumption may have a variety of negative effects on patients with liver cirrhosis. To achieve optimal salt consumption in these patients, several factors need to be considered. This article discusses several important aspects of salt consumption in patients with cirrhosis.

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INTRODUCTION

Sodium is essential for fluid balance and cellular homeostasis[1]. Under normal conditions, effective sodium balance, and hence extracellular fluid volume, is maintained by a complex interplay between various systems that regulate renal sodium excretion[2]. For example, a progressive increase in sodium intake activates natriuretic systems while suppressing the sodium retaining systems to maintain an effective sodium balance. However, in patients with liver cirrhosis, portal hypertension-related splanchnic vasodilatation reduces effective arterial blood volume, which in turn activates the sympathetic nervous system, renin-angiotensin-aldosterone system (RAAS) and, in later stages, arginine vasopressin, all of which result in renal sodium and fluid retention and, eventually, ascites[3]. Therefore, limiting sodium intake is suggested for the treatment of ascites in such patients[4]. Salt is the most common source of sodium consumed by humans; hence, the guidelines have focused on dietary salt restriction to achieve sodium limitation.

Data on the effectiveness of salt restriction for the management of ascites are not conclusive[5-7], and there are still many inconsistencies in the guidelines for salt recommendations in patients with liver cirrhosis[8-11]. Although the guidelines have suggested the upper limit, a lower limit for salt consumption was not included. Severe salt restriction could potentially result in hyponatremia and cause volume contraction, which might adversely affect kidney function in a cirrhotic patient. Salt restriction in cirrhosis compromises nutrient intake and has a negative impact on the overall outcomes[12,13]. There is a paucity of evidence regarding the dietary salt recommendation for patients with compensated cirrhosis and decompensated cirrhosis with severe hyponatremia[14,15]. The ability of salt to expand extracellular volume, known as salt sensitivity, varies among individuals; thus, a 'one size fits all' approach to dietary salt recommendations may not be appropriate[16]. Furthermore, a new finding indicating that sodium is also stored in a third compartment (interstitium and endothelial surface layer) in a non-osmotic equilibrium can have significant impact on our understanding of sodium intake and homeostasis in cirrhotic patients[17]. This opinion article covers all the aforementioned issues and emphasizes the knowledge gaps and the need for additional research in the area.

SALT AND CIRRHOSIS: IMPORTANT ISSUES

How much salt should cirrhotic patients consume, and are the current recommendations supported by data?

The recommended limits of salt consumption for cirrhotic patients with ascites varies from 4.6 g to 6.9 g/d, although most guidelines recommend around 5 g of salt, which corresponds to a teaspoon of cooking salt (Table 1). As per current recommendations, salt restriction should be considered for all cirrhotic patients with ascites, including those with refractory ascites. When it comes to the grade of ascites at which salt restriction should start, the International Ascites Club suggests grade 1, and the European Association for the Study of the Liver (EASL) suggests grade 2; however, the American Association for the Study of Liver Diseases does not provide any specific grade of ascites to this purpose[8,9,11]. The goal of salt restriction is to avoid sodium overload. Hence, the net sodium intake must be equal or lower than the sodium excretion; this can be achieved either by lowering the dietary salt intake or by increasing natriuresis using diuretics. For patients with grade 2 or higher ascites, salt restriction alone would be insufficient and diuretic therapy would need to be implemented. The first-line diuretics are often aldosterone antagonists, such as spironolactone, which can be administered alone or in conjunction with a loop diuretic such as furosemide. Restriction of fluid intake is required only in patients with dilutional hyponatremia.

Several studies, including randomized controlled trials (RCTs), have examined the role of salt restriction in patients with cirrhosis and ascites[5,6,7,12,13,18]. The amount of salt restriction ranged from a salt-free diet to 7 g of salt per day. The majority of these studies are old with varying methodology and sub-optimal quality, making it challenging to draw clear conclusions. Some of these studies found a faster elimination and better control of ascites with strict salt restriction [5,6]. Some studies found no difference in ascites control between patients with and without salt restriction[12,18]. Furthermore, two recent RCTs found that a salt-unrestricted diet (5-6.5 g/d) was superior to a salt-restricted diet (5 g/d) in resolving ascites in a larger proportion of patients (45% *vs* 16%) and in reducing the need for large volume paracentesis [7,13]. Authors proposed that worsening hyponatremia caused by salt restriction can weaken the effects of diuretics and reduces renal blood flow, both of which worsen ascites[7]. This idea is further supported by research showing that the use

Table 1 Recommendations for salt consumption in patients with liver cirrhosis

Scientific society/Guidelines	Recommended salt intake	Patient group
EASL practice guidelines (2010)[8]	4.6-6.9 g/d	Cirrhosis with grade II or more ascites
AASLD practice guidelines (2021)[9]	5.1 g/d	Cirrhosis with ascites
British Society of Gastroenterology (2021)[10]	5–6.5 g/d	Cirrhosis with ascites
International Ascites Club (2003)[11]	5.2 g/d	Cirrhosis with ascites

AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver.

of hypertonic saline solutions in conjunction with diuretics enhance fluid mobilization in patients with cirrhosis and refractory ascites[19]. The proposed mechanisms for these advantageous effects of salt loading include an increase in intravascular volume, an osmotic shift of fluid from the tissues, an increase in renal blood flow and a decrease in sympathetic tone. For the management of ascites in cirrhosis, each of these pathways is important. Therefore, although managing ascites is the fundamental premise of salt restriction in cirrhosis patients, there is conflicting evidence regarding this claim.

What adverse effects might cirrhosis patients experience with a salt-restricted diet?

The significant adverse events with salt restriction reported by various studies include hyponatremia, reduced caloric intake, higher risk of renal impairment, hepatic encephalopathy and mortality[5,6,7,12,13]. In a randomized trial, Reynolds *et al*[5] concluded that unrestricted salt intake decreased the likelihood of hyponatremia and azotemia. The risk of hyponatremia is significantly increased by concurrent use of diuretic medication. Severe salt restriction makes the food unpalatable and alters dietary patterns, which might promote protein-calorie malnutrition and increase mortality risk[4,20,21]. Notably, malnutrition and sarcopenia are already prevalent in patients with advanced cirrhosis[22,23]. In a study of cirrhotic patients with ascites requiring repeated paracentesis, salt restriction without nutritional support resulted in a 3.9-fold higher risk of mortality within one year, compared to that of unrestricted sodium intake with nutritional support [13]. In the small intestine, sodium absorption facilitates the absorption of chloride, amino acids, glucose and water[24]. Thus, severe salt restriction may affect the absorption of these substances, contributing to malnutrition.

Another issue concerns compliance with a salt-restricted diet. The average salt consumption by the general population is significantly above the levels recommended by the World Health Organization. A recent systemic review reported that most European countries consume between 4.2 g and 18.5 g of salt per day per capita[25]. Salt intake in India and China is about 10 g/d[26,27]. A cross-sectional survey indicated that only about a third of cirrhotic patients were compliant with salt restriction, with an additional 45% incorrectly stating that they were compliant[28]. Another potentially significant but unappreciated issue with low salt consumption, particularly with concurrent diuretic medication, is the development of hypochloremia, *i.e.*, a low level of serum chloride. Serum chloride, the most important anion in the blood, has received less attention in cirrhosis patients, even though hypochloremia has been recognized as an important prognostic marker in patients with advanced cirrhosis. According to the findings of two recent studies, hypochloremia may be an even better predictor of mortality in patients with decompensated cirrhosis than serum sodium[29,30]. Chloride reabsorption in the renal tubule constitutes a crucial process for the auto-regulation of the acid-base balance as well as the electrochemical equilibrium. Moreover, hypochloremia causes activation of the RAAS and the upregulation of NaCl channels in the distal convoluted tubules, which can aggravate sodium retention and contribute to diuretic resistance[31].

Thus, existing evidence tends to suggest that severe salt-restricted diets (< 5 g/d) may not significantly improve ascites control and could even lead to complications. Considering the excessive salt consumption by the general population, a moderate salt restriction, with daily salt intake of no more than 5–6.5 g may be advisable for such patients. This translates to a 'no added salt diet' with avoidance of high sodium containing meals. Table 2 provides a list of foods with high sodium content so that doctors can counsel patients on their consumption. Also, one must be mindful while recommending salt restriction in advanced cirrhosis patients with hyponatremia as such patients generally have relative hypovolemia where salt restriction could cause volume contraction and renal dysfunction.

Is unrestricted salt intake justified for patients with compensated cirrhosis?

For patients with preascitic compensated cirrhosis, guidelines do not recommend dietary salt restriction. Nonetheless, a study has found that even compensated cirrhosis patients retain sodium when faced with high salt intake[14]. However, compensatory activation of atrial natriuretic peptide and inhibition of the RAAS result in a new steady state of sodium balance in such patients, which tends to prevent ascites. Nevertheless, Jalan *et al*[15] found that the degree of portal hypertension had a significant impact on the sodium handling capacity in patients with compensated cirrhosis. In fact, it is now believed that clinically significant portal hypertension (CSPH) is the main driver of decompensation in cirrhotic patients[32]. Compensated cirrhotic patients with baseline hepatic venous portal gradient > 20 mmHg had a 47% risk of decompensation in a mean duration of just 1.6 years, compared to < 10% over 4 years when it is < 10 mmHg[33,34]. Therefore, it is reasonable to assume that the compensatory natriuretic mechanism might be overwhelmed with the rising portal pressure, and as a result, sodium retention with high dietary salt intake may result in decompensation in the form of ascites in compensated cirrhosis patients. Hence, until further data emerge, salt restriction may be considered in compensated cirrhotic patients with CSPH. This extrapolation, however, needs to be tested in a controlled trial.

Table 2 Food items with a high content of sodium

Food category	High sodium-content food[a]
Cereals, breads, and, grains	Biscuits, pancakes, pizza, sandwiches, burgers, bread with salted tops, potato crisps, and salty snack foods
Vegetables, fruits, and soups	Canned vegetables and vegetable juices, pickles, commercially prepared pasta and tomato sauces, canned soup, and cup-noodles
Dairy products	Butter milk, processed and cottage cheese
Meat products and eggs	Smoked, salted or canned meat, fish or poultry, omelettes
Fats, desserts and sweets	Salted butter or margarine, soy sauce, bottled salad dressings, instant pudding and cake, ketchup
Legumes and nuts	Salted nuts and beans

[a]The sodium content can vary significantly between similar types of foods. Therefore, the nutrition label on the products should be checked to identify foods high in sodium.

What are the implications of high salt intake for cirrhotic patients and the general population?

Studies from different countries found that dietary salt intake in the general population was approximately 10 g/d[25-27]. Directly or indirectly, high salt consumption adversely affects multiple organs in the body and may have some serious implications in patients with liver cirrhosis. Consuming excessive amounts of salt has been associated with oxidative stress, insulin resistance, vascular endothelial damage, sympathetic nerve sensitization, alteration of gut-microbiome and an increased risk of cancer (Figure 1). There is a strong positive association between dietary salt intake and cardiovascular diseases[35]. Therefore, the World Health Organization has recommended a daily salt intake of less than 5 g/d, which is approximately 2 g of sodium, for the general population[36]. According to this viewpoint, the dietary salt recommendation for advanced cirrhosis patients is similar to that of the normal healthy population. In a population-based study, subjects with intermediate salt intake (6–10 g/d) and high salt intake (> 10 g/d) were found to have a higher risk of hepatocellular carcinoma with a multivariable hazard ratio (HR) of 1.49 and 1.9, respectively, compared to those with low salt intake (< 6 g/d)[37]. A recent cohort study from Iran reported that high dietary intake of salt (9.5–15 g/d) increases the rate of mortality in patients with cirrhosis (HR 2.26). Moreover, moderate salt restriction (3–5 g/d), as compared to salt elimination, decreases the risk of death (HR 0.72)[38].

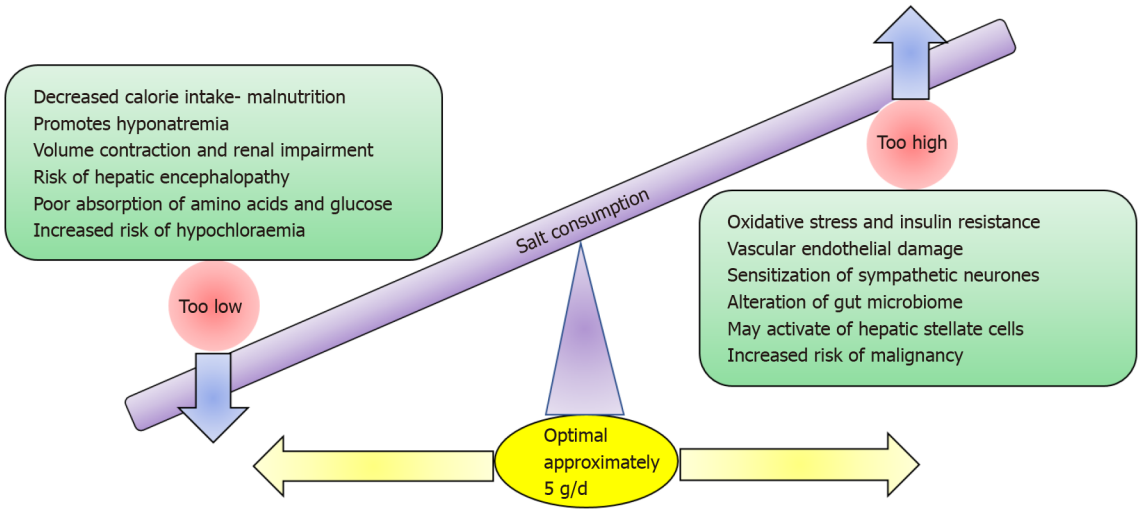
What could be the implications of salt sensitivity and third space sodium storage in patients with liver cirrhosis?

The ability of salt to expand extracellular volume, known as salt sensitivity, varies among individuals. It is estimated that around 26% of normotensive and 51% of hypertensive persons are salt sensitive[39,40]. The remaining individuals are salt resistant – their hemodynamic parameters are likely to be unaffected due to change in dietary salt consumption. Although the relevance of salt sensitivity in cirrhotic patients has never been assessed in the context of dietary salt intake, extrapolation of data from the general population suggests that between half and three-quarters of patients would be salt resistant. It would be safe to assume that a major restriction in dietary salt consumption at the cost of nutritional compromise would be undesirable in a large number of patients. Therefore, instead of having a 'one size fits all' salt recommendation for cirrhotic patients, the salt sensitivity of individual patients should also be considered.

Recently, a third compartment (skin interstitium and endothelial surface layer) of sodium storage sites, in which sodium can accumulate in a non-osmotic equilibrium and hence without concurrent water retention, have been identified [41,42]. This can have implications for sodium homeostasis, osmoregulation and the hemodynamic response to salt intake, all of which are relevant to patients with cirrhosis. In the third space, sodium can be osmotically inactivated following binding to negatively charged glycosaminoglycans[42]. Due to the changes in the dynamics of the interstitium, alterations in glycosaminoglycans and endothelial damage in cirrhotic patients, the amount of dietary salt intake can affect sodium homeostasis. Moreover, high interstitial sodium concentrations stimulate lymph angiogenesis *via* vascular endothelial growth factor-C, which helps in the mobilization of excess fluid from the skin *via* lymphatics[43]. Therefore, it would be interesting to see whether a salt-restricted diet worsens pre-existing lymphatic dysfunction in patients with advanced cirrhosis[44].

What are the practical issues with ensuring a pre-defined sodium consumption?

Measuring sodium intake in individuals is challenging as sodium is so widespread in food items. Even with extensive food labelling, it is often difficult to quantify the sodium content of food. Commonly used approaches include 24-h urine sodium measurement, 24-h dietary recall and food questionnaires[45]. Dietary sodium intake can be calculated by dividing the urine sodium excretion by 0.9, based on the assumption that 10% of sodium intake is lost through sweat and feces, and thus urinary excretion accounts for 90% of intake. Therefore, 24-h urine collection is considered the most reliable method. However, a systematic review found that measured urinary sodium varied as widely as 76%–122% of ingested sodium amount, making it a 'not so reliable' test[46]. Thus, most of these methods would only provide a rough estimate of sodium consumption. As moderate salt restriction might make foods unappealing and affect overall nutrition, some strategies need to be adopted in order to ensure adequate nutrient intake. One of the strategies is to partially substitute sodium with potassium or other minerals, such as calcium or magnesium[47]. However, there are concerns about possible negative effects of such a replacement, such as hyperkalemia with potassium-based salt, especially in



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Figure 1 Adverse consequences of too little and too high salt consumption. Salt consumption < 5 g/d appears to have no advantage on the control of ascites. Severe salt restriction (< 3 g/d) as well as very high salt consumption (> 10 g/d) can produce many deleterious consequences in cirrhosis patients. The World Health Organization has recommended a daily salt intake of approximately 5 g/d in the general population which is similar to the recommendation for cirrhosis patients with ascites.

cirrhotic patients with renal impairment or those taking potassium sparing diuretics. Additionally, flavors and sensory experiences can be imparted using herbs, spices and yeast extract. When used as salt alternatives, they have demonstrated good customer acceptance[48].

CONCLUSION

Inconsistencies in the recommendations for salt intake and conflicting evidence regarding the effectiveness of salt restriction for controlling ascites in cirrhotic patients necessitate further research. Presently, the term 'salt restriction' for cirrhotic patients appears to be a misnomer, given that the salt recommendation for normal populations is also the same. However, it is necessary to carefully evaluate the efficacy of varied levels of salt intake at various stages of cirrhosis, including those who also have concurrent hyponatremia. Studies on salt restriction must consider the patients' salt sensitivity, nutritional status, volume status, sodium storage sites and hypochloremia risk. Innovative ideas in this area would be to evaluate the efficacy and safety of low-sodium salt substitutes (such as potassium-based salt) and find ways to make low-sodium foods more palatable (by utilizing herbs, spices and yeast extract, *etc.*) to ensure appropriate nutrition.

Until further data emerge, it seems appropriate for cirrhotic patients with ascites to consume 5–6 g of salt per day, which would mean avoiding foods with added salt. To increase adherence, prevent malnutrition and avoid harmful effects of excess salt consumption, it is crucial to educate patients about the recommended salt limit. A formal consultation with a nutritionist may be sought. It is necessary to set a lower limit for salt consumption as too much salt restriction has just as many negative effects as too much consumption. Finally, personalized salt management, depending on the sodium balance, nutritional status and volume status of the patient, may be required for some of these patients.

FOOTNOTES

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REVIEW

Progress on traditional Chinese medicine in improving hepatic fibrosis through inhibiting oxidative stress

Zhen Li, Jun-Feng Zhu, Hao Ouyang

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Abstract

Hepatic fibrosis is a common pathological process that occurs in the development of various chronic liver diseases into cirrhosis and liver cancer, characterized by excessive deposition of the extracellular matrix. In the past, hepatic fibrosis was thought to be a static and irreversible pathological process. In recent years, with the rapid development of molecular biology and the continuous in-depth study of the liver at the microscopic level, more and more evidence has shown that hepatic fibrosis is a dynamic and reversible process. Therefore, it is particularly important to find an effective, simple, and inexpensive method for its prevention and treatment. Traditional Chinese medicine (TCM) occupies an important position in the treatment of hepatic fibrosis due to its advantages of low adverse reactions, low cost, and multi-target effectiveness. A large number of research results have shown that TCM monomers, single herbal extracts, and TCM formulas play important roles in the prevention and treatment of hepatic fibrosis. Oxidative stress (OS) is one of the key factors in the occurrence and development of hepatic fibrosis. Therefore, this article reviews the progress in the understanding of the mechanisms of TCM monomers, single herbal extracts, and TCM formulas in preventing and treating hepatic fibrosis by inhibiting OS in recent years, in order to provide a reference and basis for drug therapy of hepatic fibrosis.

Key Words: Hepatic fibrosis; Oxidative stress; Traditional Chinese medicine monomer; Single herbal extract; Traditional Chinese medicine formula

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Core Tip: Hepatic fibrosis is a common pathological process that occurs in the development of various chronic liver diseases into cirrhosis and liver cancer, characterized by excessive deposition of the extracellular matrix. This article reviews the progress in the understanding of the mechanisms of traditional Chinese medicine (TCM) monomers, single herbal extracts, and TCM formulas in preventing and treating hepatic fibrosis by inhibiting oxidative stress in recent years, in order to provide a reference and basis for drug therapy of hepatic fibrosis.

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INTRODUCTION

The prevalence of hepatic fibrosis ranges from 2% to 19%, and it remains a major cause of morbidity and mortality worldwide[1,2]. It leads to notorious complications such as ascites, portal hypertension, hepatic encephalopathy, and liver failure, and increases the risk of hepatocellular carcinoma, posing a heavy burden on individuals, society, and healthcare systems[3,4]. Currently, Western medical treatments for hepatic fibrosis include antiviral drugs, corticosteroids, hepatoprotective drugs, and liver support, but they have low efficacy rates, significant resistance, and side effects [5]. Traditional Chinese medicine (TCM), as a unique medical approach in China, has better safety and effectiveness and is widely used to treat hepatic fibrosis[6]. Continuous oxidative stress (OS) in the liver can induce biological changes in hepatocytes, leading to fibrotic changes in the liver[7]. Based on this, experts and scholars have conducted relevant experimental studies to demonstrate that TCM monomers, single herbal extracts, and TCM formulas can significantly improve the condition of hepatic fibrosis by regulating OS. Therefore, this article reviews the progress in the the understanding of the mechanisms of TCM in preventing and treating hepatic fibrosis by using OS as a starting point to provide references for the clinical treatment of hepatic fibrosis with TCM.

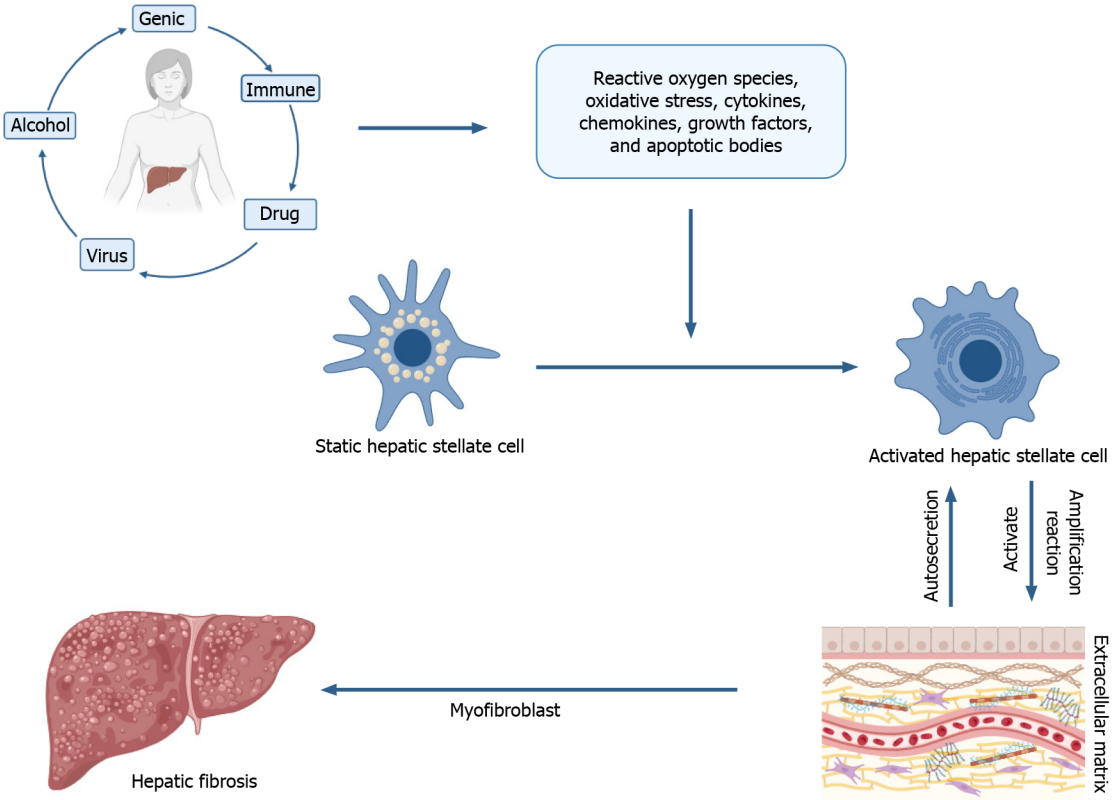
MECHANISMS OF HEPATIC FIBROSIS

Hepatic fibrosis is a chronic wound healing response to cellular damage and inflammation caused by increased synthesis and deposition of extracellular matrix (ECM) components and decreased or unbalanced ECM degradation (Figure 1)[8,9]. Various etiologies such as alcohol abuse, viral hepatitis infection, genetic abnormalities, non-alcoholic fatty liver disease, autoimmune disorders, and other non-infectious diseases can cause continuous wound healing response and liver injury, leading to hepatic fibrosis[10]. The main mechanism of hepatic fibrosis is believed to be the activation of myofibroblast precursor cells, which leads to an increase in ECM deposition surrounding the sinusoidal cell layer in the Disse space[11, 12]. ECM increase is the main feature of hepatic fibrosis, and the ECM is composed of five types of substances, collagen, non-collagenous proteins, elastic fibers, proteoglycans, and glycosaminoglycans. It is mainly divided into basement membrane and interstitial matrix according to its distribution site[13]. In human patients and rodent models of liver disease, fibrotic livers contain multiple types of collagen (types I, III, and V), non-fibrillar collagens (IV and VI), and glycosaminoglycans and proteoglycans (such as fibronectin, tenascin, laminin, basement membrane proteoglycans, decorin, biglycan, and fibrillin)[11].

In addition, hepatic stellate cells (HSCs) (Ito cells and lipocytes), portal-resident fibroblasts (portal or central veins), epithelial cells undergoing epithelial-to-mesenchymal transition, bone marrow-derived fibroblasts, vascular smooth muscle cells, and sinusoidal peri-hematopoietic stem cells are the main cell types that produce the ECM during hepatic fibrosis[14]. In human patients, HSCs constitute 5%-8% of the total liver cells involved in growth, differentiation, and regeneration. They are not only the main source of myofibroblasts but also the main cell type leading to hepatic fibrosis [15]. Portal fibroblasts are thought to play an important role in fibrosis in cholestatic liver disease[16,17]. The static HSC to myofibroblast differentiation is a multi-step process involving reactive oxygen species (ROS), cytokines, chemokines, growth factors, and apoptotic bodies from hepatocytes[18]. Chronic liver injury involves HSCs undergoing phenotypic activation towards myofibroblasts, which is characterized by increased expression of cell markers such as alpha-smooth muscle actin (α-SMA) and collagen. In addition, transforming growth factor-beta 1 (TGF-β1) inhibits liver regeneration during HSC to myofibroblast differentiation, ultimately leading to hepatic fibrosis[19].

OS-MEDIATED HEPATIC FIBROSIS

OS refers to the imbalance between the normal oxidant scavenging enzyme system [such as superoxide dismutase (SOD), catalase, and glutathione (GSH)] and the production of ROS in the cells, which is considered a key driving factor in hepatic fibrosis[20]. Oxidants, also known as ROS, include superoxide anion radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen[21]. In addition, they also contain some nitrogen oxides, lipid peroxide radicals, and



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Figure 1 The mechanism of hepatic fibrosis. Due to various factors such as alcohol abuse, viral hepatitis infection, genetic abnormalities, alcoholic fatty liver disease, autoimmune diseases, and medications, the body may produce an excessive amount of reactive oxygen species, leading to oxidative stress. At the same time, these factors may also induce cells to release cytokines, chemokines, growth factors, and apoptotic bodies, as well as activate hepatic stellate cells (HSC) and transform them into myofibroblasts. Consequently, large amounts of extracellular matrix (ECM) substances, such as collagen, non-fibrillar collagens, glycosaminoglycans, and proteoglycans, are autosecreted. The presence of these ECM substances can further stimulate the autosecretion of HSCs, ultimately resulting in fibrosis and impaired liver function.

hypochlorous acid. The process of oxidant formation begins with oxygen being reduced to water, resulting in the production of free radicals such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals[22].

Under physiological conditions, ROS are the result of normal cellular metabolism and are maintained in dynamic equilibrium with antioxidants[23]. Under pathological conditions, excess ROS can stimulate pathological oxidative-reductive signal transduction, leading to OS. Various organic compounds such as DNA, lipids, carbohydrates, and proteins are structurally damaged, resulting in cell damage and various diseases[24]. Lee *et al*[25] first demonstrated a possible molecular link between enhanced lipid peroxidation and induced collagen gene expression in cultured fibroblasts, suggesting that OS plays a direct pathogenic role in hepatic fibrogenesis. Sustained OS in the liver directly or indirectly affects hepatocytes and alters the structure of cell membranes and organelles, causing damage, necrosis, and apoptosis[26]. These processes lead to cell damage and the release of various cytokines and growth factors, inducing quiescent HSC activation into myofibroblasts expressing α-SMA as a characteristic marker[27]. Activated HSCs lose lipid droplets (vitamin A), rapidly proliferate, and upregulate many genes, especially collagen, fibronectin, laminin, and hyaluronic acid, beginning to increase the synthesis of connective tissue proteins, especially collagen, leading to fibrosis formation and further development into liver cirrhosis and even liver cancer[19,28]. In addition, excess ROS also enhance the secretion of the fibrogenic factor TGF-β1, which is highly involved in HSC activation, exacerbating ECM deposition in the liver and progressing to hepatic fibrosis[29]. The mechanism of OS-mediated hepatic fibrosis is shown in Figure 2.

UNDERSTANDING HEPATIC FIBROSIS FROM TCM PERSPECTIVE

Ancient literature did not have a clear concept of "hepatic fibrosis" as a disease name. Based on its main clinical manifestations of hypochondriac pain, palpable masses in the hypochondrium, and jaundice, modern physicians categorize it under disease categories such as distension and swelling, hypochondriac pain, accumulation, and jaundice[6]. The Ling Shu section of the Huangdi Neijing states: "If the evil is in the liver, then there is pain in both flank regions, the patient feels cold, and stagnant blood circulates within, causing restricted joint movements, and occasional foot swelling. Acupuncture at Jia Jian was used to activate the meridian around the liver area and warm up the stomach, extract blood through veins to eliminate stagnant blood, and take out the green vein by the ear to alleviate cramps[30]." The Huangdi Neijing says: "Wind, cold, and dampness combined cause obstruction of channels and collaterals, known as arthralgia.

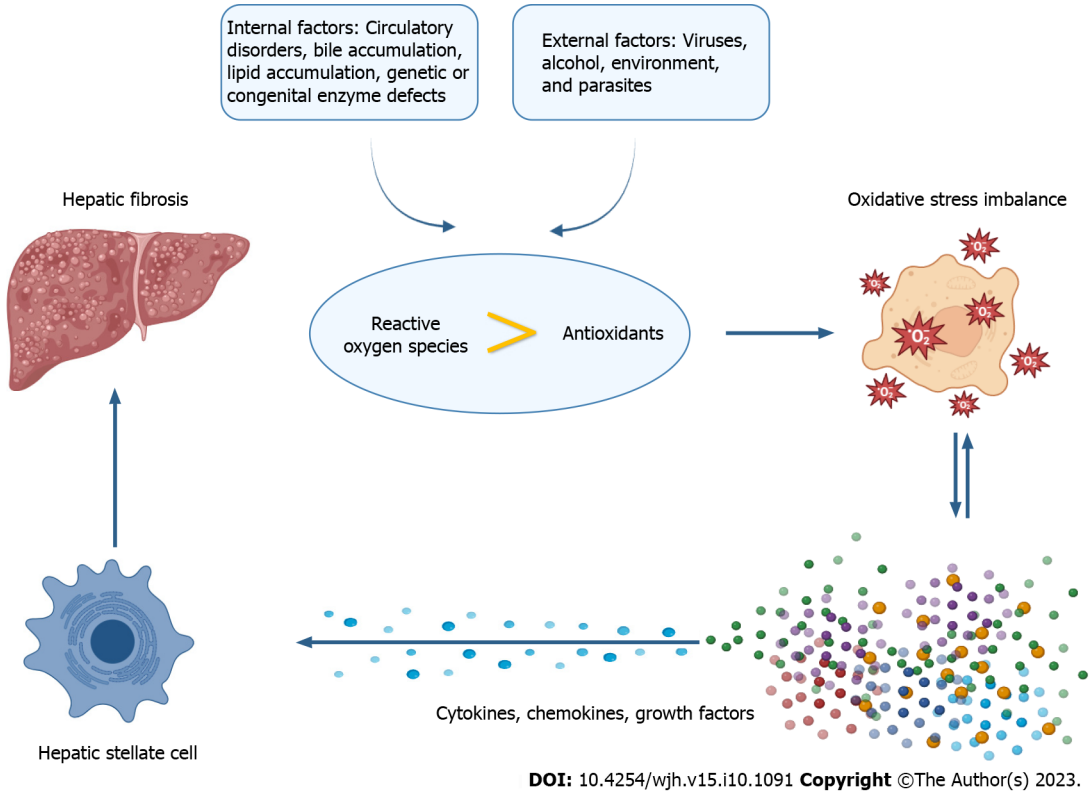
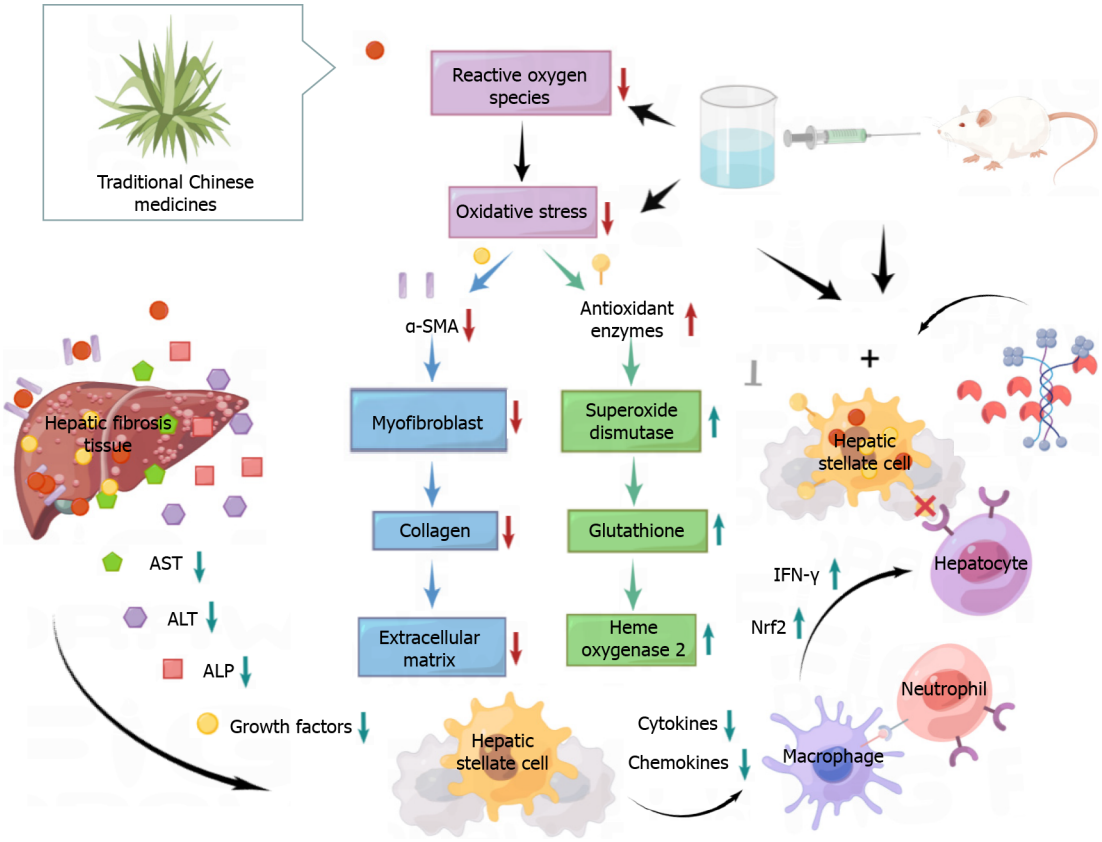


Figure 2 The mechanism of oxidative stress-mediated hepatic fibrosis. Circulatory disorders, bile accumulation, lipid accumulation, genetic or congenital enzyme defects, as well as factors such as viruses, alcohol, environment, and parasites, can all contribute to an imbalance between the production of reactive oxygen species and the body's ability to remove them, resulting in oxidative stress. This imbalance can further stimulate the secretion of cytokines, chemokines, and growth factors, which in turn activate hepatic stellate cells and contribute to the development of hepatic fibrosis.

Furthermore, if this occurs in spring, it is called tendon arthralgia. Tendon arthralgia continues stubbornly and is further affected by evil forces, which reside in the liver interior. All organs have junctions, so if an illness remains lingering, it will settle in the junction of that organ. Since the liver's junction is with the tendons, the arthralgia continues, and when affected by evil forces, it settles in the liver[31]." Therefore, the TCM understanding of the pathogenesis of hepatic fibrosis can be summarized as weakened vital qi, allowing external pathogenic factors such as excessive exposure to the "Six Pathogens" or inappropriate emotional responses from the "Seven Emotions" to invade, resulting in Qi stagnation and blood stasis[32]. This progression is often slow and persistent, ultimately leading to hepatic fibrosis. Based on classic single herbal extracts and TCM formulas, many effective preventive and therapeutic TCM formulas have been developed and applied clinically. Currently, TCM monomers, single herbal extracts, and TCM formulas can regulate HSCs and ECM expression levels by affecting OS, achieving a state of balance between yin and yang in the body, and thereby preventing and treating hepatic fibrosis[33]. Therefore, this article analyzes and discusses the research progress in preventing and treating hepatic fibrosis through the inhibition of OS by using TCM monomers, single herbal extracts, and TCM formulas, based on recent domestic and international studies on hepatic fibrosis, in order to provide a theoretical basis for future research.

MECHANISMS OF TCM IN ANTI-FIBROSIS

As TCM has shown good therapeutic effects in various chronic diseases, its role and mechanism in preventing and treating hepatic fibrosis have attracted widespread attention from scientists. Scholars from various countries have begun using modern pharmacological research methods to explore the mechanisms of TCM in anti-fibrosis. It has been confirmed that TCM monomers (such as flavonoids, glycosides, alkaloids, and polysaccharides), single herbal extracts (such as *Salvia miltiorrhiza*, *Ginkgo biloba* leaf, clove basil, *Ceratonia siliqua* pod extract, grape seed, pomegranate extract, *Taraxacum officinale* root extract, and *Myrtus communis*), and TCM formulas [such as Yin-Chen-Hao-Tang (YCHT), Xiaochaihu Tang (XCHT), Fu Zheng Hua Yu Fang, Chunggan extract, and Huangjia Ruangan Granule] can be used to inhibit OS and prevent and treat hepatic fibrosis (Figure 3).



DOI: 10.4254/wjh.v15.i10.1091 **Copyright** ©The Author(s) 2023.

Figure 3 Treatment of hepatic fibrosis by oxidative stress mediated by traditional Chinese medicine. Traditional Chinese medicine employs various mechanisms to reduce the production of reactive oxygen species in the body, thereby suppressing oxidative stress reactions. They can enhance the secretion of antioxidant enzymes such as superoxide dismutase, glutathione, and heme oxygenase 2, as well as increase the activity of interferon-γ and nuclear factor erythroid 2-related factor. Moreover, traditional Chinese medicine can inhibit the secretion of growth factors, cytokines, chemokines, macrophages, and neutrophils. They can also hinder the activation of quiescent hepatic stellate cells into myofibroblasts and facilitate the degradation of extracellular matrix components like collagen. These actions contribute to the protection of liver function and hepatocytes, ultimately mitigating and reversing hepatic fibrosis. ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate aminotransferase; α-SMA: Alpha-smooth-muscle actin; IFN-γ: Interferon-γ; Nrf2: Nuclear factor erythroid 2-related factor.

TCM MONOMERS

In recent years, numerous experimental studies have demonstrated that TCM monomers, such as flavonoids, glycosides, alkaloids, and polysaccharides, can reduce the activation rate of HSCs by inhibiting OS and reducing excessive ECM deposition. This in turn can suppress hepatic fibrosis and connective tissue proliferation or improve liver function and delay the progression of hepatic fibrosis.

FLAVONOIDS

Flavonoids are a class of compounds that belong to the family of polyphenols. They are widely found in plant-based foods such as vegetables, fruits, and grains[34]. Flavonoids are known for their beneficial effects on human health, such as antioxidant and anti-inflammatory properties[35]. The most common flavonoids include quercetin, kaempferol, and myricetin, which are found in many fruits and vegetables[34]. Flavonoids have been linked to a reduced risk of chronic diseases such as cardiovascular disease, cancer, and diabetes[36]. Briefly, the consumption of flavonoid-rich foods is an important part of a healthy diet. Extensive pharmacological research has shown that flavonoids possess the ability to inhibit the pathological production of ROS, suppress OS, boost the antioxidant capacity of the body, and offer protection against hepatic fibrosis[37].

Quercetin is an important plant chemical substance belonging to the polyphenolic flavonoid group[38]. The chemical formula of quercetin is $C_{15}H_{10}O_7$, and its structure shares a common flavonoid nucleus composed of two benzene rings linked by a heterocyclic pyran ring[39]. Quercetin is commonly found in various fruits and vegetables, including apples, berries, cherries, red leaf lettuce, onions, and asparagus, with small amounts present in pepper, broccoli, peas, and tomatoes[40]. It is well known that onions contain the highest levels of quercetin[41]. Quercetin is one of the most extensively researched flavonoids and has been found to exhibit exceptional antioxidant activity. Its effects on GSH and ROS activity, as well as its regulation of various signaling pathways including heme oxygenase 1/nuclear factor erythroid 2-related factor (Nrf2), mitogen-activated protein kinase (MAPK), Toll-like receptor 4 (TLR4)/phosphatidylin-

ositol-3-kinase (PI3K), and 5'adenosine monophosphate-activated protein kinase have been demonstrated in numerous studies[42]. Studies have also shown that quercetin can enhance the activities of antioxidant enzymes like SOD and increase GSH levels. Furthermore, it can inhibit OS, down-regulate inflammatory cytokine expression, and reduce tissue histopathological changes induced by thioacetamide, thus mitigating hepatic fibrosis[43]. In addition, Khodarahmi *et al* [44] proposed that quercetin can improve hepatic fibrosis by inhibiting ROS-related OS-mediated inflammatory cascades. Concisely, these findings suggest that quercetin has potential therapeutic benefits for hepatic fibrosis through its ability to enhance antioxidant enzyme activities, reduce OS and inflammation, and inhibit ROS-mediated cascades. However, more research is needed to fully understand the mechanism of action and optimal dosage of quercetin for treating hepatic fibrosis.

Isorhamnetin, with the chemical formula $C_{16}H_{12}O_7$, is a 3'-O-methylated gut metabolite of quercetin[45]. Isorhamnetin belongs to the flavonoid family, more specifically the flavonol group[46]. It can be found in several plants, such as sorbus, ginkgo leaves, or cactus, which have traditionally been used as medicinal plants in various cultures[47]. *In vitro*, isorhamnetin can scavenge diphenylpicrylhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radicals, inhibit liver mitochondrial lipid peroxidation, and exhibit antioxidant activity[48]. Furthermore, isorhamnetin can block TGF-β1-induced ROS production and GSH depletion, reduce phosphorylated Smad3, TGF-β1, α-SMA, and plasminogen activator inhibitor-1 expression, and collagen expression in primary mouse HSCs and LX-2 cells, alleviate OS, and inhibit HSC activation, thereby preventing hepatic fibrosis[49]. These findings suggest that isorhamnetin may have potential therapeutic applications for hepatic fibrosis.

Naringin is a natural organic compound with the molecular formula $C_{15}H_{12}O_5$[50]. Naringin and its glycosides are present in various herbs and fruits, including grapefruit, Buddha's hand citron, lime, tart cherry, tomato, cocoa, Greek hay, water mint, and legumes[51]. Due to its hydroxyl substituents, naringin exhibits high reactivity towards ROS and reactive nitrogen species and has strong inhibitory effects on lipid peroxidation in mouse liver, brain, and heart tissues [52]. In 2017, Hernández-Aquino *et al*[53] reported that administration of naringin could potentially prevent an increase in liver enzymes such as alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase, and GSH peroxidase (GSH-Px). Additionally, naringin was shown to enhance the body's antioxidant capacity and effectively prevent liver inflammation, hepatocyte necrosis, and hepatic fibrosis induced by carbon tetrachloride (CCl_4)[53]. Building on this work, Hernández-Aquino *et al*[54] conducted further research in 2019 and found that naringin has the potential to inhibit OS and exert its anti-fibrotic effect by blocking the nuclear transcription factor-κB (NF-κB), TGF-β-Smad3, and c-Jun N-terminal kinase-Smad3 pathways. These findings suggest that naringin could be a promising candidate for treating human fibrosis.

Additionally, *Mallotus apelta* (Lour.) Muell.Arg. leaf and *Bidens bipinnata* L. contain total flavonoids along with other beneficial compounds such as puerarin, hesperidin, alpinetin, fisetin, glabridin, morin, and astilbin. These compounds have been shown to inhibit OS and promote a stable internal environment within the body, thereby improving hepatic fibrosis (Table 1).

GLYCOSIDES

Glycosides are a diverse group of biologically active compounds that are widely distributed in the plant kingdom[55]. They consist of a sugar molecule linked to a non-sugar compound, such as a flavonoid or terpenoid. Glycosides have been extensively studied for their pharmacological properties, which include anti-inflammatory, antibacterial, antifungal, and antioxidant effects[56]. In particular, glycosides have been found to possess potent antioxidant properties that can prevent or reduce OS in various tissues and organs, including the liver[57].

Saikosaponin-D is a type of glycoside monomer component extracted from the dried roots of *Bupleurum chinense* DC. and *Bupleurum scorzonerifolium* Willd, both plants belonging to the Umbelliferae family[58]. Its molecular formula is $C_{42}H_{68}O_{13}$[58]. Saikosaponin-D possesses various pharmacological effects such as antioxidant, sedative, antiviral, anti-inflammatory, immune-regulatory, hepatoprotective, and anticancer activities[59]. Saikosaponin-D can delay the development of hepatic fibrosis by alleviating liver cell damage caused by OS[57]. Researchers such as Que *et al*[60] have proposed that saikosaponin-D may downregulate the ROS/MAPK signaling pathway. This not only significantly inhibits the proliferation and activation of HSC-T6 cells induced by OS but also reduces the deposition of the ECM, such as tissue inhibitors of TGF-β1, hydroxyproline, collagen-1, and matrix metalloproteinase (MMP)-1, which indicates its potential as a therapeutic agent for hepatic fibrosis[60]. However, despite its promising pharmacological properties, the clinical use of saikosaponin-D is limited due to its low bioavailability and poor water solubility. Further research is needed to develop effective delivery systems and optimize its pharmacokinetic properties to allow for its use in clinical settings.

Resveratrol glucoside is a polyphenol and monocrystalline natural compound belonging to the stilbene class[61]. Vitaceae, Liliaceae, and Leguminosae families are the important sources of resveratrol glucoside extraction[62]. It is mainly isolated from the rhizome and roots of *Polygonum cuspidatum*, and also found in daily foods such as grapes and red wine[62]. Various studies have shown that resveratrol glucoside has a variety of pharmacological activities, such as anti-inflammatory, anti-apoptotic, anti-tumor, lipid-lowering, and cardiovascular protective effects, particularly strong antioxidant pharmacological activity[63]. Resveratrol glucoside has been shown to have antioxidant biological activity and therapeutic action on many liver diseases, including hepatic fibrosis. For example, resveratrol glucoside has been found to inhibit the production of 4-hydroxydecenoic acid in the liver and the expression of nicotinamide adenine dinucleotide phosphate oxidase 4, thereby reducing OS and inflammation and improving chronic liver injury and fibrosis [64,65]. Moreover, research has demonstrated that resveratrol glucoside can also downregulate the nicotinamide adenine dinucleotide phosphate oxidase 4 enzyme, thus decreasing TLR4/NF-κB p65 signaling pathway-related inflammatory

Table 1 Flavonoids improve hepatic fibrosis by inhibiting oxidative stress

No.	Flavonoids	Model	Upregulated molecules	Downregulated molecules	Mechanism	Ref.
1	*Mallotus apelta* (Lour.) Muell.Arg. leaf	Rat	IkBa, SOD, GSH-Px	HYP, PC-III, Col-I, HA, LN, α-SMA, Col-III, MDA, TNF-α, IL-1β, IL-6, MMP-2/9, TIMP-1, TGF-β1/Smad, IKKβ, NF-κB p65, pNF-κBp65, IκBα, p-IκBα	Improving hepatic fibrosis and reducing ECM accumulation are related to inhibiting OS and regulating the TGF-β1/Smad signaling pathway and NF-κB-dependent inflammatory response	Zhang *et al* [117]
2	*Bidens bipinnata* L.	Rat	SOD, GSH-Px	NF-κB, α-SMA, TGF-β1	Inhibiting OS to improve liver injury and protect rats from CCl4-induced hepatic fibrosis	Yuan *et al* [118]
3	Puerarin	Rat	SOD, CAT	TG, TCHO, LDL, α-SMA, Col-I/III, MDA, IL-1β, IL-6, TNF-α, MCP-1, NF-κB/p65, TGF-β, TGF-β/Smad2/3	Inhibiting OS and inflammation associated with NF-κB signal inactivation, thereby blocking the upregulation of pro-inflammatory cytokines (IL-1β and TNF-α) and chemokines (MCP-1), ultimately improving hepatic fibrosis	Hou *et al* [119]
4	Hesperidin	Mice		ROS	Regulating liver OS and alleviating mitochondrial dysfunction to reduce fibrosis	Li *et al*[120]
5	Alpinetin	Mice	IL-10, CAT, GSH-Px, SOD, GSH, Nrf2	HYP, α-SMA, fibronectin, α1(I) procollagen, IL-1β, IL-6, TNF-α, Cox-2, iNOS, MDA, VEGF, VEGFR2, PDGF-βR, HIF-1α	Preventing and treating hepatic fibrosis by inhibiting the anti-inflammatory activity mediated by NLRP3 and the anti-OS activity mediated by Nrf2	Zhu *et al* [121]
6	Fisetin	Rat	GSH, GSK-3β	MDA, TNF-α, IL-6, TGF-β1, Col-I, TIMP-1, Wnt3a, β-catenin, α-SMA, Cyclin D1	Inhibiting liver OS can suppress Wnt/β-catenin pathway and inhibit HSC activation and proliferation, regulate MMP-9 and TIMP-1, and suppress multiple pro-fibrotic factors, to prevent and treat hepatic fibrosis	El-Fadaly *et al*[122]
7	Glabridin	Mice	PPAR-γ, GSH, T-AOC	α-SMA, fibronectin, Col-α1(I), MDA	Activation of PPARγ can inhibit inflammation and OS, thereby inhibiting HSC activation and hepatic fibrosis	Zhang *et al* [123]
8	Morin	Rat	GSH	NO, HYP, TNF-α, iNOS, NF-κB (p65)	Receiving MDA and nitric oxide levels elevation, and restoring GSH to normal levels can inhibit OS and improve hepatic fibrosis	Heeba and Mahmoud [124]
9	Astilbin	Rat	SOD, GSH, GCLC, GCLM, HO-1, NQO1, Nrf2	TGF-β, Col-I/III, HYP, TNF-α, IL-1β, IL-6, MDA	Significant reduction in collagen production, inflammation and OS *in vivo* can prevent CCL4-induced hepatic fibrosis in rats	Sun *et al* [125]

α-SMA: Alpha-smooth-muscle actin; ECM: Extracellular matrix; OS: Oxidative stress; TGF-β1: Transforming growth factor-beta 1; NF-κB: Nuclear transcription factor-κB; SOD: Superoxide dismutase; GSH: Glutathione; GSH-Px: Glutathione peroxidase; IL: Interleukin; TNF: Tumor necrosis factor; ROS: Reactive oxygen species; Nrf2: Nuclear factor erythroid 2-related factor; HSC: Hepatic stellate cell.

reactions and macrophage expression, which suggests that it could be an effective therapeutic agent for preventing and treating hepatic fibrosis[66]. To sum up, resveratrol glucoside shows promise as a natural compound for preventing and treating chronic liver injury and fibrosis. Further research is needed to fully understand its mechanisms of action and optimal dosage for therapeutic use.

Geniposide is an organic compound with the molecular formula $C_{17}H_{24}O_{10}$[67]. It is derived from the dried mature fruit of *Gardenia jasminoides Ellis*, a plant belonging to the Rubiaceae family[68]. Geniposide is mainly found in *Gardenia jasminoides*, but has also been detected in other commonly used Chinese herbal medicines such as *Eucommia ulmoides*, *Rehmannia glutinosa*, and *Scutellaria baicalensis*[68]. Geniposide not only upregulates endogenous antioxidant enzymes to slow down cell damage, but also increases the activity of antioxidant enzymes and pathways such as liver lipid peroxidation, GSH S-transferases, GSH, GSH-Px, and copper- and zinc-containing SOD, which can prevent OS damage, protect hepatocytes, and improve hepatic fibrosis[69]. The study by Yang *et al*[70] investigated the protective effects of geniposide on hepatic fibrosis in a rat model induced by CCl_4 administration. The researchers found that geniposide treatment significantly reduced hepatic fibrosis and improved liver function, as evidenced by decreased levels of serum ALT, aspartate aminotransferase (AST), and ALP. Further analysis revealed that geniposide exerted its anti-fibrotic effects through multiple mechanisms[70]. First, geniposide increased the activities of two important antioxidant enzymes, SOD and GSH-Px, which scavenge free radicals and protect cells from oxidative damage. This was accompanied by a reduction in the levels of malondialdehyde (MDA), a biomarker of lipid peroxidation, in the liver tissue[70]. In brief, the study suggested that geniposide has potential as a therapeutic agent for hepatic fibrosis by targeting OS. However, further studies are needed to confirm these findings in human subjects and to explore the optimal dosage and duration of geniposide treatment.

In addition, glycoside compounds such as baicalin, vitexin, and forsythoside A (Table 2) can also improve hepatic fibrosis by inhibiting the body's OS, regulating intestinal flora bile acid metabolism, increasing antioxidant and phase II

Table 2 Glycosides improve hepatic fibrosis by inhibiting oxidative stress

No.	Glycosides	Model	Upregulated molecules	Downregulated molecules	Mechanism	Ref.
1	Wogonoside	Rat	SOD, GSH, IL-10	HA, LN, HYP, MDA, TNF-α, IL-1β, IL-6, TGF-β, MMP-2/9, Smad-3, PI3K/Akt/mTOR, NF-κB p65, PI3K/Akt/mTOR/p70S6K, α-SMA, Col-I	Inhibition of OS can improve hepatic fibrosis	Wang *et al* [126]
2	Hyperoside	Mice	SOD, GSH-Px, CAT, GSH, Nrf2	GOT, GPT, MDA, TGF-β, Col-I/III	Activating Nrf2 nuclear translocation, inhibiting OS to increase antioxidant and phase II detoxifying enzyme activity can improve hepatic fibrosis	Zou *et al*[127]
3	Forsythiaside A	Mice	SOD, GSH, CAT, GSH-Px, p450 7a1, FXR, SHP, LRH-1, BSEP, NTCP, OATP-1	HA, LN, PC-III, Col-I/IV, HYP, α-SMA, TNF-α, IL-1β, IL-6, LCA, DCA, HDCA, β-MCA, alloLCA, NorDCA, NorCA, CA, FGF-R4, MRP-2	Inhibiting OS and inflammation, regulating gut microbiota and BA metabolism can improve hepatic fibrosis	Fu *et al* [128]

α-SMA: Alpha-smooth-muscle actin; OS: Oxidative stress; SOD: Superoxide dismutase; GSH: Glutathione; GSH-Px: Glutathione peroxidase; IL: Interleukin; Nrf2: Nuclear factor erythroid 2-related factor.

detoxification enzyme activity. To recap, the evidence suggests that glycosides can prevent or reduce hepatic fibrosis by inhibiting ROS production, reducing OS, and modulating HSC activation. These findings highlight the potential of glycosides as therapeutic agents for hepatic fibrosis and other OS-related diseases. However, further studies are needed to elucidate the molecular mechanisms underlying the protective effects of glycosides and identify optimal doses and treatment regimens.

ALKALOIDS

Alkaloids are a class of naturally occurring organic compounds that are characterized by their bitter taste and basic properties[71]. They are found in many plants and have a wide range of biological activities, including analgesic, anti-inflammatory, and anti-cancer properties. Alkaloids have complex interactions with ROS and hepatic fibrosis[72].

Some alkaloids have protective effects against hepatic fibrosis by reducing ROS production and promoting antioxidant activity. Further research is needed to better understand the mechanisms underlying these effects and to identify new alkaloids with potential therapeutic applications in hepatic fibrosis.

Berberine is a naturally occurring compound found in various plants such as goldenseal, barberry, and oregon grape [73]. It has been widely used in traditional medicine for its anti-inflammatory, anti-microbial, and anti-diabetic properties [74]. In recent years, there has been growing interest in the potential of berberine as a treatment for hepatic fibrosis. Studies have shown that berberine has potent antioxidant properties that help reduce OS and ROS levels in the liver[75]. It achieves this by activating various cellular defense mechanisms that protect liver cells from oxidative damage[75]. Furthermore, studies suggest that berberine can prevent the activation of HSCs, which are responsible for producing the scar tissue that leads to hepatic fibrosis[76]. Domitrović *et al*[77] demonstrated that the administration of high-dose berberine (9 mg/kg) is effective in reducing OS, decreasing the expression of tumor necrosis factor-alpha (TNF-α) and TGF-β1, increasing MMP-2 levels, and promoting the removal of fibrous deposits to ameliorate hepatic fibrosis. In essence, these findings suggest that berberine has great potential as a therapeutic agent in the treatment of hepatic fibrosis. By reducing OS and preventing the activation of HSCs, berberine may help slow or even reverse the progression of hepatic fibrosis. However, further research is necessary to fully understand the mechanisms through which berberine exerts its beneficial effects on hepatic fibrosis.

Betaine, also known as trimethylglycine, is a naturally occurring compound found in many foods, including spinach, beets, and whole grains[78]. It is used as a dietary supplement to improve athletic performance, promote liver health, and reduce the risk of liver disease[79]. Research suggests that betaine may help to alleviate hepatic fibrosis by reducing OS. In a study conducted by Bingül *et al*[80], betaine supplementation was found to significantly reduce ROS levels, decrease lipid peroxidation, and increase antioxidant enzyme activity in rats with hepatic fibrosis induced by CCl_4 exposure. Additionally, betaine treatment reduced collagen deposition and improved liver function in these rats, indicating that it may have therapeutic potential for hepatic fibrosis[81]. Another study conducted by Kim *et al*[82] investigated the effects of betaine on OS and fibrosis in liver cells. The researchers found that betaine treatment reduced ROS levels and lipid peroxidation, increased GSH levels (an important antioxidant in the body), and inhibited the expression of fibrotic markers in HSCs, the primary cells responsible for hepatic fibrosis. These results suggest that betaine may exert its anti-fibrotic effects by modulating OS and reducing fibrogenic signaling pathways in liver cells[82]. In conclusion, the relationship between betaine and hepatic fibrosis is complex, but emerging evidence suggests that betaine may help to alleviate hepatic fibrosis by reducing OS, inhibiting fibrogenic signaling pathways, and promoting liver function. Further research is needed to fully elucidate the mechanisms underlying betaine's therapeutic effects on hepatic fibrosis and determine optimal dosages and treatment durations for clinical use.

Lycorine is a natural alkaloid that is found in various plant species such as the Amaryllidaceae family[83]. It has been known to possess multiple pharmacological properties, including anti-cancer, anti-inflammatory, and antiviral activities [84]. Recently, lycorine has also been studied for its effect on hepatic fibrosis, a chronic condition that occurs due to the accumulation of ECM proteins in the liver tissue. Lycorine has been shown to inhibit ROS production and reduce OS, thereby reducing HSC activation and regulating the fibrotic process[83]. Furthermore, lycorine may also have a protective effect against liver injury caused by OS. In a study conducted on animal models of acute liver injury induced by CCl_4, lycorine was found to prevent liver damage by reducing OS and inflammation in liver tissue[85]. To conclude, the relationship between lycorine, ROS, OS, and hepatic fibrosis is complex and multifaceted. More research is needed to explore the potential therapeutic effects of lycorine in hepatic fibrosis and other related liver diseases. However, the current evidence suggests that lycorine may hold promise as a natural therapeutic agent for hepatic fibrosis.

To put it briefly, alkaloids have shown potential as therapeutic agents for the treatment of hepatic fibrosis. However, more research is needed to determine their efficacy and safety, especially at higher doses. It is important to work with a healthcare professional to determine the best course of action for managing hepatic fibrosis and to monitor liver function regularly.

POLYSACCHARIDES

Polysaccharides are complex carbohydrates that play an important role in the body's physiological processes[86]. They are found in a variety of sources, including plants, fungi, and animals, and are known for their ability to confer a range of health benefits[86]. One area where polysaccharides have shown particular promise is in the treatment of hepatic fibrosis. Recent research has suggested that polysaccharides may offer a promising alternative for managing hepatic fibrosis. In particular, studies have shown that certain polysaccharides have antioxidant properties, which can help reduce OS in the liver. OS is known to play a key role in the development and progression of hepatic fibrosis.

Cordyceps is a type of fungus that has been used for centuries in traditional medicine to treat a variety of ailments[87]. In recent years, researchers have been investigating the therapeutic potential of Cordyceps polysaccharides, one of the main bioactive compounds found in the fungus[88]. Cordyceps polysaccharides have been shown to have antioxidant and anti-inflammatory properties, making them a promising candidate for treating hepatic fibrosis, a condition characterized by scarring and damage to the liver[89]. Cordyceps polysaccharides protect hepatocytes from hydrogen peroxide-induced mitochondrial dysfunction by reducing ROS production and regulating mitochondrial apoptotic signaling *via* cytochrome C, apoptosis regulator Bax, and mitochondrial-related apoptotic proteins in HepG2 cells[90]. In addition, studies have shown that Cordyceps polysaccharides can reduce the release of pro-inflammatory cytokines and cell apoptosis by regulating TLR4/myeloid differentiation factor 88/NF-κB, Bcl-2/Bax, and caspase family signaling pathways, thereby reducing OS, serum enzymes, α-SMA, Col-III, TGF-β1, p-Smad3, and collagen volume fraction, inhibiting OS, enhancing the body's antioxidant defense system, and improving hepatic fibrosis[91]. Recapitulating, Cordyceps polysaccharides have demonstrated hepatoprotective effects against liver injury caused by OS. They can regulate various signaling pathways to promote liver cell survival, reduce fibrosis, and improve liver function. These findings suggest that polysaccharides may be a promising therapeutic agent for liver diseases.

The relationship between polysaccharides, OS, and hepatic fibrosis is complex, and additional research is needed to fully understand the mechanisms underlying this relationship. However, these findings suggest that polysaccharides may offer a promising new avenue for the treatment of hepatic fibrosis.

SINGLE HERBAL EXTRACTS

Single herbal extracts such as *Salvia miltiorrhiza*, *Ginkgo biloba* leaf, clove basil, *Ceratonia siliqua* pod extract, grape seed, pomegranate extract, *Taraxacum officinale* root, and *Myrtus communis* have demonstrated significant potential in combating hepatic fibrosis. These extracts target OS pathways, regulate HSC activation and apoptosis, and inhibit collagen deposition in liver tissue, as outlined in Table 3.

Salvia miltiorrhiza, also known as Danshen, is a TCM that has been used for centuries to treat various ailments, including liver disease[92]. Recent studies have shown that one of the mechanisms by which *Salvia miltiorrhiza* exerts its hepatoprotective effects is through the modulation of OS pathways in the liver[93]. Research has demonstrated that *Salvia miltiorrhiza* can alleviate hepatic fibrosis by reducing the generation of ROS and the consequent activation of HSCs[94]. In a recent study, Zhang *et al*[94] investigated the effect of *Salvia miltiorrhiza* on ROS-induced hepatic fibrosis in rats. The researchers found that treatment with *Salvia miltiorrhiza* significantly reduced the levels of ROS and lipid peroxidation products in the liver tissue, and also inhibited the activation of HSCs[94]. Furthermore, they observed that *Salvia miltiorrhiza* treatment increased the expression of antioxidant enzymes, such as SOD and catalase, which help protect against OS in the liver[95]. Another study by Wang *et al*[96] explored the potential molecular mechanisms underlying the anti-fibrotic effects of *Salvia miltiorrhiza*. The researchers found that *Salvia miltiorrhiza* could inhibit the expression of fibrogenic genes in HSCs, such as *Col-I* and *α-SMA*, by downregulating the activity of the TGF-β signaling pathway, which is known to be a key regulator of fibrogenesis in the liver[96]. Moreover, they observed that *Salvia miltiorrhiza* treatment reduced the production of ROS and enhanced the activity of antioxidant enzymes in HSCs, which may contribute to its anti-fibrotic effects[97]. Summarily, these studies provide evidence that *Salvia miltiorrhiza* can protect against hepatic fibrosis by modulating OS pathways in the liver. Further research is needed to fully understand the molecular mechanisms underlying this effect and to explore the potential of *Salvia miltiorrhiza* as a therapeutic agent for

Table 3 Single herbal extracts improve hepatic fibrosis by inhibiting oxidative stress

No.	Single herbal extracts	Model	Upregulated molecules	Downregulated molecules	Mechanism	Ref.
1	*Salvia miltiorrhiza*	Mice	SOD, GSH	Col-1/III, TGF-β, MDA, IL-1α, TNF-α	Reducing MDA content, increasing SOD activity and GSH content, and suppressing OS while also reducing TNF-α and IL-1α can improve hepatic fibrosis	Zhang *et al* [94]
2	*Ginkgo biloba* leaf	Rat		MDA, TGF-β1, Col-1	Effectively regulating the OS thioacetamide-induced hepatic fibrosis	Al-Attar *et al* [100]
3	Clove basil	Rat	CAT	α-SMA, Col-α	Inhibiting OS can significantly suppress HSC activation, as well as the expressions of α-SMA and Col-α, exhibiting its anti-hepatic fibrosis properties	Chiu *et al* [103]
4	*Ceratonia siliqua* pod	Mice	GSH, SOD, CAT, GST, GPx, GR	TIMP-2, NO	Inhibiting the production of LPO and NO, increasing the content of GSH, and restoring the activity of antioxidant enzymes can suppress OS, granuloma formation, and hepatic fibrosis	Al-Olayan *et al*[129]
5	Grape seed	Rat	AOC, GSH	TNF-α, MDA, MPO	Inhibiting neutrophil infiltration and lipid peroxidation, suppressing OS, restoring the oxidative and antioxidant status in tissues can help protect against hepatic fibrosis	Dulundu *et al*[130]
6	Pomegranate	Rat	SOD, GST, CAT, Nrf2	NF-κB, α-SMA, Cox-2	Reducing OS by regulating Nrf2 and NF-κB can eliminate hepatic fibrosis	Husain *et al* [131]
7	*Taraxacum officinale* root	Mice	SOD, mt I/II	α-SMA	Inhibiting OS, inducing HSC inactivation and enhancing liver regeneration capacity are effective in the treatment of hepatic fibrosis	Domitrović *et al*[132]
8	*Myrtus communis*	Rat	GSH, SOD	TNF-α, IL-1β, MDA, MPO, TGF-β	Its antioxidant and free radical scavenging activities protect liver tissue from OS damage after bile duct ligation , thereby exerting its anti-fibrotic effects	Sen *et al*[133]

α-SMA: Alpha-smooth-muscle actin; OS: Oxidative stress; SOD: Superoxide dismutase; GSH: Glutathione; IL: Interleukin; Nrf2: Nuclear factor erythroid 2-related factor; HSC: Hepatic stellate cell.

hepatic fibrosis.

Ginkgo biloba leaf is a popular herb that has been used for centuries for various health benefits[98]. Ginkgo contains flavonoids and terpenoids, which are known to have antioxidant and anti-inflammatory properties[45]. These compounds make ginkgo a potential treatment option for hepatic fibrosis[99]. *Ginkgo biloba* leaf has the potential therapeutic effect of effectively regulating OS induced by thioacetamide and exerting an effect on thioacetamide-induced hepatic fibrosis[100]. Studies have demonstrated that *Ginkgo biloba* leaf can inhibit HSC activation and reduce collagen deposition in liver tissue by regulating OS pathways[100]. In addition, *Ginkgo biloba* leaf has been found to upregulate the expression of Nrf2, a transcription factor that plays a key role in the cellular response to OS[101]. This suggests that *Ginkgo biloba* leaf may be a promising therapeutic agent for hepatic fibrosis.

Clove basil, also known as *Ocimum gratissimum*, is a species of basil that is native to parts of Africa and Asia[102]. It has been used in traditional medicine to treat a variety of conditions, including liver diseases. Recent studies have demonstrated the potential of clove basil extract in preventing the development of hepatic fibrosis through its antioxidant properties. Clove basil contains high levels of phenolic compounds, such as rosmarinic acid, which have potent antioxidant activity. Studies have shown that the clove basil extract possesses anti-hepatic fibrosis properties by inhibiting serum-induced HSC activation and reducing the expression of α-SMA and Col-a[103]. This effect is attributed to the antioxidant components in the extract, which suppress OS and prevent hepatic fibrosis[103]. Additionally, studies have shown that clove basil extract can maintain levels of ALT and AST, as well as MDA, catalase, and α-SMA levels induced by CCl_4, indicating its ability to protect the liver from oxidative damage and promote healing of hepatic fibrosis [104]. Synthetically, clove basil extract has the potential to be used as a therapeutic agent in the treatment of hepatic fibrosis due to its antioxidant and anti-HSC activation properties. However, further studies are needed to explore its mechanism of action and its potential use in clinical settings.

These findings suggest that single herbal extracts may be a potential treatment option for hepatic fibrosis. However, more research is needed to fully understand their effectiveness and potential side effects. It is important to consult with a healthcare professional before taking any herbal supplements, including clove basil.

TCM FORMULAS

The TCM formula stands out for its multi-component, multi-target, and low adverse reaction properties. It employs syndrome differentiation treatment and holistic therapy to regulate the body functions and status, improves prognosis, and eliminates pathogenic factors without harming healthy ones. As a result, TCM formulas show remarkable potential in preventing and treating hepatic fibrosis. This article presents a summary of TCM formulas (Table 4) which have been

Table 4 Traditional Chinese medicine formulas improve hepatic fibrosis by inhibiting oxidative stress

No.	Traditional Chinese medicine formulas	Composition	Efficacy	Model	Upregulated molecules	Downregulated molecules	Mechanism	Ref.
1	Yin-Chen-Hao-Tang	*Artemisia annua* L. 18 g, *Gardenia jasminoides Ellis* 12 g, *Rheum palmatum* 6 g	Clearing heat and promoting diuresis to relieve dampness and jaundice	Rat		Col-I, α-SMA, TGF-β, HYP, ROS	Alleviating hepatic fibrosis by enhancing antioxidant capacity, reducing ROS levels, suppressing OS, and inhibiting inflammatory response	Lee *et al* [106], Mountford *et al*[108], Chen *et al* [109]
2	Xiaochaihu Tang (XCHT)	Bupleurum root 12 g, *Scutellaria baicalensis* 9 g, Ginseng 6g, *Pinellia ternata* 9 g, Roasted Licorice 5 g, Fresh Ginger 9 g, 4 Red Dates	Soothing liver and relieving depression, regulating stomach and lowering qi counterflow, tonifying qi and strengthening spleen, clearing liver and purging fire	Rat	Nrf2, Nqo1, HO-2, GCLC, GCLM	α-SMA, HA, PCIII, LN	Activating the Nrf2 pathway can attenuate OS and further suppress activated HSCs, thereby improving hepatic fibrosis	Li *et al*[111], Hu *et al*[112]
3	Fu Zheng Hua Yu Fang	*Salvia miltiorrhiza*, Fermented *Cordyceps sinensis* powder, Peach kernel, Pine pollen, *Lycopodium clavatum*, *Schisandra chinensis* (processed)	Activating blood circulation and removing blood stasis, nourishing essence and nurturing liver	Mice		α-SMA, Nqo1, HO-2, GCLC, GCLM, TIMP1, TGF-β1, TGF-RI, PDGF-Rβ, MDA, Col-1aI, TGF-βRI, PDGF-β, MCP-1, MIP-1 mRNA	Effectively suppressing the production of liver ROS, inhibiting OS, and exerting anti-fibrotic effects	Jiang *et al* [116]
4	CGX	*Artemisia annua* 5g, *Trionyx sinensis* shell 5g, Radish seed 5g; White Atractylodes 3g, Pogostemon cablin 3g, Alisma orientale 3g, White Atractylodes 3g, *Salvia miltiorrhiza* 3g; *Poria cocos* 2g, Aurantium fruit 2g, Amomum cardamomum 2g, Licorice 1g, *Saussurea costus* 1g	Clearing liver and activating blood circulation, strengthening spleen and transforming dampness	Rat	GSH-Px, GSH-Rd, GST, SOD, IFN-γ	MDA, PDGF-BB, TGF-β1, CTGF	It inhibits the levels of liver tissue HYP and MDA, while increasing the total GSH content and the activity of GSH-oxidation reduction system enzymes, which suppresses OS and exerts its anti-hepatic fibrosis effects	Kim *et al* [134]
5	Huangjia Ruangan Granule	*Astragalus membranaceus*, *Trionyx sinensis* shell, *Pueraria lobata* root, Bupleurum root, *Ganoderma lucidum*, *Paeonia lactiflora*, *Salvia miltiorrhiza*, *Panax notoginseng*, *Lycopodium* clavatum, *Phyllanthus urinaria*	Regulating qi and soothing liver, activating blood circulation and relieving hardness	Rat	SOD, GSH	PC-III, Col-IV, LN, HA, α-SMA, MDA, MPO, TNF-α, IL-1β, IL-6, Cox-2, iNOS, TNFR1, p-IκBα, p-P65/P65, p-ERK/ERK, p-JNK/JNK, MAPK p-P38/P38	Antagonizing OS effectively inhibits hepatic fibrosis by regulating TNF/MAPK and NF-κB signaling pathways to suppress liver inflammation	Cai *et al* [135]

α-SMA: Alpha-smooth-muscle actin; OS: Oxidative stress; SOD: Superoxide dismutase; GSH: Glutathione; IL: Interleukin; IFN: Interferon; Nrf2: Nuclear factor erythroid 2-related factor; HSC: Hepatic stellate cell.

reported to prevent and improve hepatic fibrosis by inhibiting OS.

YCHT is a TCM formula that has been used for centuries to treat liver diseases[105]. It is composed of three herbs, *Artemisia annua L.* (Qing Hao), *Gardenia jasminoides Ellis* (Zhi Zi), and *Rheum palmatum L.* (Da Huang), and is commonly used in clinics in China and other Asian countries[105]. Studies have suggested that YCHT can decrease ROS production and alleviate hepatic fibrosis. One of the mechanisms by which YCHT works is by regulating the balance between the pro- and anti-inflammatory cytokines in the liver[106]. Inflammatory cytokines such as TNF-α, interleukin (IL)-6, and IL-1 β are known to promote ROS production and hepatic fibrosis[107]. YCHT inhibits the expression of these cytokines and promotes the secretion of anti-inflammatory cytokines such as TGF-β and IL-10, which can reduce ROS production and hepatic fibrosis[108]. Another mechanism by which YCHT inhibits ROS production is by increasing the expression of antioxidant enzymes, such as SOD[109]. SOD converts superoxide radicals, one of the primary ROS, into hydrogen peroxide, which is less toxic. This conversion reduces OS in the liver and improves liver function[109]. In short, the

beneficial effects of YCHT on hepatic fibrosis may be attributed to its antioxidant properties. Further studies are needed to fully elucidate the underlying mechanisms and to establish its clinical efficacy and safety for the treatment of hepatic fibrosis.

XCHT is a TCM formula that has been used for centuries to alleviate various ailments, including hepatic fibrosis[110]. Recent studies have shown that XCHT may effectively reduce hepatic fibrosis by inhibiting ROS and OS pathways. Recent research has suggested that XCHT can suppress the production of ROS and reduce OS, leading to improvement in antioxidant capacity[111]. XCHT contains various active compounds such as baicalin, baicalein, and saikosaponin[111]. These compounds have all been shown to possess potent antioxidant properties, which may help to explain XCHT's ability to reduce oxidative liver damage and improve hepatic fibrosis[111]. Furthermore, XCHT has shown promising results in treating hepatic fibrosis by increasing levels of Nqo1, HO-2, GCLC, and GCLM - key components of the Nrf2 pathway in the liver[111]. This mechanism of action is likely responsible for its effectiveness in improving hepatic fibrosis. XCHT has been found to upregulate OS through the Nrf2 pathway, while also inhibiting the proliferation and activation of HSCT6 cells, which contributes to its ability to improve hepatic fibrosis[112]. To put it briefly, XCHT has shown promise in reducing hepatic fibrosis by inhibiting ROS and OS pathways, enhancing antioxidant capacity, and modulating the immune system and ECM remodeling processes. Further research is needed to fully understand the molecular mechanisms underlying XCHT's therapeutic effects in hepatic fibrosis and to optimize its clinical application in treating hepatic fibrosis.

Fuzheng Huayu Fang is a formula used in TCM for treating liver diseases and related complications[113]. This formula aims to strengthen the body's immune system, promote blood circulation, and reduce inflammation, hence helping to alleviate the symptoms associated with liver disorders[114]. Fuzheng Huayu Fang contains several herbs with antioxidant and anti-inflammatory properties that help to regulate ROS levels and protect liver cells from damage[115]. For instance, the herb Danshen (*Salvia miltiorrhiza*) has been shown to reduce ROS levels and inhibit the production of pro-inflammatory cytokines in liver cells, thereby improving liver function and reducing hepatic fibrosis[96]. Moreover, some studies have found that Fuzheng Huayu Fang can also help to lower OS and inflammation by regulating the expression of genes involved in these processes[116]. To cut a long story short, Fuzheng Huayu Fang is a useful formula for treating hepatic fibrosis due to its antioxidant and anti-inflammatory properties. By reducing ROS levels and promoting liver regeneration, this formula can help to alleviate the symptoms associated with liver disorders and improve the overall health of the liver.

In essence, TCM formulas have shown great potential in preventing and treating hepatic fibrosis. These formulas can regulate the body's functions and status and improve prognosis without causing adverse reactions. The mechanisms underlying their anti-fibrotic effects involve the suppression of ROS production, inhibition of HSC activation, and regulation of cytokine expression. Further studies are needed to validate their efficacy and safety in clinical practice.

DISCUSSION

Hepatic fibrosis is a compensatory response to liver injury and is also a risk factor for liver cirrhosis, liver cancer, and liver failure. Although the pathogenesis of hepatic fibrosis is complex and not fully understood, OS has been shown to play an important role in the development of hepatic fibrosis diseases. Therefore, the inhibition of OS can prevent hepatic fibrosis and related liver diseases. TCM is a treasure of Chinese traditional medicine, with unique advantages and profound connotations. TCM monomers, single herb extracts, and TCM formulas are increasingly being studied by researchers for their low adverse reactions, cost-effectiveness, and broad targeting of multiple pathways in the prevention and treatment of hepatic fibrosis. Based on the development of modern scientific technologies such as pharmacology and genomics, TCM monomers, single herb extracts, and TCM formulas have been extensively studied for their molecular mechanisms of anti-hepatic fibrosis by targeting OS and intracellular signal transduction processes, achieving good results and having broad application prospects.

However, there are also some issues that need to be addressed at present: Clinical trials are still lacking, and current studies on the role of TCM monomers, single herb extracts, and TCM formulas in inhibiting OS to treat hepatic fibrosis are mainly focused on animal experiments, with limited large-scale clinical trials, which limits the further research and transformation of these TCM monomers, single herb extracts, and TCM formulas. Therefore, how to apply the results of basic experiments to clinical practice and verify the anti-fibrosis effects of TCM monomers, single herb extracts, and TCM formulas in large-scale clinical trials has become another challenge in the treatment of hepatic fibrosis.

In addition, the structure-activity relationship study is not sufficient. Since most polysaccharides, single herb extracts, and TCM formulas are mixtures, their pharmacological mechanisms are difficult to fully elucidate, and they may be limited to the accumulation of effective components of single herb extracts or may be the result of the formation of a systemic pharmacological mechanism of mixtures. This limits the practical application of these TCM in clinical practice. Therefore, TCM theory should be combined with modern medical theory. Based on the summary of previous research, individual differences in pathogenesis and disease progression should be analyzed to further explore how TCM inhibits OS to address hepatic fibrosis.

As well as, some people may be allergic to certain TCMs. If symptoms such as rash, itching, difficulty breathing, or throat swelling occur, medication should be immediately discontinued and medical help should be sought. Additionally, individuals with pre-existing health conditions or those taking other medications should consult healthcare professionals before starting any TCM treatment. This is crucial to ensure the absence of potential drug interactions or worsening of current health issues. Besides, it is important to purchase Chinese medicine products from reputable suppliers to ensure their quality and safety. Counterfeit or adulterated products can pose serious health risks and should be avoided. In

summary, while TCM can provide therapeutic benefits, understanding and addressing potential adverse reactions and preventive measures associated with its use are crucial. By following proper guidelines, consulting healthcare professionals, and using legitimate products, risks can be minimized and benefits can be maximized. This will help find better inducing effects, with fewer adverse reactions and lower prices for TCM, providing a more reliable theoretical basis for achieving true and effective reversal of hepatic fibrosis.

CONCLUSION

Hepatic fibrosis is a condition characterized by inflammation and excessive growth of fibrous tissue in the liver, resulting from long-term exposure to harmful factors. OS is considered a key mechanism in the development and progression of hepatic fibrosis. TCM monomers, single herb extracts, and TCM formulas play important roles in treating hepatic fibrosis by inhibiting OS. This review summarizes the role and effectiveness of TCM monomers, single herb extracts, and TCM formulas in inhibiting OS for the treatment of hepatic fibrosis, based on relevant research. The findings demonstrate that TCM monomers, single herb extracts, and TCM formulas possess significant antioxidant properties, effectively reducing OS levels in the liver and alleviating the occurrence and progression of hepatic fibrosis.

FOOTNOTES

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REVIEW

Challenges and dilemmas in pediatric hepatic Wilson's disease

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Abstract

Wilson disease is an autosomal recessive disorder affecting the ATP7B gene located on chromosome 13q. This leads to copper deposition in various organs, most importantly in the liver and brain. The genetic mutations are vast, well reported in the West but poorly documented in developing countries. Hence the diagnosis is made with a constellation of clinico-laboratory parameters which have significant overlap with other liver diseases and often pose a significant dilemma for clinicians. Diagnostic scoring systems are not fool-proof. The availability and affordability of chelators in developing countries impact the drug compliance of patients. While D-penicillamine is a potent drug, its side effects lead to drug discontinuation. Trientine is cost-prohibitive in developing countries. There is no single test to assess the adequacy of chelation. Exchangeable urinary copper is an essential upcoming diagnostic and prognostic tool. In the presence of cirrhosis, hypersplenism clouds the assessment of myelosuppression of drugs. Similarly, it may be difficult to distinguish disease tubulopathy from drug-induced glomerulonephritis. Neurological worsening due to chelators may appear similar to disease progression. Presentation as fulminant hepatic failure requires rapid workup. There is a limited window of opportunity to salvage these patients with the help of plasmapheresis and other liver-assisted devices. This review addresses the challenges and clinical dilemmas faced at beside in developing countries.

Key Words: Wilson's disease; Children; Hepatic Wilson disease; D-penicillamine; Trientine; Exchangeable copper

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Core Tip: Wilson's disease is an important and common cause of chronic liver disease among children with multi-organ affection. There are various biochemical parameters that aid in making the diagnosis but are nonetheless fraught with limitations. Treatment is lifelong and it's important to maintain adequate chelation while monitoring for its adverse effects. It is important to distinguish the adverse effects of chelators from the disease manifestations per se, as the management is diagonally opposite, for example, differentiating D-penicillamine-induced paradoxical neurological worsening from neurological manifestations of Wilson disease. This review discusses these challenges in making a diagnosis and treating pediatric hepatic Wilson's disease.

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INTRODUCTION

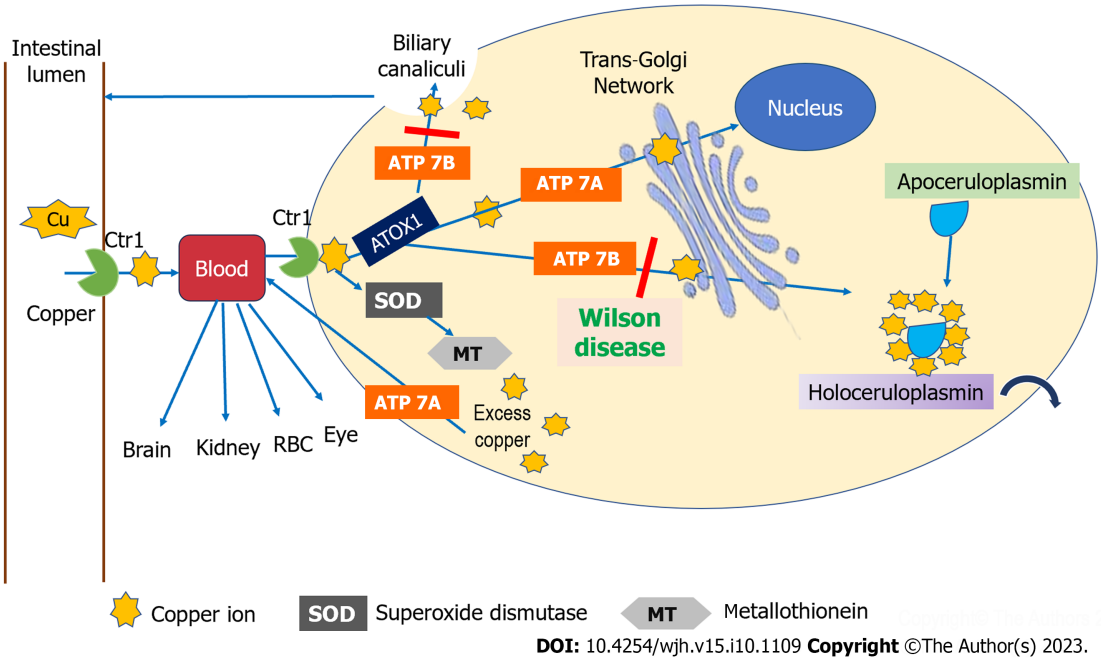
Wilson disease (WD) is a genetic disorder of copper metabolism, with an autosomal recessive mode of inheritance. The estimated prevalence of WD is approximately 30 per million population[1]. Among all causes of hepatobiliary disorders in children, in a 3.5-year study period, in a tertiary care centre in India, WD accounted for 7.6% of total cases[2]. It is important to make an early diagnosis for the prevention of disease progression and the identification of pre-symptomatic cases. This paper addresses the management difficulties in Wilson disease in terms of the identification of varied atypical clinical presentations, diagnostic dilemmas, and pharmacotherapy-related problems.

Pathogenesis

Figure 1 depicts the etiopathogenesis of WD. WD is caused by a mutation in the ATP7B gene, located on chromosome no. 13 Long arm, (13q14.3). This gene codes for an ATPase (a metal-transporting P-type adenosine triphosphatase), which is membrane-bound and mediates trans-Golgi migration of copper within the hepatocyte and its subsequent excretion into the biliary canaliculi[3]. Copper (Cu^{+}) from the diet enters the hepatocyte *via* the copper transport receptor 1 (CTR1) located in the apical membrane. Then it binds to Atox1 (antioxidant protein1) and is then handed over to ATPase (gene product of ATP7B) for its transport into the trans-Golgi network (TGN)[4]. Ceruloplasmin has two functions- i. it transports copper to the TGN where apoceruloplasmin binds to 6 atoms of copper to form holo-ceruloplasmin ii. in copper loaded condition, ATP7B goes to the endocytic vesicles to mediate copper excretion into the bile canaliculi[5]. Thus, in normal conditions, ATP7B is present in the trans-Golgi network and helps in the synthesis of holo-ceruloplasmin. But, in the copper-excess state, the protein moves towards the canalicular membrane to promote copper excretion[5]. Apo-ceruloplasmin is the inactive form of ceruloplasmin and is less stable. In the absence of this transporter, apoceruloplasmin degrades, and non-TGN-transported copper accumulates in the liver and gets bound to metallothioneins (endogenous chelators). When the copper binding capacity of metallothioneins is overwhelmed, the free copper spills into the lysosomes and causes free radical-mediated cell damage. Ultimately, this free copper comes to the peripheral circulation, where it is weakly bound to albumin and gets deposited in other tissues (brain, kidneys, cornea) (Figure 1). Apoceruloplasmin is a plasma protein and carrier of copper, mainly produced by the liver. In normal physiology, after copper reaches the liver *via* the portal circulation, 20% of copper is re-excreted into the GI tract *via* bile and the remaining 80% is transported to the periphery, bound to ceruloplasmin (Apoceruloplasmin + copper = holo-ceruloplasmin). However, in WD, holoceruloplasmin is reduced, and free copper (non-ceruloplasmin bound copper) is elevated, which leads to high urinary excretion of copper[6,7]. Hepatic involvement is the most common presentation in children with WD, as liver is the major organ responsible for copper homeostasis. Initially, copper binds with metallothionein and accumulates in lysosomes and progressively causes mitochondrial damage leading to hepatic steatosis, fibrosis, and cirrhosis. Subsequently, non-ceruloplasmin-bound excess and toxic copper leaks into the blood and accumulates in the other tissues like brain, leading to neurological manifestations, which is the commonest presentation in older children and adults.

DIAGNOSTIC DIFFICULTIES

Hepatic Wilson disease may present as acute hepatitis, persistent or intermittent elevations of transaminases, organomegaly, fluctuating or worsening jaundice, resembling autoimmune hepatitis, fatty liver, acute liver failure or as decompensated CLD[6,8,9]. Other major presentations of WD include neurological presentation with extrapyramidal symptoms and psychiatric manifestations in the form of tremors, dystonia, dysarthria, gait disturbances, and psychiatric manifestations, like behavioral abnormalities and psychiatric manifestations, like bipolar affective disorder and psychosis with cognitive decline. It is hypothesized that it is because of copper which gets deposited in the basal ganglia by penetrating through the choroid plexus (fenestrated endothelium)[4]. Minor presentations include renal tubular acidosis presenting with microscopic hematuria, osseo-muscular involvement in the form of pathological fractures, arthralgia,



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Figure 1 Dietary copper (Cu) is taken up in the small intestine by intestinal enterocytes by copper transport receptor 1 (Ctr1) on the apical site. Most of the newly absorbed copper is taken up by the liver, mediated primarily by Ctr1. Accumulated copper is sequestered by superoxide dismutase and stored in metallothioneins. Specialized chaperons shuttle copper to its specific cellular targets: Antioxidant protein 1 (Atox1) to the copper-transporting ATPases ATP7A and ATP7B. Both ATP7A and ATP7B transport copper into the trans-Golgi network for subsequent incorporation into copper-dependent enzymes. ATP7B provides copper for incorporation into ceruloplasmin as well as for biliary excretion of copper, whereas ATP7A has role in copper mobilization from hepatic stores into the bloodstream during peripheral copper deficiency. Mutation in ATP7B gene leads to impaired biliary excretion of copper, causing copper overload in liver and other tissues as well as failure of loading of ceruloplasmin with copper. MT: Metallothioneins.

proximal muscle weakness.), and importantly haematological presentation in the form of acute fulminant liver failure with Coomb's negative haemolytic anemia.

To make the diagnosis of Wilson disease, we use the presence of a Kayser-Fleischer (K-F) ring on slit lamp exam, 24-h urine copper of > 100 micrograms (mcg) per day, and ceruloplasmin < 20 milligrams/decilitre (mg/dL) commonly. These biochemical parameters are not to be interpreted in isolation; it is prudent to remember various factors that affect them. Table 1 summarizes all the diagnostic tests and their interpretation. Table 2 enlists various diagnostic tests used in suspected WD patients and for family screening.

Ceruloplasmin

Apo-ceruloplasmin combines with copper and is secreted from the liver in the form of holo-ceruloplasmin. Serum ceruloplasmin is measured by two methods; either enzymatically by measuring its copper-dependent ferroxidase activity (holo-ceruloplasmin) or by antibody-based tests such as radioimmunoassay, radial immunodiffusion, or nephelometry[10]. The enzymatic method measures holo-ceruloplasmin, whereas immunologic assays measure both apo ceruloplasmin and holo-ceruloplasmin and thus can overestimate ceruloplasmin levels[10,11]. It has been suggested that the enzymatic method is better than the immunologic method to diagnose Wilson's disease[12]. In those patients with impaired synthetic functions due to causes other than WD, the specificity of both these tests dropped, but the enzymatic test had better specificity as compared to immunologic assays (84.5% *vs* 68.9%)[12]. However, in routine practice, immunological assays are more readily available, rapid and commonly used. The normal value of ceruloplasmin ranges between 20-40 mg/dL. Ceruloplasmin level < 20 mg/dL with the presence of Kayser Fleischer (KF) ring is considered consistent with WD[8]. Ceruloplasmin < 5 mg/dL is strongly suggestive of WD[6]. Also, ceruloplasmin level < 14 mg/dL has a 100% positive predictive value for the diagnosis of WD and a value < 5 mg/dL is strongly suggestive of WD[8,13]. Serum ceruloplasmin is also an acute phase reactant and may be falsely elevated in the presence of sepsis and it may be falsely low in conditions like fulminant hepatic failure of any etiology, advanced liver disease, celiac disease and aceruloplasminemia, nephrotic syndrome, and 20% of heterozygotes[10,11,14].

24-h urine copper excretion

It is another indirect marker of non-ceruloplasmin-bound copper, which is elevated in WD. 24-h urine copper excretion of more than 100 mcg per day is diagnostic of Wilson's disease[15]. However, certain other conditions like autoimmune hepatitis, chronic cholestatic liver disease and acute fulminant liver failure of any aetiology may give rise to high 24-h urine copper excretion[10]. Basal copper excretion may be low in children and asymptomatic siblings, thus a normal value doesn't rule out WD. The test may give rise to false results in case of improper urine collection and copper contamination if kept in certain types of containers. 24-h urine copper of more than 40 mcg per day may indicate WD in asymptomatic individuals. In addition to making a diagnosis, this is also a valuable test in monitoring the adequacy of

Table 1 Various laboratory investigations used in the diagnosis and monitoring of Wilson disease

Diagnostic tests	Cut-off values in diagnosis	Disease monitoring	Problems in interpretation
Serum cerulo-plasmin	< 10 mg/dL (strong evidence), 10-20 mg/dL (needs further evaluation), 20-40 mg/dL (normal value, but does not exclude diagnosis)	Not helpful	Can be normal in fulminant presentation and acute inflammation as it is an acute phase reactant
24-h urine copper	> 100 mcg/d (virtually diagnostic in symptomatic patients), > 40 mcg/d (may indicate disease, needs further evaluation)	> 500 mcg/d during initial phase, 200-500 mcg/d in the maintenance phase	Difficult to perform, to be done in reliable laboratories
Hepatic copper	> 250 mcg/g dry weight (diagnostic), 50-250 mcg/g dry weight (needs further evaluation), < 50 mcg/g dry weight (normal)	Not recommended	Inhomogenous distribution of copper (sampling error), elevated in long-standing cholestasis
Serum total copper	> 25 micromol/L (needs further evaluation), 14-24 micromol/L (90-150 mg/dL) normal	N/A[a]	
Non-ceruloplasmin copper	10-15 mcg/dL (normal person), > 25 μg/dL (untreated patients), Not recommended for diagnosis	> 15 mg/dL (poor compliance)[a] < 5 mg/dL (over-chelation)	
Exchangeable copper	> 2.08 micromol/L (more likelihood of extrahepatic organ involvement), 0.62 and 1.15 micromol/L (normal)	Experimental	Requires equipped laboratories and expertise
Relative exchangeable copper	> 15% (100% sensitivity and specificity for diagnosis)	Experimental	Requires equipped laboratories and expertise

[a]The serum free copper or NCC has a role in monitoring: Initial phase: > 25 mcg/dL, maintenance phase: 10-15 mcg/dL, under-chelation: > 15 mcg/dL, over-chelation: < 5 mcg/dL.

Table 2 Diagnostic tests for suspected Wilson disease patients and asymptomatic siblings

Parameters	Tests for suspected patients	Tests for asymptomatic siblings
Clinical examination	Yes	Yes
Liver function test	Yes	Yes
Slit lamp examination for Kayser–Fleischer ring	Yes	Yes
Serum ceruloplasmin	Yes	Yes
24 h urine copper	Yes	Yes
Genetic analysis for ATP7B gene mutation analysis	Yes, if feasible	Yes (if proband sample is available)
Liver biopsy	Yes, ancillary test	No
Hepatic copper	Yes, to be done in cases of ambiguity	No

chelation, a 24-h urine copper of 200-500 mcg per day is considered the target[8].

Liver biopsy and hepatic copper

On liver biopsy, early changes include steatosis, and fibrosis and late changes include changes of chronic active hepatitis with bridging fibrosis, occasionally resembling autoimmune hepatitis and, Mallory-Denk bodies[16]. Copper staining is demonstrated by orcein, which stains the copper-associated proteins, and rhodanine which stains the elemental copper. Copper-associated proteins may be elevated in various causes of advanced cholestatic liver disease (*e.g.*, Primary biliary cirrhosis or Chronic hepatitis B) other than Wilson's disease, thus histochemical staining for copper is not specific for Wilson's disease[17]. Also, negative histochemical staining for copper does not rule out the possibility of WD[18]. Copper deposition also depends on the stage of disease, early in the disease, copper staining is diffuse in the cytoplasm in the periportal region, and later in the disease, it is mainly seen within the lysosomes of hepatocytes in the periphery of the regenerating nodules. This pattern of distribution suggests that the cytoplasmic distribution leads to hepatocyte damage while copper in the lysosomes is likely to be less toxic. Initial cytoplasmic deposition of copper is due to the high affinity of the sulfhydryl-rich proteins (metallothionines) present in the cytoplasm as compared to lysosomes[19]. Hepatic copper of more than 250 mcg/gram (gm) dry weight of the liver is strongly suggestive of copper deposition in the liver but it may be also found in any advanced liver disease. Liver copper < 50 mcg/gm dry weight almost excludes the diagnosis of WD[8,20]. Also, the regional variation due to regenerative nodules and tissue fibrosis is to be kept in mind while interpreting hepatic copper[10].

Serum copper

It is important to measure the serum copper simultaneously while measuring ceruloplasmin, as it helps in the calculation of the "free copper" also known as "non-ceruloplasmin bound copper (NCC)". Total serum copper comprises of holo-ceruloplasmin (70%), albumin-bound (20%), peptide-bound, and free copper. Holo-ceruloplasmin level is low in WD. So, in most patients of WD, serum copper is low, unless there is a massive release of free copper from the liver due to liver necrosis[21]. This free copper, which is not bound to ceruloplasmin is known as "non-ceruloplasmin bound copper", is elevated in WD and is toxic. Serum copper is estimated by either atomic absorption or emission spectrometry or by inductively coupled plasma mass spectrometry (ICP-MS) methods[22]. Total serum copper (in mcg/L) = serum copper (in micromol/L) × 63.5[10]. The normal range of serum copper is 14- 24 micromol/L (90- 150 mg/dL)[11]. Holo-ceruloplasmin contains 3.15 mcg of copper per mg of ceruloplasmin, hence, ceruloplasmin-bound copper may be calculated as serum ceruloplasmin (mg/dL) x 3.15 (mcg copper per mg ceruloplasmin)[11]. Free copper or NCC = (Serum copper-ceruloplasmin bound copper). Non-ceruloplasmin-bound copper has been proposed as a diagnostic test for WD[23]. In normal individuals, the NCC (free copper) is 10-15 mcg/dL[3]. In untreated patients, it is > 25 mcg/dL[3]. The estimation of NCC depends on the methods used to estimate serum ceruloplasmin and copper levels. Falsely high ceruloplasmin calculated by immunological tests may lead to the calculation of a falsely low or negative value of free copper. In normal conditions in WD, serum copper is low. In acute fulminant WD, due to a massive release of copper from the liver, free copper may rise up to ten times and can cause intravascular hemolysis[23]. NCC is not validated for diagnosis of WD but is helpful in monitoring treatment response. With adequate chelation, the NCC decreases to < 5 mcg/dL. Among patients who are on chelation therapy, the target 24-hour urine copper excretion is 200-500 mcg/d. However, patients with poor compliance to chelators can also have low 24-h urine copper excretion as there is adequate chelator in the circulation to leach out copper. Such patients can be differentiated by estimating the NCC. Those with high NCC > 15 mg/dL have poor compliance while those with NCC < 5 mg/dL are over-chelated[10].

Exchangeable copper and relative exchangeable copper

Exchangeable copper is measured by adding ethylenediaminetetraacetic acid (EDTA) to the serum sample. As EDTA is a copper chelator, it binds with copper, and EDTA-bound copper is measured as exchangeable copper. It measures copper bound to albumin, and other amino acids, which easily gets exchanged with EDTA (copper-chelating agents)[21]. Unlike NCC, it doesn't depend on serum ceruloplasmin levels. Normal values for exchangeable copper (CuEx) are between 0.62 and 1.15 micromol/L[24]. Poujois *et al*[25] demonstrated that CuEx is normal to moderately increased in WD, but elevated in those with acute fulminant WD and WD with extrahepatic involvement. In patients with extra-hepatic manifestations, CuEx was the only biological marker to be positively correlated with the neurologic disease burden (assessed by the Unified Wilson Disease Rating Score). CuEx determination is, in consequence, useful when diagnosing WD with a value > 2.08 micromol/L, and is indicative of the severity of the extra-hepatic involvement. However, CuEx did not indicate the severity of liver damage. CuEx is an interesting experimental biomarker but needs to be interpreted with caution, especially in WD patients with hepatic manifestation. In this study, it was postulated that there may be a threshold concentration of CuEx above which organs like the brain and eyes (and possibly others such as kidneys and heart) may be affected by copper overload[25]. Relative exchangeable copper (REC) is the ratio of exchangeable copper to total serum copper. El Balkhi *et al*[26] showed that REC > 18.5% can be used to diagnose WD with a sensitivity and specificity of 100%. Trocello *et al*[27] showed that REC can also be used to diagnose WD in asymptomatic siblings, by taking REC cut-off of 15%, it can distinguish asymptomatic WD from heterozygotes accurately.

KF ring

It represents copper deposition of copper in Descemet's membrane of the cornea, visible on slit lamp examination, it is pathognomonic of WD. It is not specific to Wilson's disease and may be found in various cholestatic conditions. It is present in 44%-62% of hepatic WD and 95% of neurological WD[3]. KF-like rings have been described in primary biliary cirrhosis, autoimmune hepatitis and severe cholestasis where the total bilirubin is > 10 g/dL. They are called bilirubin rings and disappear with the resolution of jaundice[28]. It is important to note that the appearance of the KF ring is not related to disease severity and its resolution may take variable time, months to years[29,30]. Anterior segment-optical coherence tomography (AS-OCT) can also be used to diagnose the KF ring, where the KF ring is visualized as an intense reflectivity in Descemet's membrane and it may have better accuracy in diagnosing KF ring as compared to slit-lamp examination[31]. Broniek-Kowalik *et al*[32] showed that AS-OCT detected copper deposition in 15 additional patients of WD, in whom the KF ring was not seen in slit lamp examination.

Coomb's negative hemolytic anemia with a disproportionate elevation of aspartate transaminase (AST) as compared to alanine transaminase (ALT) is suggestive of acute fulminant WD. The ratio of AST/ALT > 2.2 and ALP/total bilirubin ratio < 4, when combined is suggestive of acute fulminant Wilson disease with a sensitivity and specificity of 100%[33]. Alkaline phosphatases are metalloenzymes and zinc is a co-factor. In fulminant WD, during the period of massive release of copper into circulation, copper may compete with zinc for incorporation into alkaline phosphatase apoenzymes. Copper-containing enzymes released into the circulation would have little or no enzymatic activity, and serum alkaline phosphatase values would be low[34].

Neurological imaging

The face of the giant panda sign, which is caused by the normal intensity of red nuclei, preservation of signal intensity in the lateral portion of pars reticulata of substantia nigra and hypointensity in the superior colliculus with hyperintense surrounding tegmentum, is considered pathognomonic of neurological WD[35]. This finding has been shown to reverse with chelation[36,37]. Other findings include lesions in the putamen, globus pallidus, caudate, thalamus, midbrain, pons,

and cerebellum as well as cortical atrophy and white matter changes. Usually, the lesions are hyperintense in T2 and hypointense in T1 weighted images. Magnetic resonance imaging (MRI) changes correlate with disease severity. Diffuse atrophy of the brain is one of the most common features[35]. MRI findings are universal in symptomatic patients and occasionally reported in pre-symptomatic patients[38].

Genetic analysis

Lastly, genetic analysis can be done in patients having ambiguity in diagnosis or for sibling screening. More than 600 pathogenic variants have been identified[39]. Most common mutations are single-nucleotide missense and nonsense mutations[39]. There is no genotype-phenotype correlation. Siblings may have varied presentations[40]. On screening the proband, the chances of a sibling being affected is 25% while either parent being affected is 0.5%[6].

Leipzig score

A diagnostic score named Leipzig score was proposed in 2001, which took into account various clinical, and biochemical, parameters to diagnose WD. A Leipzig score of more than 4 is highly suggestive of WD, while a score of 2-3 would merit further investigations and a score of less than 1 makes the diagnosis of WD unlikely[41]. There are practical difficulties in implementing the Leipzig score in developing countries. Hepatic copper is not universally available and inconsistent in reporting. Genetic testing is cumbersome and region-specific. Most regions do not report the mutations. The d-penicillamine challenge is not reliable. A revised Leipzig score was devised for the Asian setting, which gave importance to family history, greater points for the KF ring and an additional score for a ceruloplasmin level < 5 mg/dL[6].

Family screening

First-degree relatives of newly diagnosed patients should be screened by clinical examination, LFT, slit-lamp examination, serum ceruloplasmin, 24-h urine copper estimation and genetic testing, especially if the mutation is known in the proband. Screening by laboratory tests is usually deferred till 2 years of age though genetic screening is possible[6]. Preservation of the DNA sample of the proband is essential.

MANAGEMENT DIFFICULTIES

Differentiating manifestations of WD from chelation-related complications/Atypical symptoms of WD masquerading as chelation-related complications:

Kidney-related issues in WD

The renal manifestation of WD can be categorized into (1) renal involvement of underlying disease, or (2) treatment-related nephrotoxicity. The differentiating features in pathogenesis, laboratory investigations and management of these two conditions are summarized in Table 3. The renal involvement of WD can be in the form of renal calculi, hypercalciuria or tubulopathy. Renal manifestations of WD are due to copper deposition in the renal tubular cells. It has been shown that glomerular and tubular functions improve and normalize after starting D-penicillamine[42]. It is vital to differentiate the renal manifestations from the drug-induced glomerulonephritis as the management differs. Renal involvement in WD was first reported by Litin *et al*[43] in 1959 in the form of hypercalciuria. Acute renal failure, which is the most severe form, can be precipitated by massive intravascular hemolysis seen in WD[44]. Renal involvement can manifest with glomerular or non-glomerular (tubular) injury. Aminoaciduria was first detected in patients with Wilson disease by Uzman and Denny-Brown in 1948[45]. Tubular causes are tubulopathies [proximal or distal renal tubular acidosis (RTA)] presenting as renal rickets, polyuria, polydipsia, or macroscopic hematuria (nephrocalcinosis or renal stone), aminoaciduria, glucosuria, proteinuria, hyperphosphaturia, hypercalcemia and defective urinary acidification [46]. Sözeri *et al*[42] in their study of 10 patients of Wilson's disease, for whom excretion of tubular markers could be repeated between 3 mo to 12 years, N-acetyl-β-d-glucosaminidase (NAG), beta 2 microglobulin and low molecular weight proteins were higher in the first year, as compared to the subsequent period. This is in contrast to high molecular weight proteinuria, which increases after 1 year of treatment with D-penicillamine. Renal impairment may be mild to severe. It may present as sub-nephrotic or nephrotic range proteinuria, microscopic hematuria, hypercalciuria, renal stones, renal failure, and lastly D-penicillamine-associated glomerulonephritis[47].

Tubular damage/ RTA

The disease per se can cause glomerular as well as tubular dysfunction, however, tubulopathy is more common. Excess copper deposits in the epithelium of the proximal and distal convoluted tubules, and the thickening of the basement membrane interfere with reabsorptive function of the renal tubules, thereby leading to RTA[47]. Distal, as well as proximal RTA, has been reported in WD. In a cross-sectional study by Kapoor *et al*[48], done over 1 year duration, 14 out of 25 patients (56%) of WD, had renal tubular acidosis of which 24% (6/25) had distal RTA, 16% (4/25) had mixed RTA, and another 16% (4/25) had proximal RTA. Wolff *et al*[49] described post-mortem kidney biopsies of 5 patients, wherein, on histopathology, glomeruli were normal in all 5 patients, focal areas of degeneration and necrosis of tubular epithelial cells along with copper staining (rubeanic-acid staining) were seen in all patients. Elsas *et al*[50] showed progressive renal impairment in an adolescent with Wilson's disease for whom D-penicillamine was stopped for almost 18 mo (owing to D-penicillamine induced lupus nephritis), with simultaneous renal biopsy showing an increased number of conspicuous electron-dense bodies in the subapical areas of cytoplasm suggestive of metalloprotein complexes. Azizi *et al*[51]

Table 3 Differentiating features between Wilson disease-related renal tubular acidosis and D-penicillamine induced glomerulonephritis

	Wilson disease-related renal tubular acidosis	D-penicillamine induced glomerulonephritis
Mechanism	Copper induced tubular damage	Immune complex deposition
Presentation	Prior to starting chelation/during chelation	After starting chelation
Tests to differentiate	Normal anion gap metabolic acidosis, Urine pH, Urine for glucosuria, aminoaciduria, acidification test of urine	Urine for proteinuria, autoantibodies for glomerulonephritis, renal biopsy
Chelation	To be continued	To be stopped

described a 9-year-old boy, who first presented with renal colic due to hypercalciuria, only to be diagnosed as WD 1 year later. There are few case reports of children presenting with renal rickets who were finally diagnosed as WD[52]. In a retrospective study of 85 children with WD by Zhuang *et al*[47], renal impairment was found in 25 (29.4%) of the treatment-naïve patients. Seven of the twenty-five WD patients (28%) had symptoms of renal impairment (one each had acute nephritis, persistent glomerulonephritis, hemolytic uremic syndrome, and 2 had nephrotic syndrome). Five children had evidence of the glomerular cause of hematuria while the remaining two had non-glomerular hematuria. Twelve of the 25 (48%) had proteinuria, 14/25 (56%) had hematuria, and 5/25 (20%) had both proteinuria and hematuria, 4/25 (16%) had glucosuria[47]. Thus, by characterizing the type of hematuria (glomerular or non-glomerular) and proteinuria (low molecular weight or high molecular weight), the site of renal involvement can be ascertained. In the treatment -naïve patients, albuminuria with elevated serum creatinine, and low creatinine clearance suggest glomerular involvement. On the other hand, glycosuria, LMW proteinuria, non-glomerular hematuria, an increase in NAG and beta2 microglobulin, nephrocalcinosis, hypercalciuria, and non-anion gap metabolic acidosis indicate tubular involvement.

Glomerular involvement can be due to (1) copper deposition in the mesangium, leading to membranoproliferative glomerulonephritis, (2) IgA nephropathy caused by IgA deposits in the glomerulus, due to the loss of scavenging capacity of the liver, and (3) chelation (D-penicillamine) induced. IgA nephropathy: Gündüz *et al*[53] have reported a 13-year-old boy who presented with nephritic syndrome after 4 mo of diagnosis of Wilson disease, and was diagnosed as membranoproliferative glomerulonephritis on renal biopsy with positive immunofluorescence for IgA, suggestive of IgA nephropathy. Similar case of IgA vasculitis is described by Acharya *et al*[54] in an 11-year-old boy with Wilson disease with F2 fibrosis on liver biopsy (METAVIR staging), who presented with palpable purpura without any arthritis or gastrointestinal involvement. He was diagnosed with IgA nephropathy and mild tubular epithelial degeneration and atrophy on renal biopsy and had complete resolution of the rash and achievement of normal renal function within 3 to 6 mo of chelation. This is similar to primary IgA nephropathy, abnormally glycosylated IgA1 form large soluble IgA1 immune complexes by combining with IgG and IgA, which deposit in the mesangium and lead to mesangial injury. Clinical and histopathological changes may reverse after liver transplantation.

Treatment-related nephrotoxicity

D-penicillamine-induced nephrotic syndrome was first reported by Fellers and Shahidi in 1959[55]. D-penicillamine can cause inhibition of enzymes required for collagen synthesis, thus can damage the glomerular basement membrane and reduce GFR or it can act as a hapten and induce the formation of immune complexes leading to membranous glomerulonephritis[56]. D-penicillamine-induced proteinuria occurs in < 10% of patients of Wilson disease and usually begins after 1 year of treatment[57,58]. The spectrum of D-penicillamine-induced nephrotoxicity ranges from membranous glomerulonephritis, tubule-interstitial disease, crescentic glomerulonephritis, Goodpasture's syndrome and renal-limited vasculitis[59,60].

Proteinuria is the most common manifestation and is the first abnormality to be detected followed by progressive renal disease if D-penicillamine is not stopped[60]. In a case series reported by Sternlieb, all the patients were started on racemic penicillamine, the clinical features resembled nephrotic syndrome, 3 of whom improved following discontinuation of drugs, and another 5 required steroids and there was a recurrence of nephrotic syndrome in all 3 patients in whom penicillamine was re-started while 2 of them didn't have any recurrence after they were started on D isomer of penicillamine[61]. Initially, the racemic mixture of penicillamine was incriminated in the causation of nephrotic syndrome, however, later on, it was shown that even the D- isomer can cause proteinuria. Since 1960, the D-isomer of penicillamine has been available and was approved by the FDA in 1963[61]. Histological examination shows membranous nephropathy (most commonly) minimal change disease or membranoproliferative or rarely crescentic nephropathy[62-64]. D-penicillamine causes nephrotoxicity by immunological mechanism, ultrastructural changes include immune-complex deposits, subepithelial deposits and IgG deposits on immunofluorescence.

The incidence of nephrotoxicity due to D-penicillamine was more in conditions unrelated to copper metabolism, *e.g.* rheumatoid arthritis, cystinurias, and scleroderma[65,66]. In a case series of 33 patients, studied by Hall *et al*[59], who were followed up serially for a mean duration of 74 mo, it was found that the onset of proteinuria peaked after the first 6 mo, with 27/33 developing proteinuria within 1 year of starting D-penicillamine. There are reports to show that the time period from exposure of D-penicillamine to proteinuria may range from a few weeks to years[60]. Siafakas *et al*[67] report a 12-year-old boy who presented with nephrotic syndrome (nephrotic range proteinuria without hematuria, with renal biopsy showing minimal change disease and a negative immunofluorescence study) after 2 wk of D-penicillamine and showed resolution of proteinuria in 3 wk, after being started on steroids. There are studies to show that proteinuria increases even after stoppage of D-penicillamine (within 1-5 mo), and then gradually improves over a few months, which

can be explained by Wilson disease-related tubulopathy, as it improves over time with chelation[42,60,63]. In the study by Hall *et al*[59] the median first and last creatinine clearance almost remained the same, and none went into renal failure or required corticosteroids. The spontaneous resolution was seen within 2-32 mo of the stoppage of the drug[63]. According to Hall *et al*[59], among 33 patients of patients on D-penicillamine who had proteinuria, the number of patients in whom proteinuria resolved by 6, 12 and 18 mo were 12 (36%), 21(63%), 29 (88%) respectively. Renal biopsies done in all patients, 29/33 showed ultrastructural changes characteristic of membranous glomerulonephritis with IgG deposits and complement deposits on immunofluorescence, the remaining 4 patients' biopsies showed mesangial proliferation without any deposition of immunoglobulins or complement on immunofluorescence[59]. The severity of proteinuria is not related to the duration or dose of d-penicillamine or HLA typing[59]. In a retrospective study of 63 patients who developed nephrotic syndrome after being started on D-penicillamine, (75% had rheumatoid arthritis, 10% had Wilson disease), the mean duration of D-pen exposure to proteinuria was 7.6 (± 3.90) months and mean duration of drug exposure until the diagnosis of nephrotic syndrome was 11.9 (± 18.8) mo. Fifty-five percent of them had membranous glomerulonephritis and 27% had minimal change disease[63].

In a study of 8 patients of rheumatoid arthritis, who developed proteinuria while on D-penicillamine of variable duration, ranging from 3 to 48 mo and dosage ranging from 250 mg/d to 1.2 gm per day, 5 patients had scanty subepithelial deposits (spikes), 1 had plenty of sub-epithelial deposits, 2 had associated findings of glomerulosclerosis, interstitial scarring and, on immunofluorescence, 3 showed a positive granular pattern of fluorescence along basement membrane for IgG and C3[68]. After stopping D-penicillamine, proteinuria resolved in 3, progressive proteinuria was seen in one patient, mild proteinuria persisted in 4 others, with the persistence of proteinuria being seen up to 27 mo after stoppage of D-penicillamine[68]. Some studies have been done to see whether D-penicillamine can be resumed after the resolution of proteinuria[69]. In one of the studies in rheumatoid arthritis patients, D-penicillamine was re-introduced after 3 mo of resolution of proteinuria at a low dose of 50 mg/d, escalated to a maximum of 250 mg/d, showed that none of them had a relapse of proteinuria. However, this low dose is not enough to treat WD. In another study by Bacon *et al* [64] in 14 patients of rheumatoid arthritis, 3 developed proteinuria, and 11 developed nephrotic syndrome in a mean duration of 7.5 mo of starting D-penicillamine. Renal biopsies showed a picture similar to membranous nephropathy on light microscopy, granular deposits on immunofluorescence, positive for IgG and C3 and sub-epithelial dense deposits on electron microscopy, even after 3- 12 mo of stoppage of treatment. A renal biopsy done soon after stopping D-penicillamine showed effacement of podocytes with discrete electron-dense deposits in the subepithelial region and positive fine granular immunofluorescence with IgG and C3. When the biopsy was repeated after 6 mo of discontinuation, it showed normal foot processes with occasional dense deposits and focal weak immunofluorescence with IgG and C3. Among 3 patients in whom proteinuria persisted at a high level even after stoppage of the drug, biopsy done at 6, 8, 12 mo of discontinuing D-penicillamine, electron microscopy still showed discrete but much smaller electron-dense deposits, and brilliant fluorescence with IgG[64]. D-penicillamine-induced glomerulonephritis and Goodpasture syndrome have also been described[70].

To summarize, diagnosis of D-penicillamine-induced nephropathy can be made with routine urine microscopy, 24-h urine estimation for proteinuria, and type of proteinuria (tubular or glomerular), and if feasible, histopathological and electron microscopic examination (renal biopsy) especially in the first 18 mo. Membranous nephropathy is the most common nephropathy caused by D-penicillamine[59,63,68]. Serum MPLA2R antibodies (an immunofluorescence test) can be done as a supportive test. These antibodies are prevalent in primary membranous glomerulonephritis in up to 68.5% of cases and their absence on the face of renal biopsy picture of membranous nephropathy suggests the secondary cause of membranous glomerulonephritis[71]. Kumar *et al*[72] reported nephrotic syndrome in a 24-year-old lady, a case WD on D-penicillamine for 18 mo. Renal biopsy showed membranous nephropathy with positive immunofluorescence for IgG and C3, and with negative serum auto-antibodies to M-type phospholipase A2 receptor ($MPLA_2R$, which is usually seen in primary membranous nephropathy), thus supporting the diagnosis of secondary membranous glomerulonephritis.

Management of D-penicillamine nephrotoxicity includes stoppage of the drug and use of trientine as an alternative. In the study done by Neild *et al*[63], glucocorticoids had no effect on the natural history of nephropathy. In another case series by De Silva, among 35 patients who developed proteinuria while on D-penicillamine, 60% developed nephrotic syndrome (NS). Among NS patients, D-penicillamine was continued for 62%, stopped at a variable interval, and it was shown that within 4 mo of stopping D-penicillamine, proteinuria decreased to < 2 g/d and 12 mo after stopping D-penicillamine, proteinuria was 0-0.3 g/d[73]. AASLD recommends prompt withdrawal of D-penicillamine immediately.

Haematological manifestations

These include Coomb's negative hemolytic anaemia and acute renal failure, D-penicillamine-induced myelosuppression and lastly hypersplenism (splenomegaly).

Acute fulminant WD

Acute fulminant Wilson disease usually presents with acute intravascular hemolysis, which is due to free copper-mediated damage to the RBC membranes, mortality is 95%[74]. It needs to be tackled with plasmapheresis while awaiting liver transplantation[75].

Drug-induced cytopenia

The rate of myelotoxicity varies from D-penicillamine 0 to 7% and it is one of the most fatal adverse effects[76]. It has been hypothesized that marrow toxicity could be of two types: The first being an idiosyncratic reaction, leading to cytopenias occurring within the first year of treatment, and the other being a dose-dependent gradual fall[77]. Day *et al*[78] showed that of the 69 patients of rheumatoid arthritis, who were on D-penicillamine for more than 1 year, 15 (21.7%) had

developed dose-dependent hematological adverse effects; while those who were on < 500 mg per day, had no cytopenias. However in another case series of 10 patients of rheumatic arthritis with D-penicillamine induced myelosuppression, 7 had sudden onset myelosuppression, of these, 6 patients died, and the remaining showed gradual recovery of marrow over 1 year[79]. These studies are to be interpreted keeping in mind 2 points; first, both the studies were in rheumatoid arthritis patients, and myelosuppression was documented even with a low dose of D-penicillamine (in contrast to WD, where a higher dose of D-penicillamine is required), secondly, these patients didn't have concomitant hypersplenism (to confound the picture), thereby, cytopenia could easily be attributed to D-penicillamine, leading to faster decision making in terms of drug-discontinuation. Once myelosuppression is there, D-penicillamine is to be stopped. European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) has suggested monitoring blood counts initially for 1-3 monthly and later 3-6 monthly[80]. Table 4 enlists the risk factors that may complicate/mimic drug toxicity features.

Neurologic symptoms

Neurological symptoms include dystonia, dysarthria, gait abnormalities, and tremors, with dysarthria being the most common[8]. Other manifestations include abnormal gait, musculoskeletal symptoms, seizures, behavioral problems, drooling of saliva, and chorea[81]. Pediatric patients present with hepatic WD whereas neurological manifestations are more common among adults[82]. Neurological manifestations of WD are due to high concentrations of copper in brain tissue as well in CSF and anti-copper therapy leads to improvement in symptoms[83]. It is essential to characterize the disease extent in order to monitor the evolution of symptoms carefully. It is important to identify the subtle neurologic manifestations at the time of diagnosis as it has a bearing on choosing the medical therapy. Multiple studies have shown that neurological worsening on starting chelation is more commonly seen in those having neurological manifestations, within 1-3 mo of starting therapy. In one of the early studies, Brewer *et al*[84] showed that neurological worsening post-D-penicillamine occurred in 50% of neurologic WD, with more than half of the patients showing deterioration within 4 wk of chelation. Ranjan *et al*[85] performed an MRI brain at the time of neurological worsening after D-penicillamine and showed the appearance of new lesions in MRI in white matter, thalamus, pons, and midbrain which showed diffusion restriction with simultaneous blood investigations revealed increased free serum copper, malondialdehyde and reduced glutathione. Litwin *et al*[86] showed neurological worsening in 11% of neuro-hepatic WD in a mean time of 2.3 ± 1.9 mo from the time of treatment initiation independent of the type of chelation used. One hypothesis proposed is that there is a sudden release of free copper from the liver, which enters the blood-brain barrier and causes brain damage[84]. The second hypothesis is that the free copper is generated by the chelators within the brain tissue itself. This was substantiated by an increase in free copper concentration in serum and brain tissue, within 3 d of starting on D-penicillamine, measured by ultrafiltration in an animal model of WD. It was noted that at the time of the increase in brain tissue-free copper, there was a fall in protein-bound copper in the brain tissue. A simultaneous increase in immunofluorescent staining of ATP7A (copper transporting protein in neurons) and CTR1 (mediates copper uptake in mammalian tissue) in the cortex and basal ganglia, and not in the blood-brain barrier, suggests that the free copper is generated probably by mobilization of copper within the brain parenchymal cells and not *via* peripherally released free copper entry *via* the blood-brain barrier[87]. The expression of ATP7A present in the subcellular level to mediate biliary excretion of copper (in this animal model) correlated with the free copper in the cortex and basal ganglia. The third hypothesis proposed by Miki *et al*[88] is that the penicillamine-copper complexes that are generated are non-toxic, so, shouldn't cause tissue damage. However, these complexes can catalyze oxidation of the ghost membranes, due to changes in the redox potential of copper, Cu^{2+} to Cu^{1+}), thereby causing neurological symptoms.

TREATMENT

Once the diagnosis is established, the patient is to be started on lifelong pharmacotherapy, depending on the stage of the disease. WD patients can be broadly classified into three subtypes; symptomatic WD, asymptomatic WD with active disease (on biochemical, histological or imaging findings), and pre-symptomatic WD (mostly those detected on family screening). Those with symptomatic or asymptomatic active disease need to be started on chelation. Patients who are asymptomatic or without active disease can be treated with lower maintenance doses of chelators or zinc alone. It takes around 6-18 mo of consistent chelation therapy to improve the organ function and then patients can be shifted to a lower dose of chelator and zinc[8].

Table 5 summarizes all the drugs used in the treatment of WD, their mechanism of action, dose, storage and important side effects. There is a fundamental difference in the mechanism of action of chelators and zinc. Chelators decrease the copper load in the body by chelating the copper in the liver, enterocytes and extrahepatic circulation. Whereas zinc induces the synthesis of metallothioneins, which in turn bind to copper and sequester it in the enterocyte (copper is lost as the enterocytes shed off). Thus, the action of zinc is slow. In scenarios, which demand fast and immediate action, for example, advanced liver disease, chelators are a better choice than zinc.

D-penicillamine

D-penicillamine (D-3β,3β-dimethylcysteine; C5H11NO2S) has been the first line of treatment, since its discovery in 1956. It acts by chelating divalent metal ions using its thiol (-SH) group and forming a water-soluble complex which is excreted in urine. It chelates extracellular copper and mediates its excretion in urine. Also, it chelates the intracellular copper from tissue complexes[81]. In WD, the excess free copper in the cytoplasm of hepatocytes, after saturating the metallothioneins, deposits in the lysosomes and causes free radicle-mediated cell damage. D-penicillamine, trientine, as well as zinc, increase the expression of metallothioneins, which bind to copper ions. Metallothionein is a cysteine-rich protein that is

Table 4 Risk factors for side effects of drug therapy in Wilson's disease

Side effect of drugs	Risk factor	How to differentiate
Neurological worsening with D-penicillamine or trientine	Pre-existing neurological Wilson disease	Exchangeable copper and relative exchangeable copper
Cytopenia due to D-penicillamine	Co-existing hypersplenism due to portal hypertension	Bone marrow biopsy

Table 5 Drugs, their mechanism of action, dosage and side effects

Name of drug	Mechanism of action	Dose	When to start	Side effects
D-penicillamine	Induces cuprieuresis, induces hepatic metallothionine synthesis, reduces fibrosis (by preventing collagen formation)	20 mg/kg/d (maximum induction dose of 1500 mg/d and maintenance dose of 1000 mg/d), to be taken 1 h before or 2 h after meal, storage at room temperature	Chelator of choice in all hepatic phenotype	**Early (1-3 wk):** Fever, rash, arthralgia, cytopenia, proteinuria; **Late:** (1) Skin: degenerative dermatoses elastosis perforans serpingosa, cutis laxa, pseudoxanthoma elasticum, bullous dermatoses, psoriasiform dermatoses, lichen planus, seborrheic dermatitis alopecia, aphthous ulcerations, hair loss; (2) Connective tissue disorders: Lupus like syndrome, arthralgia, Rheumatoid arthritis, polymyositis; (3) Renal: proteinuria, hematuria, glomerulonephritis, nephrotic syndrome, renal vasculitis, Goodpasture's syndrome; (4) Nervous system: Paradoxical neurological worsening, neuropathies, myasthenia, hearing abnormalities, serous retinitis; (5) Gastrointestinal: Nausea, vomiting, diarrhea, elevated transaminases, cholestasis, hepatic siderosis; (6) Respiratory: Pneumonitis, pulmonary fibrosis, pleural effusion; (7) Hematological: Cytopenia, agranulocytosis, aplastic anemia, hemolytic anemia; and (8) Others: Immunoglobulin deficiency, breast enlargement, pyridoxine deficiency
Trientine	Induces cuprieuresis, induces hepatic metallothionine synthesis	20 mg/kg/d (maximum induction dose of 1500 mg/d and maintenance dose of 1000 mg/d), to be taken 1 h before or 2 h after meal, storage at 2^0-8^0 temperature	Indicated if intolerant to D-penicillamine	Paradoxical neurological worsening (10%-50%), sideroblastic anemia, bone marrow suppression, gastritis, skin rash, arthralgia, myalgia, hirsutism
Zinc	Induces intestinal synthesis of metallothioneins, prevents copper absorption	25 mg thrice daily (weight < 50 kg), 50 mg thrice daily (weight > 50 kg), taken in empty stomach	Maintenance phase of symptomatic hepatic WD; First-line induction treatment in selected patient subgroups (neurologic WD, intolerant to chelators, pre-symptomatic patients)	Gastric irritation (30%-40%)
Ammonium Tetra-thiomolybdate	Forms complexes with copper in blood, binds the copper present in food		Neurological WD	Neurological dysfunction (rare), hepatotoxicity, bone marrow suppression

WD: Wilson disease.

an endogenous chelator of copper[5,81]. D-penicillamine also solubilizes the copper deposited in the lysosomes, without much affecting the metallothionein-bound copper and reduces cell damage[89]. Thus, D-penicillamine causes the excretion of copper but can also lead to the sequestration of free intracellular copper with metallothioneins[5]. D-penicillamine also has an anti-inflammatory effect, which is why it is a second-line drug in rheumatic diseases[90]. The standard dose of D-penicillamine is 20 mg/kg/d in 2-3 divided doses, it is usually started at a dose of 1000-1500 mg/d, and after liver functions improve it can be reduced to a maintenance dose of 10-15 mg/kg/d or 750-1000 mg/d[8]. Around 40%-70% of D-penicillamine is absorbed in the proximal intestine, and peak plasma concentration is reached within 1-3 h. After long-standing treatment, it is eliminated within 4-6 d (slow pool of drug bound reversibly to the tissue). Eighty percent of the drug circulates in bound form with D-penicillamine and the rest in free form. The drug is eliminated *via* the kidneys[91]. In neurohepatic WD, the dictum is to start at a low dose and increase slowly. Indian National Association for the Study of the Liver (INASL) recommends starting D-penicillamine at 250 mg on alternate days and increasing by 250 mg every 2-3 wk, until the maximum dose of 1000-1500 mg per day, while keeping a watch on neurological symptoms

[6]. As D-penicillamine causes depletion of pyridoxine by direct effect, pyridoxine needs to be supplemented at a dose of 25-40 mg per day. D-penicillamine should be given either 1 hour before or 2 h after meals, as it may get bound to the copper in the diet before its absorption into the intestine[92]. Antacids and iron significantly decrease its absorption[8]. Brewer *et al*[93] showed that fecal excretion of copper is significantly less with penicillamine and zinc combination than with zinc alone.

Adverse effects of D-penicillamine are acute (early onset) and late-onset

Acute adverse effects include hypersensitivity reaction, in the form of fever, rash, urticaria, arthralgia, proteinuria and leukopenia, usually within the initial 3 wk of starting D-penicillamine, seen in up to 15% of patients[6,94]. Delayed hypersensitivity include pemphigus, lupus-like syndrome[6]. Other delayed adverse effects include various organ systems, such as hematological (both idiosyncratic and dose-dependent marrow suppression), renal (membranous glomerulonephritis, and neurological (worsening of neurological symptoms). Renal and hematological adverse effects have already been discussed in the article earlier. In case of early hypersensitivity reactions, the drug is to be stopped immediately. In case of isolated dermatological involvement, it may be re-started under steroid cover[6]. Other dermatological adverse effects include dermatopathies due to elastic fibre abnormalities *e.g.* elastosis perforans serpiginosa, and pseudo-pseudoxanthoma elasticum. About 15%-30% of patients develop these adverse effects due to high doses and a long duration of drug intake. These skin changes are caused due to inhibition of the aldol crosslinking of tropocollagen and take months to years to manifest (as it takes a long time for new weakened collagen to be synthesized)[95]. Penicillamine-induced autoimmune dermatoses include pemphigus, epidermolysis bullosa and lupus-erythematosus-like syndrome. Immune-mediated toxicities like Goodpasture syndrome, systemic lupus erythematosus, and nephrotic syndrome warrant immediate drug withdrawal[8,58] (Table 5).

In some cases, paradoxical neurological worsening is seen after starting d-penicillamine, likely due to peripheral mobilization of copper from the liver to the bloodstream and its subsequent deposition in CNS, seen in 11%-50%[84,86,96,97]. Large-scale comparative studies should be done to study the effect of chelators on neurological WD by correlating the dose of chelators with MRI changes and copper transporting molecules by *in vivo* studies. Reversal of neurologic deterioration was seen in only 50% of those affected as D-penicillamine was being continued[84]. In another retrospective study, partial/complete reversal was seen in 53% (8/15) and a further partial response in 13% (2/15) of cases during 9.2 ± 5.2 mo[86]. Thus, the solution to this problem is to start at a low dose and increase it gradually.

Trientine

It is also known as triethylenetetramine. It is an alternative to D-penicillamine for chelation, particularly for those who do not tolerate D-penicillamine. One molecule of trientine combines with copper in a 1:1 ratio to form a stable complex, which is excreted in the urine. Trientine dihydrochloride is the oral preparation, which requires cold storage at 2^0-8^0 Celsius to maintain its stability, failure to maintain cold chain is a common cause of drug discontinuation[98]. Since 2018, trientine is also available as trientine tetrahydrochloride. Woimant *et al*[99] showed that there was no difference in efficacy of the two drugs and in terms of adverse effects, there was a case of recurrence of lupus erythematosus-like syndrome with trientine dihydrochloride. Trientine tetrahydrochloride is stable at room temperature, has slightly more rapid absorption and higher bioavailability and greater systemic exposure. Also, it is a cheaper alternative. It is poorly absorbed with a bioavailability of 8-30%. It reaches maximum plasma concentration in 1-3 h[100,101]. Plasma concentration of trientine is significantly reduced when given after food[95]. Trientine has fewer side effects. Pancytopenia is rarely caused. It should not be given with oral iron because trientine-iron complexes are toxic. There are no hypersensitivity reactions reported. Other minor adverse effects include hemorrhagic gastritis, loss of taste and nausea, sideroblastic anemia and allergic rash[6,102,103]. In a multicenter retrospective study of 77 patients with WD, where patients were treated with trientine for an average duration of 8 years (range: 5 mo to 32.5 years), 49.4% had improved hepatic functions, 10.4% of patients remained unchanged, 5.2% showed worsening, and remaining were asymptomatic to begin with. Twenty-two percent of patients had trientine-associated minor adverse effects, with only one patient requiring treatment discontinuation due to anaemia[104]. Neurologic deterioration is reported with trientine, hence, it should be started at a low dose and increased gradually in patients having neurologic manifestations[8]. In a large retrospective cohort of 471 patients (326 receiving D-penicillamine vs. 141 receiving trientine), it was shown that hepatic and neurologic improvement was comparable in either group. Stable neurologic disease as first-line drug was comparable in either group (27.2% for D-penicillamine *vs* 20% for trientine). A higher rate of neurologic worsening was reported with trientine as the first-line agent (20% *vs* 5.3%)[105]. In another randomized control trial among neurologic WD patients, for comparison between tetrathiomolybdate, 6/23 (26%) in trientine had neurologic worsening as compared to 1/23 in the tetrathiomolybdate group[106]. The dose is 750-1000 mg per day or 20 mg/kg/d in 3 divided doses. It is to be given 1 h before or 2 h after food[100].

Zinc

It induces synthesis of metallothioneins, and thereby promotes copper-binding to metallothionein in the enterocyte and ultimately hinders its absorption (as copper is lost when the enterocyte is shed). It is a slow de-coppering agent, it decreases the copper absorption but doesn't lead to a sudden massive increase in free copper, hence, it is of choice in neurological WD. However, because of the slow reduction in copper, it is not suitable for treating florid symptoms[8]. Disease may show progression in the initial few months, because of its slow onset of action[106]. In the first pediatric study, in which trientine was used as the initial chelator, it was shown that once adequate chelation was achieved, zinc combination therapy and subsequently zinc monotherapy maintained normal ALT/AST levels[102]. Among those having weight < 50 kg, it is to be given in a dose of 25 mg thrice a day and in those weighing > 50 kg, 50 mg thrice a day[6].

Various zinc preparations are available; zinc acetate, zinc gluconate, and zinc sulfate. There is no statistically significant difference in various zinc preparations in terms of improvement of liver function[107]. There are few adverse effects, gastric irritation is the most common side effect, in 30%-40% of patients. It can also cause an asymptomatic elevation in amylase and lipase[8]. It is recommended to use zinc in pre-symptomatic patients or in the maintenance phase of treatment in symptomatic patients[8]. In a systematic review and meta-analysis, zinc showed better improvement as compared to D-penicillamine in neurologic WD, however, there was no difference in hepatic WD. Also, the incidence of adverse effects and neurologic deterioration was higher with D-penicillamine as compared to zinc (RR: 2.42, 95%CI: 1.20%–4.88%; P = 0.014) and RR: 1.96, 95%CI: 1.31%–2.93%; P = 0.001, respectively)[108].

Ammonium tetra-thiomolybdate

It acts by forming a tripartite complex with copper and protein, which is stable. It binds the copper present in food and prevents its absorption. When given without food, it is absorbed into the blood and forms complexes with copper bound to albumin, thus preventing its deposition in various organs[109]. Also, it enters the blood-brain barrier and enters neuronal cells[110]. In an open-label study, 55 neurologic WD were treated with tetrathiomolybdate (120-410 mg for 8 wk), followed by zinc maintenance therapy. Only 3.6% (2/55) showed neurologic deterioration. Among the other adverse effects, 5/22 (23%) of treatment-naïve patients had bone marrow suppression and 3/22 (14%) had an elevation in liver enzymes and both these adverse effects quickly responded to drug dose reduction[111]. Bone marrow suppression is caused by the depletion of copper and is reversed by decreasing the dose of tetrathiomolybdate. The rise in the liver enzymes could be due to the mobilization of hepatic copper (from hepatic pools including metallothioneins) in a heavily copper-loaded liver, which is reversible on dose reduction[111]. In a subsequent double-blind RCT between tetrathiomolybdate (120 mg per day) and trientine (1000 mg/d) among 48 patients of neurologic WD, as mentioned above, tetrathiomolybdate was better than trientine, only 4% worsened neurologically *vs* 26% in trientine arm[106]. Ammonium tetrathiomolybdate is unstable for routine use, bis-choline tetrathiomolybdate is a more stable complex and has a better availability. Recently, bis-choline tetrathiomolybdate underwent a phase II trial among 28 neurologic WD, where it was given for a span of 24 wk, with the target to achieve a primary end-point of normal value of non-ceruloplasmin bound $\text{copper}_{\text{corrected}}$ ($\text{NCC}_{\text{corrected}}$ stands for NCC corrected for copper contained in tetrathiomolybdate-copper-albumin complexes) or achievement of 25% reduction from baseline $\text{NCC}_{\text{corrected}}$. Twenty-two patients completed the study up to 24 wk and by week 24, 20 (71%) had achieved the primary end-point (treatment success) accompanied by improvement in neurologic status (without any paradoxical neurological worsening, being reported). There were 11 (25%) serious adverse effects which included 6 events of psychiatric disorders in 4 patients, gait disturbance in one patient and two events of raised aminotransferases, one agranulocytosis and a decline in neurologic functioning[112]. Also, Brewer *et al*[113] analysed free copper levels in patients treated with tetrathiomolybdate *vs* trientine and found that the mean free copper was significantly less in the tetrathiomolybdate group at week 4 and week 8 of treatment. Thus, tetrathiomolybdate is a fast copper-lowering agent with minimal adverse effects. The dose recommended is initially 120 mg per day for the first 2 wk, as, 20 mg thrice a day with meals and 20 mg daily between meals followed by 60 mg daily as 10 mg thrice a day with meals and 10 mg thrice daily between meals[113]. Currently, a phase 3 trial is going on and results are keenly awaited (NCT03403205).

COMPARISON BETWEEN VARIOUS CHELATORS

D-penicillamine vs trientine

In a large retrospective cohort of median follow-up of 13.3 years, both D-penicillamine and trientine were comparable in terms of improvement in hepatic WD (> 90% cases) as well as in neurologic WD (> 55% cases) while neurologic worsening was more common in trientine group as compared to D-penicillamine group (20% *vs* 5.3%, P = 0.042). Treatment discontinuation due to adverse effects was more common in the D-penicillamine group as compared to trientine (28.8% *vs* 7.1%, P = 0.039)[105]. In the most recent randomized open-label non-inferiority trial of D-penicillamine *vs* trientine, for maintenance therapy after 1 year of chelation with D-penicillamine, trientine was found non-inferior to D-penicillamine[114].

D-penicillamine vs zinc

There are studies to show that zinc was found to be more effective in ameliorating neurological symptoms in 90% of patients in the zinc arm as compared to 25% alone in the D-penicillamine arm[115]. In a head-to-head comparison between D-penicillamine and zinc, among 67 new patients, of whom the majority were asymptomatic or had neurological disease, 44% discontinued D-penicillamine owing to its adverse effects as compared to 12% in the zinc arm[116]. In Another retrospective study of 288 patients for a period of 17.1 years by Weiss *et al*[117] showed that zinc monotherapy led to 15.9% (14/88) hepatic treatment failure as compared to 1.2% (4/313) in the D-penicillamine group without any statistically significant difference in the adverse effects in either group. Further, these zinc non-responders responded to chelators (either D-penicillamine or trientine). This supports the use of chelators in asymptomatic patients with active disease.

Dhawan *et al*[118], Askari *et al*[119] and Santos *et al*[120] have shown favourable outcomes with combination therapy but earlier studies by Brewer *et al*[93] show no added advantage. A systematic review of 17 articles involving 1056 on combination therapy showed that combination therapies are significantly less effective than individual therapies (47.1 *vs* 78.6%)[121].

Adequacy of treatment

Treatment targets on chelators: Asymptomatic patients should remain asymptomatic and symptomatic patients should show improvement in liver functions in the initial 6-18 mo[8]. Patients with decompensation might take longer to improve. Patients are to be monitored clinically for symptom improvement/new symptom onset. Initially, LFTs are to be done, every 3 mo, and thereafter 3-6 mo depending on the disease severity[8]. Adequacy is monitored by monitoring liver function tests and by quantifying 24-h urine copper excretion or free copper estimation. Free copper estimation, is not a full-proof test if the ceruloplasmin is calculated by the immunologic method as it calculates both apoceruloplasmin and holoceruloplsmin[6]. Also, urinary copper excretion is to be interpreted carefully after taking proper treatment history. Urinary copper excretion may be high if chelation has been re-started after a period of non-adherence or it can be falsely low, in case of poor drug absorption or inadequate dosing itself. Another way to monitor adequacy is to measure REC.

For de-coppering agents, free copper is a marker of the adequacy of chelation, while for zinc, urine copper levels below a certain cut-off is recommended for good copper control (Table 1).

When to decrease chelation?

Transition to maintenance dose or zinc can be used once liver function improves. AASLD recommends a transition to maintenance after clinical and biochemical parameters improve (usually seen after 1 year of therapy). The drug of choice drug could be low-dose chelating agents or full-dose zinc. In a retrospective study of 31 symptomatic hepatic WD who were transitioned from D-penicillamine to zinc (28 due to financial constraints and 3 due to adverse effects), wherein the majority of patients belonged to Child's class C (54%) patients, the average duration of zinc therapy on follow-up was 363 (35–728) weeks. In the Child C cirrhosis group at presentation, who received D-penicillamine for 111 (2-230) weeks followed by zinc for 344 (41–652) weeks, 15 had significant improvement in liver function and disease severity scores [122]. In a prospective study of 44 hepatic WD patients, who received D-penicillamine plus zinc combination therapy for more than 2 years, and were in biochemical remission (defined by AST and ALT > 1.5 times upper limit of normal, serum albumin > 3.5 gm/dL and INR < 500 mcg/d and NCC < 15 mcg/dL, were shifted to zinc monotherapy and biochemical parameters were assessed on follow-up. They showed that 9/44 (20.4%) relapsed till the last follow-up[123]. More prospective studies are needed to establish the correct time of transition and establish guidelines for the same.

CONCLUSION

Wilson's disease is a treatable metabolic liver disease. Early diagnosis is imperative. Wilson's disease has varied manifestations. Certain disease manifestations need to be differentiated from drug-toxicities, *e.g.*, tubulopathies due to WD vs. D-penicillamine-induced nephrotoxicity and hypersplenism *vs* D-penicillamine-induced myelosuppression. Though there are difficulties in making the correct diagnosis, with the help of non-ceruloplasmin bound copper, relative exchangeable copper and newer methods to detect early Kayser-Fleischer ring (AS-OCT), diagnosis can be made in resource-limited conditions, where mutational analysis is cost-forbidding. Chelation remains the mainstay of treatment and is to be preferred in active disease whether symptomatic or asymptomatic. Regarding the transition to maintenance therapy, the exact timeline is not yet defined, but depends on the liver function and is to be decided on a case-to-case basis with close follow-up of the copper load in the body. Trientine has been shown to have a good clinical response in a recent clinical trial and its availability as tetrahydrochloride, which is cheaper and doesn't require cold storage gives hope in resource-limited conditions. Bis-choline tetrathiomolybdate is the new addition in the armamentarium, which rapidly decreases the free copper load and is undergoing phase III trials. In fulminant hepatic failure, plasmapheresis has shown (while awaiting liver transplantation) some hope.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Liver disease epidemiology and burden in patients with alterations in plasma protein metabolism: German retrospective insurance claims analysis

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Abstract

BACKGROUND

Alpha-1 antitrypsin deficiency is a rare genetic disease and a leading cause of inherited alterations in plasma protein metabolism (APPM).

AIM

To understand the prevalence, burden and progression of liver disease in patients with APPM including alpha-1 antitrypsin deficiency.

METHODS

We conducted a retrospective analysis of anonymized patient-level claims data from a German health insurance provider (AOK PLUS). The APPM cohort comprised patients with APPM (identified using the German Modification of the

International Classification of Diseases-10th Revision [ICD-10-GM] code E88.0 between 01/01/2010-30/09/2020) and incident liver disease (ICD-10-GM codes K74, K70.2-3 and K71.7 between 01/01/2012-30/09/2020). The control cohort comprised patients without APPM but with incident liver disease. Outcomes were incidence/prevalence of liver disease in patients with APPM, demographics/baseline characteristics, diagnostic procedures, progression-free survival (PFS), disease progression and mortality.

RESULTS

Overall, 2680 and 26299 patients were included in the APPM (fibrosis, 96; cirrhosis, 2584) and control (fibrosis, 1444; cirrhosis, 24855) cohorts, respectively. Per 100000 individuals, annual incidence and prevalence of APPM and liver disease was 10-15 and 36-51, respectively. In the APPM cohort, median survival was 4.7 years [95% confidence interval (CI): 3.5-7.0] and 2.5 years (95%CI: 2.3-2.8) in patients with fibrosis and cirrhosis, respectively. A higher proportion of patients in the APPM cohort experienced disease progression (92.0%) compared with the control cohort (67.2%). Median PFS was shorter in the APPM cohort (0.9 years, 95%CI: 0.7-1.1) compared with the control cohort (3.7 years, 95%CI: 3.6-3.8; $P < 0.001$). Patients with cirrhosis in the control cohort had longer event-free survival for ascites, hepatic encephalopathy, hepatic failure and esophageal/gastric varices than patients with cirrhosis in the APPM cohort ($P < 0.001$). Patients with fibrosis in the control cohort had longer event-free survival for ascites, cirrhosis, hepatic failure and esophageal/gastric varices than patients with fibrosis in the APPM cohort ($P < 0.001$). In the APPM cohort, the most common diagnostic procedures within 12 mo after the first diagnosis of liver disease were imaging procedures (66.3%) and laboratory tests (51.0%).

CONCLUSION

Among patients with liver disease, those with APPM experience substantial burden and earlier liver disease progression than patients without APPM.

Key Words: Alpha-1 antitrypsin deficiency; Epidemiology; Germany; Liver diseases; Retrospective study

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Core Tip: This was a retrospective analysis of anonymized, patient-level, insurance claims data from a German health insurance provider (AOK PLUS), which demonstrated that a diagnosis of alterations in plasma protein metabolism (APPM) (E88.0) in patients with liver disease was associated with a substantial burden and higher rate of liver disease progression compared with patients with liver disease but without APPM. To enable accurate diagnosis and inform disease management, it is important to have specific diagnostic codes that differentiate between genetic liver disease and liver manifestations from other causes.

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INTRODUCTION

Alterations in plasma protein metabolism (APPM) can either be inherited or acquired[1]. As hepatocytes are responsible for the majority of protein production (approximately 10-20 g/d)[2], liver disease is frequently observed among patients with APPM[1].

Alpha-1 antitrypsin (AAT) deficiency (AATD) is the most common form of inherited APPM and is caused by mutations in serpin family A member 1 (*SERPINA1*), which encodes AAT, a serum protein produced primarily by hepatocytes that protects the lungs from protease-mediated degradation[3]. A homozygous mutation (Glu342Lys) in *SERPINA1,* named protease inhibitor (Pi) ZZ, is estimated to affect approximately 1 in 2000-5000 newborn infants in Europe and North America[4]. Mutations in *SERPINA1* cause a reduction in serum AAT levels and promote the development of respiratory diseases, such as emphysema or chronic obstructive pulmonary disease[5]. In addition, AATD can result in liver diseases such as liver cirrhosis or hepatocellular carcinoma (HCC) due to the accumulation of hepatic AAT, which can trigger proteotoxic stress and lead to hepatocyte death and liver injury[6,7]. Approximately 20%-36% of patients with a PiZZ genotype develop significant fibrosis and approximately 10%-15% develop advanced fibrosis [8]. The only available curative treatment for end-stage liver disease in patients with AATD is liver transplantation[9]. Liver transplants in Germany have been allocated based on urgency according to the model for end-stage liver disease scoring system[10].

Given the lack of information on the natural history and epidemiology of AATD, we conducted a retrospective analysis of insurance claims data from Germany to better understand overall prevalence, burden and progression of liver disease in patients with APPM, including AATD. To improve our understanding of the natural history of APPM and liver disease, we compared patients with APPM and liver disease with a control cohort of patients with liver disease but without APPM.

MATERIALS AND METHODS

Data source and sample selection

This retrospective study used anonymized, patient-level, insurance claims data from 01 January 2010 to 30 September 2020, provided by the German regional health insurance provider, AOK PLUS. This data set covers approximately 3.3 million individuals from the federal states of Saxony and Thuringia, accounting for approximately 4.5% of the German statutory health insured (SHI) population in 2020. The age and comorbidity characteristics of patients insured by AOK PLUS are similar to those in the general German population who are insured by sickness funds[11-14].

The cohort of patients with APPM and incident liver disease was identified using the German Modification of the International Classification of Diseases-10th Revision (ICD-10-GM) code E88.0 for disorders of plasma protein metabolism (which includes AATD and other metabolic disorders such as plasminogen deficiency and bisalbuminaemia), and codes K74, K70.2-3 and K71.7 for liver disease.

Patients were included in the APPM cohort if they had APPM (diagnosed between 01 January 2010 and the end of the study) and incident liver disease (diagnosed between 01 January 2012 and the end of the study; Supplementary Figure 1). Patients were excluded if their liver disease was diagnosed in 2010 or 2011, to guarantee a liver disease-free period of 2 years. Continuous insurance coverage (no interruption of insurance for > 30 d) in 2010 and/or 2011 was required. Patients were observed from the date of the first diagnosis of liver disease (index date) until death, loss to follow-up due to end of insurance or end of the study. In patients with incident fibrosis who developed cirrhosis after 01 January 2012, the index date was defined as the date of the first diagnosis of cirrhosis for analyses of the subgroup of patients with cirrhosis. Patients were included in the control cohort if they did not have a diagnosis of APPM, but had incident liver disease. All patients with incident liver disease in both cohorts were further divided into two sub-cohorts: (1) Those with fibrosis at index date (ICD-10-GM codes: K74.0-2 and K70.2); and (2) those with cirrhosis at index date or those with fibrosis who developed cirrhosis during the study period (ICD-10-GM codes: K74.3-7, K70.3 and K71.7).

Outcomes

The study evaluated point prevalence and cumulative incidence of patients with APPM and liver disease, stratified by sex. In addition, demographics and baseline disease characteristics, including the Charlson Comorbidity Index, were measured at index date, with comorbidities identified during the previous 12 mo. Comorbidities were identified based on three-digit ICD-10-GM codes and evidence of confirmatory diagnosis/diagnoses (at least one inpatient or at least two outpatient diagnoses). Diagnostic procedures (liver biopsy, imaging, laboratory tests, AAT phenotyping and liver function tests) in the 12 mo after the index date were identified based on German procedure codes (Operationen-und Prozedurenschlüssel and einheitlicher Bewertungsmaßstab). A composite endpoint of progression-free survival (PFS) was defined as the time from index date until the first date with selected liver disease-related clinical events (acute peritonitis, ascites, cirrhosis, only among patients with fibrosis), esophageal/gastric varices, HCC, hepatic encephalopathy, hepatic failure, liver transplantation or all-cause death (used to assess mortality). Disease progression events were also analyzed separately, and comprised the following (one inpatient or one confirmed outpatient diagnosis): Ascites, esophageal/gastric varices, acute peritonitis, hepatic encephalopathies, gastrointestinal bleeding (*e.g.*, melaena or hematemesis), hepatic failure, malignant neoplasm of the liver and intrahepatic bile ducts and HCC. Procedure-related events were analyzed, which comprised liver incision, liver resection, other operations on the liver, failure or rejection of transplant, and infection due to prosthesis, implant or graft.

Statistical analysis

To calculate the point prevalence of liver disease in patients with APPM, the denominator was the number of individuals insured by AOK PLUS on 01 January of the respective calendar year (2011-2020) and during the preceding 12 mo. The numerator was the number of patients alive on the 1st January of each year, with evidence of confirmatory diagnosis/diagnoses of APPM (made in two different quarters within the same year) and a diagnosis of liver disease during the previous year, and with continuous insurance coverage during that year.

The cumulative incidence of liver disease in patients with APPM was estimated for 2012-2019 by dividing the number of new cases in a calendar year by the total number of insured patients at risk of liver disease (*i.e.*, those with no current evidence of liver disease) at the beginning of the same year. The numerator was the number of patients with APPM diagnosed at any point and with liver disease diagnosed during the year of the index date, but without any liver disease diagnosis within the previous 2 years and with continuous insurance coverage during this period. The denominator was the number of patients alive at the beginning of the respective calendar year for whom no liver disease diagnosis was documented in the 24 mo before index date, and with continuous insurance over this period.

Point prevalence and cumulative incidence were adjusted for age and sex differences compared with the German SHI population.

Patient demographics and baseline characteristics were analyzed using summary statistics (mean, standard deviation, median and interquartile range) for continuous variables and frequency statistics for categorical variables.

Time to disease progression was estimated using the Kaplan-Meier method. Patients were censored if they were lost to follow-up or had reached the end of the study. In addition to the number of patients with a progression event, the following Kaplan-Meier estimates were reported: median follow-up time in patients without an event, the 25th, 50th and 75th percentiles of time without an event, and event rates at years 1, 3 and 5 post-baseline. All time to event analyses were compared *via* a log-rank test.

Time from index date to all-cause death was estimated for patients with APPM and incident fibrosis or cirrhosis, and separately for patients with APPM and cirrhosis with and without a previous documented diagnosis of fibrosis.

RESULTS

Overview of analyzed patients

In total, 45503 patients had confirmatory diagnosis(es) of liver disease between 01 January 2012 and the end of the study (Figure 1). Of these, 2680 fulfilled the criteria to be included in the APPM cohort. In total, 96 of these patients had fibrosis and 2584 had cirrhosis at their index date (between 01 January 2012 and the end of the study).

In total, 26299 patients were included in the corresponding control cohort with no diagnosis of APPM. Of these, 1444 had fibrosis and 24855 had cirrhosis at their index date (Figure 1).

Epidemiology

Between 2012 and 2019, the annual cumulative incidence of liver disease per 100000 individuals with APPM was 10-15, and was higher in males (15-22) than females (5-9; Supplementary Figure 2). When adjusted for age and sex differences *vs* the SHI population, the cumulative incidence was 8-13 per 100000 individuals. Between 2011 and 2020, the point prevalence of liver disease per 100000 individuals with APPM was 36-51 and was again higher in males (52-74) than females (22-32; Supplementary Figure 3). When adjusted for age and sex differences compared with the SHI population, the point prevalence was 33-47 per 100000 individuals.

Demographics and baseline characteristics

Demographics and baseline characteristics were similar between cohorts (Table 1). In the APPM and control cohorts, respectively, 840 patients (31.3%) and 8595 patients (32.7%) were female (P = 0.159 between cohorts). Patients in the APPM cohort were significantly younger than in the control cohort, with a median age (interquartile range) of 63 years (54-73) and 65 years (56-76; P < 0.001), respectively. The most common liver-related comorbidities in the APPM and control cohorts, respectively, were "other liver disease" [679 (25.3%) and 6298 (23.9%)], non-alcoholic steatohepatitis [17 (0.6%) and 150 (0.6%)] and chronic hepatitis [12 (0.5%) and 163 (0.6%)]. The proportion of patients with respiratory system-related comorbidities was similar between the APPM [827 (30.9%)] and control [8333 (31.7%)] cohorts.

Minor differences in demographics and baseline characteristics were observed when the APPM cohort was stratified by the presence of fibrosis or cirrhosis. There was a higher proportion of females with fibrosis [41 (42.7%)] compared with cirrhosis [815 (31.0%)] and alcohol-related disorders were more common in patients with cirrhosis [649 (24.7%)] compared with fibrosis [17 (17.7%)].

Diagnostic procedures

The most common diagnostic procedures within 12 mo after the index date in the APPM cohort were imaging procedures [1778 (66.3%)] and laboratory tests [1366 (51.0%); Table 2]. Only 55 patients (2.1%) underwent AAT phenotyping.

Disease progression and mortality

A higher proportion of patients in the APPM cohort experienced disease progression [2465 (92.0%)] compared with the control cohort [17682 (67.2%)]. Median PFS (composite endpoint) was significantly shorter in the APPM cohort [0.9 years (95%CI: 0.7-1.1)] compared with the control cohort [3.7 years (95%CI: 3.6-3.8); P < 0.001; Supplementary Figure 4]. The most common disease progression events were ascites, hepatic failure, esophageal/gastric varices, and hepatic encephalopathies (Figure 2). Patients with cirrhosis but without APPM had significantly longer event-free survival for ascites, hepatic failure, esophageal/gastric varices, and hepatic encephalopathy compared with those with APPM and cirrhosis (all P < 0.001; Figure 3). Similarly, patients with fibrosis but without APPM had significantly longer event-free survival for ascites, cirrhosis, hepatic failure and esophageal/gastric varices compared with those with APPM and fibrosis (all P < 0.001; Supplementary Figure 5). In the subgroup of 96 patients in the APPM cohort with fibrosis, median time from fibrosis to cirrhosis was 2.9 years (95%CI: 1.2-not reached; Supplementary Figure 5B). In the control cohort, median time from fibrosis to cirrhosis was not reached.

The most common procedures and procedure-related events indicating disease progression were liver resection, liver transplantation, failure and rejection of liver transplant and other operations on the liver (Supplementary Figure 6). A significantly higher proportion of patients with APPM and fibrosis had a liver resection [15 (15.6%) *vs* 93 (6.4%); P = 0.025] and a liver transplantation [6 (6.3%) *vs* 3 (0.2%); P < 0.001] compared with patients without APPM but with fibrosis (Table 3). Similarly, a significantly higher proportion of patients with APPM and cirrhosis had a liver resection [87 (3.3%) *vs* 488 (1.9%); P < 0.001] and a liver transplantation [78 (3.0%) *vs* 31 (0.1%); P < 0.001] indicating greater disease progression compared with patients without APPM but with cirrhosis (Table 3; Supplementary Figure 6).

Table 1 Demographics and baseline characteristics, *n* (%)

	Patients with APPM and liver disease: APPM cohort, *n* = 2680			Patients without APPM and with liver disease: Control cohort, *n* = 26299
	All patients, *n* = 2680	Patients with fibrosis, *n* = 96	Patients with cirrhosis, *n* = 2626[1]	
Total observed patient-years	6118	283	5944	70261
Median follow-up, yr (IQR)	1.5 (0.4-3.7)	2.3 (0.8-4.7)	1.5 (0.4-3.7)	2.0 (0.5-4.4)
Female sex[2]	840 (31.3)	41 (42.7)	815 (31.0)	8595 (32.7)
Median age, yr (IQR)[3]	63.0 (54.0-73.0)	64.5 (53.0-76.5)	63.0 (54.0-73.0)	65.0 (56.0-76.0)
0-14 yr	14 (0.5)	6 (6.3)	10 (0.4)	49 (0.2)
15-29 yr	10 (0.4)	0	10 (0.4)	156 (0.6)
30-44 yr	183 (6.8)	10 (10.4)	177 (6.7)	1420 (5.4)
45-59 yr	818 (30.5)	21 (21.9)	814 (31.0)	7516 (28.6)
60-74 yr	1046 (39.0)	31 (32.3)	1029 (39.2)	9564 (36.4)
75-89 yr	577 (21.5)	25 (26.0)	557 (21.2)	7090 (27.0)
≥ 90 yr	32 (1.2)	3 (3.1)	29 (1.1)	504 (1.9)
With care needs[4]	547 (20.4)	33 (34.3)	528 (20.1)	4934 (18.8)
Median CCI[5] (IQR)	3.0 (1.0-5.0)	4.0 (1.5-6.0)	3.0 (1.0-5.0)	3.0 (1.0-5.0)
Most common liver-related comorbidities				
"Other" liver disease[6]	679 (25.3)	26 (27.1)	669 (25.5)	6298 (23.9)
Non-alcoholic steatohepatitis	17 (0.6)	1 (1.0)	16 (0.6)	150 (0.6)
Chronic hepatitis	12 (0.5)	0	12 (0.5)	163 (0.6)
Respiratory system-related comorbidities	827 (30.9)	41 (42.7)	806 (30.7)	8333 (31.7)
Most common comorbidities				
Hypertension	1751 (65.3)	64 (66.7)	1710 (65.1)	17278 (65.7)
Type 2 diabetes mellitus	1103 (41.2)	40 (41.7)	1082 (41.2)	10766 (40.9)
Dyslipidaemia	832 (31.0)	35 (36.5)	813 (31.0)	8932 (34.0)
Alcohol-related disorders[7]	650 (24.3)	17 (17.7)	649 (24.7)	6127 (23.3)
Heart failure	605 (22.6)	21 (21.9)	591 (22.5)	6071 (23.1)

Data are *n* (%) unless otherwise indicated.

[1]In total, 42 patients with fibrosis progressed to cirrhosis during follow-up and are included in both fibrosis and cirrhosis subgroups.

[2]Patients in the alterations in plasma protein metabolism (AAPM) cohort were significantly younger than patients in the control cohort (Wilcoxon rank-sum test: $P < 0.001$).

[3]There was no statistically significant difference in the proportion of females between cohorts (Wilcoxon rank-sum test: $P = 0.159$).

[4]Dummy variable for patients who were classified in any of the five care levels which determine the benefits from long-term care insurance in Germany.

[5]Assessed based on diagnoses observed in the 12 mo before the index date.

[6]Captured using the K76 code in the German Modification of the International Classification of Diseases-10th Revision, and includes central hemorrhagic necrosis of the liver, chronic congestive liver, fatty liver (not otherwise classified), hepatorenal syndrome, liver infarction, peliosis hepatis, portal hypertension, veno-occlusive liver disease and other/unspecified liver disease.

[7]Patients with alcohol-related disorders diagnosed before [1172 (43.7%)] and after [1042 (38.9%)] diagnosis of APPM were detected in the cohort with APPM and liver disease.

CCI: Charlson comorbidity index; IQR: Interquartile range.

Median survival was shorter in the APPM cohort [2.6 years (95%CI: 2.3-2.8)] than in the control cohort [4.3 years (95%CI: 4.2-4.5)]. Median survival was 4.7 years (95%CI: 3.5-7.0) in patients with APPM and fibrosis and 2.5 years (95%CI: 2.3-2.8) in patients with APPM and cirrhosis (Figure 4). In the 42 patients in the APPM cohort with fibrosis who developed cirrhosis during the follow-up period, median survival was 4.1 years (95%CI: 2.2-7.1). In the 2584 patients in the APPM cohort with cirrhosis and without previous fibrosis, the median survival was 2.5 years (95%CI: 2.2-2.7).

Table 2 Diagnostic procedures within 12 mo after the index date, *n* (%)

Diagnostic procedure	Patients with APPM and liver disease: APPM cohort, *n* = 2680
Imaging procedure[1]	1778 (66.3)
Laboratory test[2]	1366 (51.0)
Liver biopsy	207 (7.7)
AAT phenotyping	55 (2.1)
Liver function test[3]	13 (0.5)

Data are *n* (%).
[1]Included sonographies, computed tomography scans, magnetic resonance imaging procedures, angiographies of the abdomen and magnetic resonance elastographies.
[2]Included albumin and bilirubin measurements.
[3]Liver function test with intravenous application of a ^{13}C-labeled substrate.
AAT: Alpha-1 antitrypsin; APPM: Alterations in plasma protein metabolism.

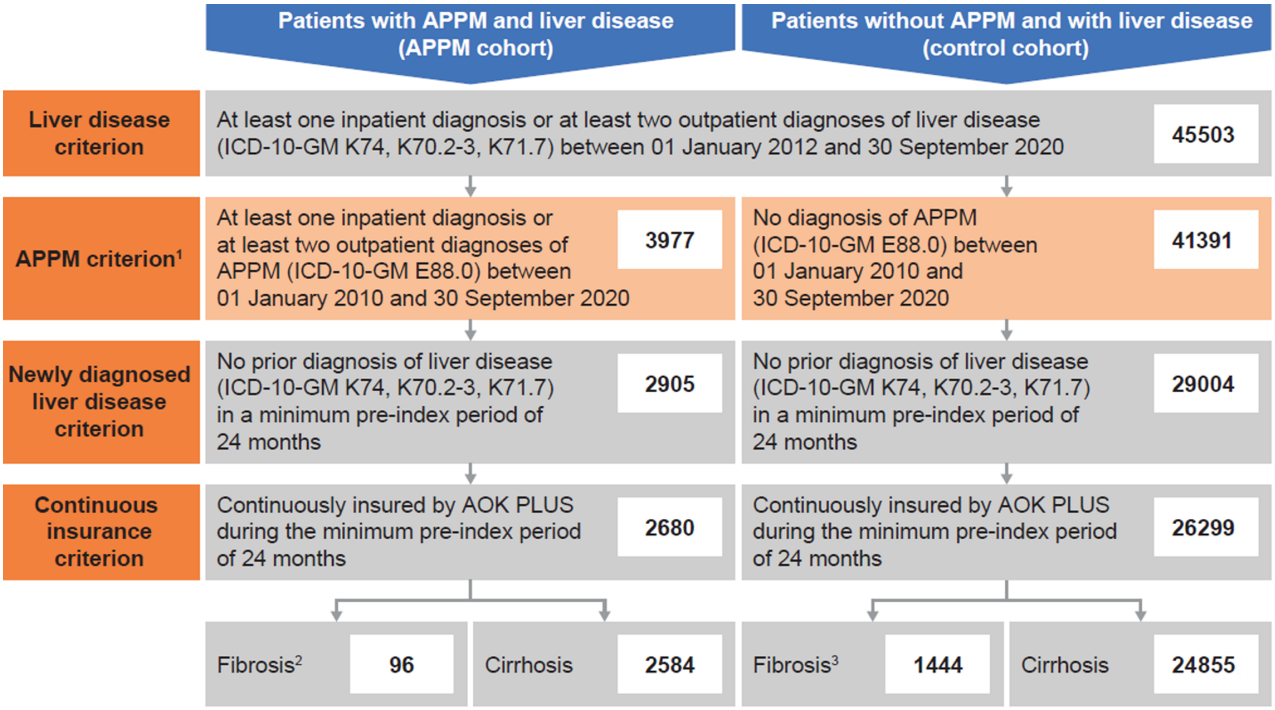
Table 3 Procedures indicating disease progression stratified by fibrosis or cirrhosis

Parameter	Patients with fibrosis				Patients with cirrhosis			
	Liver resection		Liver transplantation		Liver resection		Liver transplantation	
	Patients with APPM, *n* = 96	Patients without APPM, *n* = 1445	Patients with APPM, *n* = 96	Patients without APPM, *n* = 1445	Patients with APPM, *n* = 2626	Patients without APPM, *n* = 25134	Patients with APPM, *n* = 2626	Patients without APPM, *n* = 25134
Patients with event during follow-up	15 (15.6)	93 (6.4)	6 (6.3)	3 (0.2)	87 (3.3)	488 (1.9)	78 (3.0)	31 (0.1)
Patients included in the KM analysis[1]	85 (88.5)	1373 (95.0)	94 (97.9)	1442 (99.8)	2606 (99.2)	24849 (98.9)	2621 (99.8)	25034 (99.6)
Median follow-up in patients without an event, yr (IQR)[2]	2.3 (0.7-5.0)	3.0 (1.2-5.2)	2.3 (0.7-4.7)	2.8 (1.2-5.1)	1.5 (0.4-3.7)	1.9 (0.5-4.4)	1.5 (0.4-3.7)	1.9 (0.5-4.4)
Failure rate								
After 1 yr	3 (4.2)	15 (1.2)	1 (1.2)	1 (0.1)	40 (2.0)	198 (1.0)	26 (1.4)	8 (< 0.1)
After 3 yr	3 (4.2)	18 (1.5)	3 (4.8)	1 (0.1)	56 (3.4)	258 (1.5)	53 (3.6)	23 (0.2)
After 5 yr	3 (4.2)	20 (1.9)	4 (7.3)	1 (0.1)	64 (4.7)	289 (1.9)	66 (5.6)	25 (0.2)
P value (log-rank test)	0.025		< 0.001		< 0.001		< 0.001	

Data are *n* (%) unless otherwise indicated.
[1]Patients with the event on the index date were excluded from the Kaplan-Meier (KM) analysis. As there were no patients with liver incision in the alterations in plasma protein metabolism cohort, and only one patient in the control cohort, this outcome is not reported.
[2]There were no patients with fibrosis who had an infection/inflammatory reaction due to prothesis/implant/graft. For one patient in the control cohort, the index date and end date were the same; this patient was automatically excluded from the KM analysis. Median follow-up in patients without the event was estimated *via* the reverse KM method.
IQR: Interquartile range; APPM: Alterations in plasma protein metabolism.

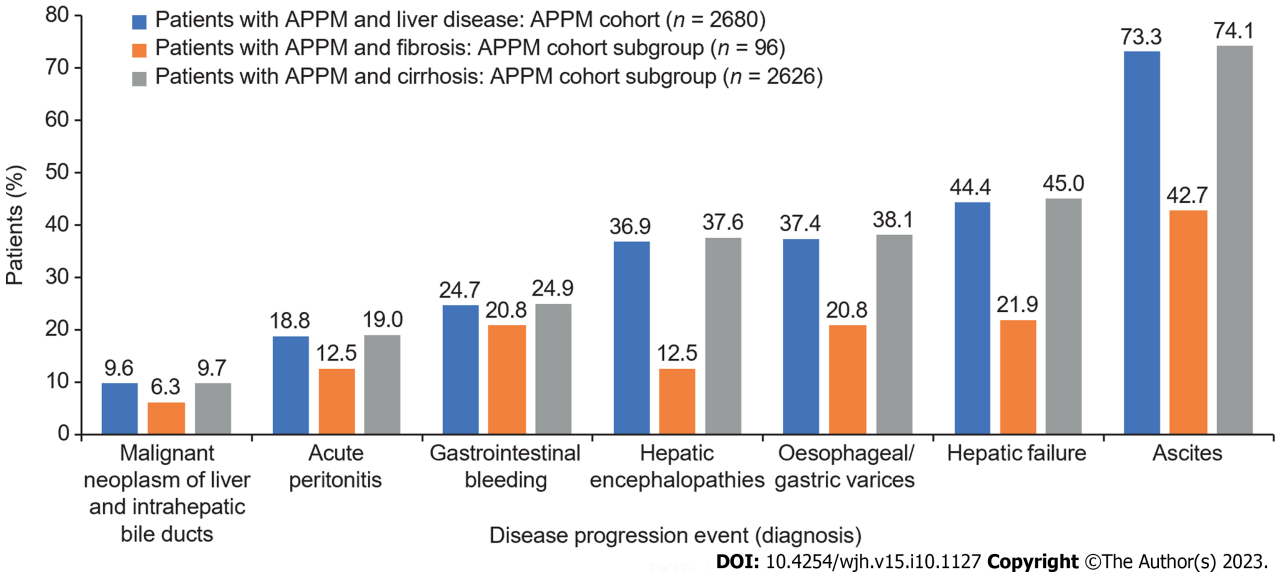
DISCUSSION

This retrospective insurance claims-based study demonstrated that, per 100000 individuals, the annual incidence (2012-2019) and point prevalence (2011-2020) of APPM was 10-15 and 36-51, respectively, with higher rates in males than in females. Patients in the APPM cohort experienced shorter PFS, higher mortality and a higher rate of liver decompensation events compared with patients in the control cohort. In addition, patients with fibrosis in the APPM cohort had significantly shorter cirrhosis-free survival compared with patients with fibrosis in the control cohort ($P < 0.001$). This may have been because APPM is indicative of a more advanced stage of fibrosis that is more likely to decompensate, or alternatively may reflect how the E88.0 code is used in clinical practice. Liver cirrhosis is often associated with decreased plasma levels of hepatocyte-derived proteins[15]. Some of these proteins, such as albumin and transferrin, are well-



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Figure 1 Selection of patient cohorts. [1]In total, 135 patients had only one outpatient alterations in plasma protein metabolism (APPM) diagnosis and therefore did not meet these criteria; [2]In total, 42 patients with fibrosis in the APPM cohort progressed to cirrhosis during follow-up; and [3]In total, 280 patients with fibrosis in the control cohort progressed to cirrhosis during follow-up. ICD-10-GM: German Modification of the International Classification of Diseases-10th Revision.



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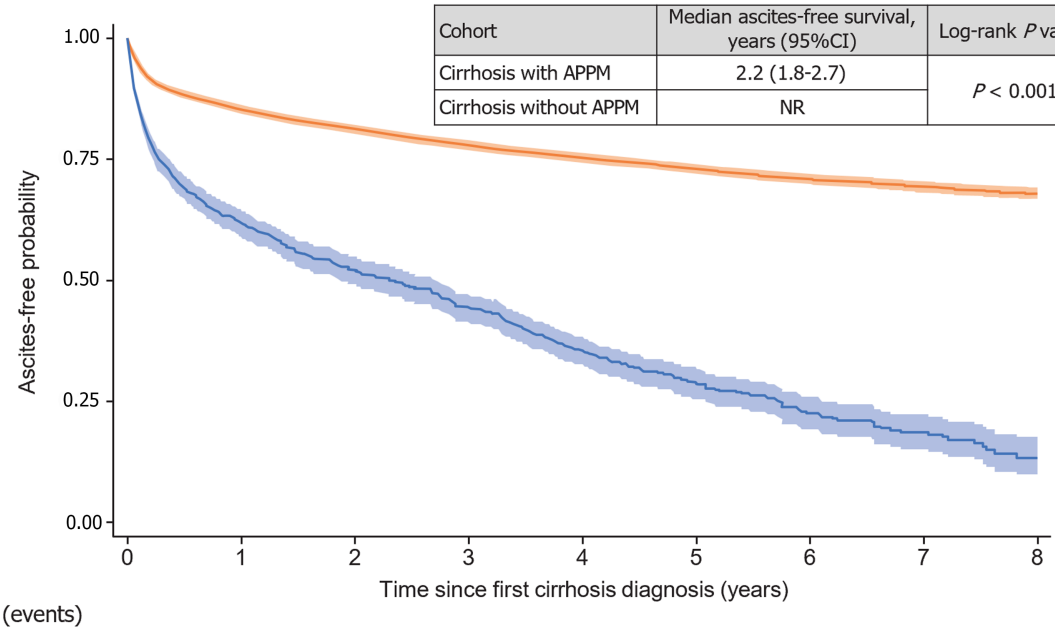
Figure 2 Disease progression events. Disease progression events that occurred after the index date until the end of the study. APPM: Alterations in plasma protein metabolism.

established indicators of poor prognosis[1]. Previous studies have demonstrated that patients with cirrhosis and a heterozygous Pi mutation, PiMZ, in *SERPINA1* decompensate faster than patients with cirrhosis but without AATD[16,17]. However, only 2.1% of patients in the APPM cohort underwent AAT phenotyping in our study, and therefore we were unable to further evaluate the association between decompensation and AATD genotype. The median age of patients in the APPM cohort was 2 years younger than in the control cohort, yet the APPM cohort had a higher risk of liver disease-related clinical events. This supports that patients with APPM are at a higher risk of liver disease-related clinical events than patients without APPM irrespective of age.

As AATD is also associated with the development of respiratory system comorbidities such as chronic obstructive pulmonary disease[18], it was anticipated that the APPM cohort would have a higher incidence of such comorbidities compared with the control cohort. However, we observed a similar incidence in the APPM and control cohorts (30.9%

A

Cohort	Median ascites-free survival, years (95%CI)	Log-rank *P* value
Cirrhosis with APPM	2.2 (1.8-2.7)	*P* < 0.001
Cirrhosis without APPM	NR	



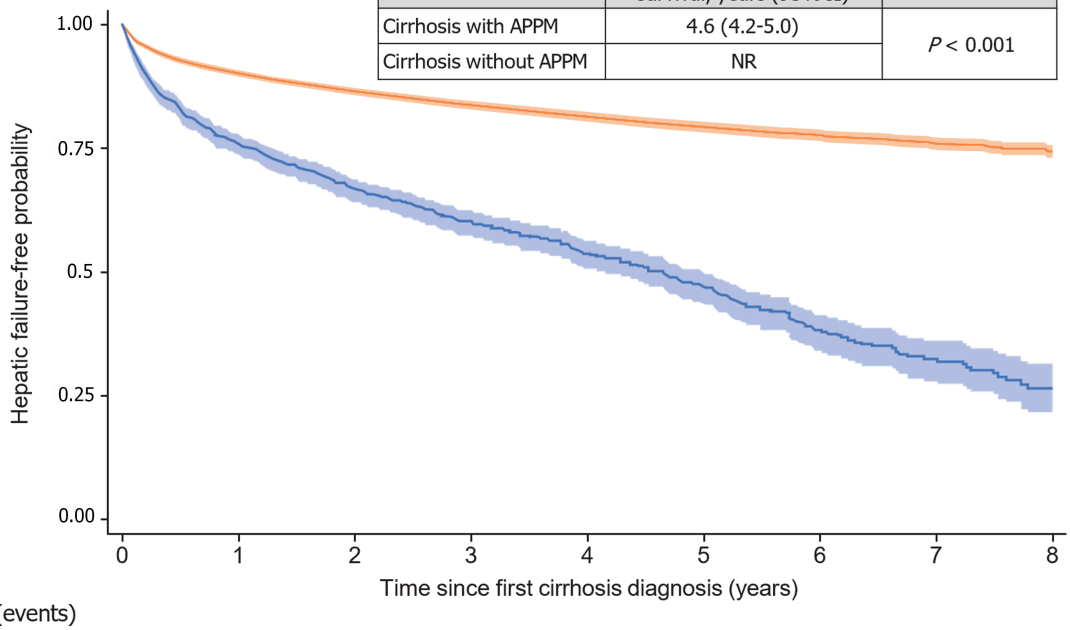
Number at risk (events)

Cirrhosis with APPM	1556	(544)	740	(112)	524	(71)	364	(66)	234	(40)	144	(26)	74	(11)	36	(8)	12
Cirrhosis without APPM	19318	(2566)	12229	(530)	9359	(343)	7175	(230)	5377	(146)	3834	(92)	2575	(48)	1462	(20)	535

95%CI Cirrhosis with APPM 95%CI Cirrhosis without APPM

B

Cohort	Median hepatic failure-free survival, years (95%CI)	Log-rank *P* value
Cirrhosis with APPM	4.6 (4.2-5.0)	*P* < 0.001
Cirrhosis without APPM	NR	



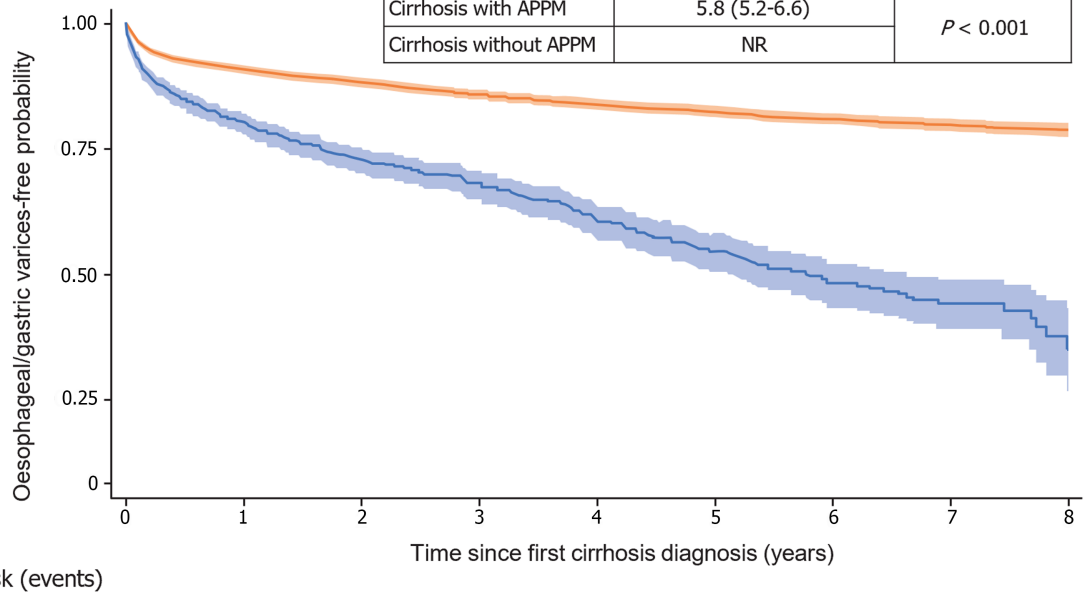
Number at risk (events)

Cirrhosis with APPM	2253	(460)	1160	(124)	806	(70)	558	(53)	382	(40)	243	(38)	135	(16)	63	(10)	25
Cirrhosis without APPM	22915	(1908)	14289	(523)	10774	(321)	8174	(196)	6080	(129)	4315	(87)	2873	(46)	1637	(22)	595

95%CI Cirrhosis with APPM 95%CI Cirrhosis without APPM

C

Cohort	Median oesophageal/gastric varices-free survival, years (95%CI)	Log-rank *P* value
Cirrhosis with APPM	5.8 (5.2-6.6)	*P* < 0.001
Cirrhosis without APPM	NR	



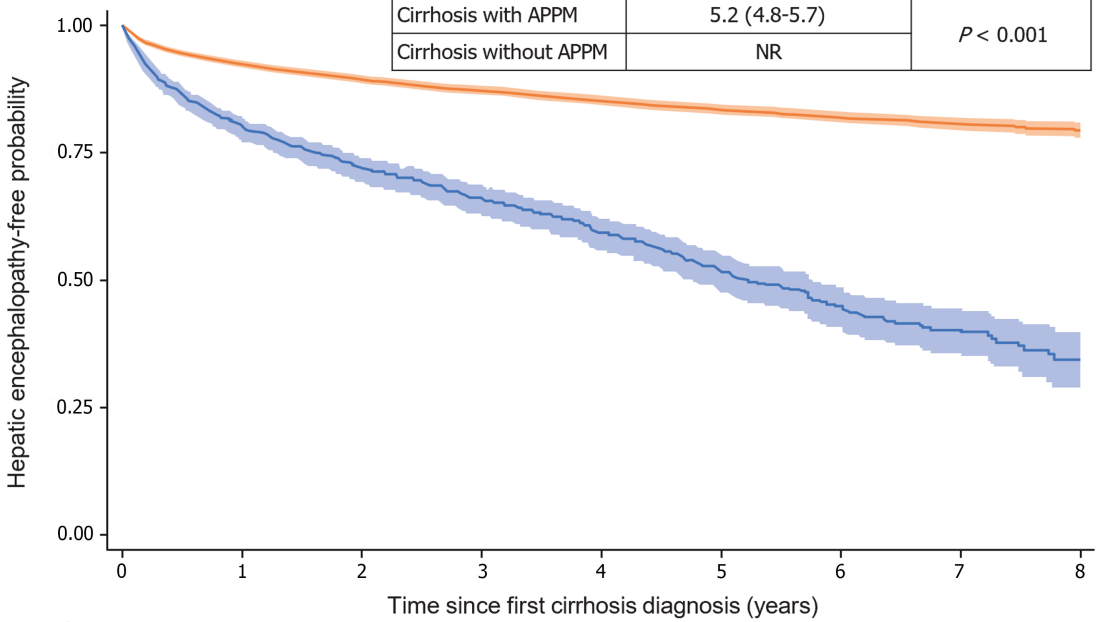
Number at risk (events)

Cirrhosis with APPM	2218	(359)	1065	(86)	717	(42)	496	(45)	332	(29)	213	(20)	121	(7)	50	(5)	14
Cirrhosis without APPM	22620	(1758)	13576	(340)	10255	(261)	7767	(161)	5808	(86)	4142	(61)	2774	(33)	1568	(14)	574

95%CI — Cirrhosis with APPM; 95%CI — Cirrhosis without APPM

D

Cohort	Median hepatic encephalopathy-free survival, years (95%CI)	Log-rank *P* value
Cirrhosis with APPM	5.2 (4.8-5.7)	*P* < 0.001
Cirrhosis without APPM	NR	



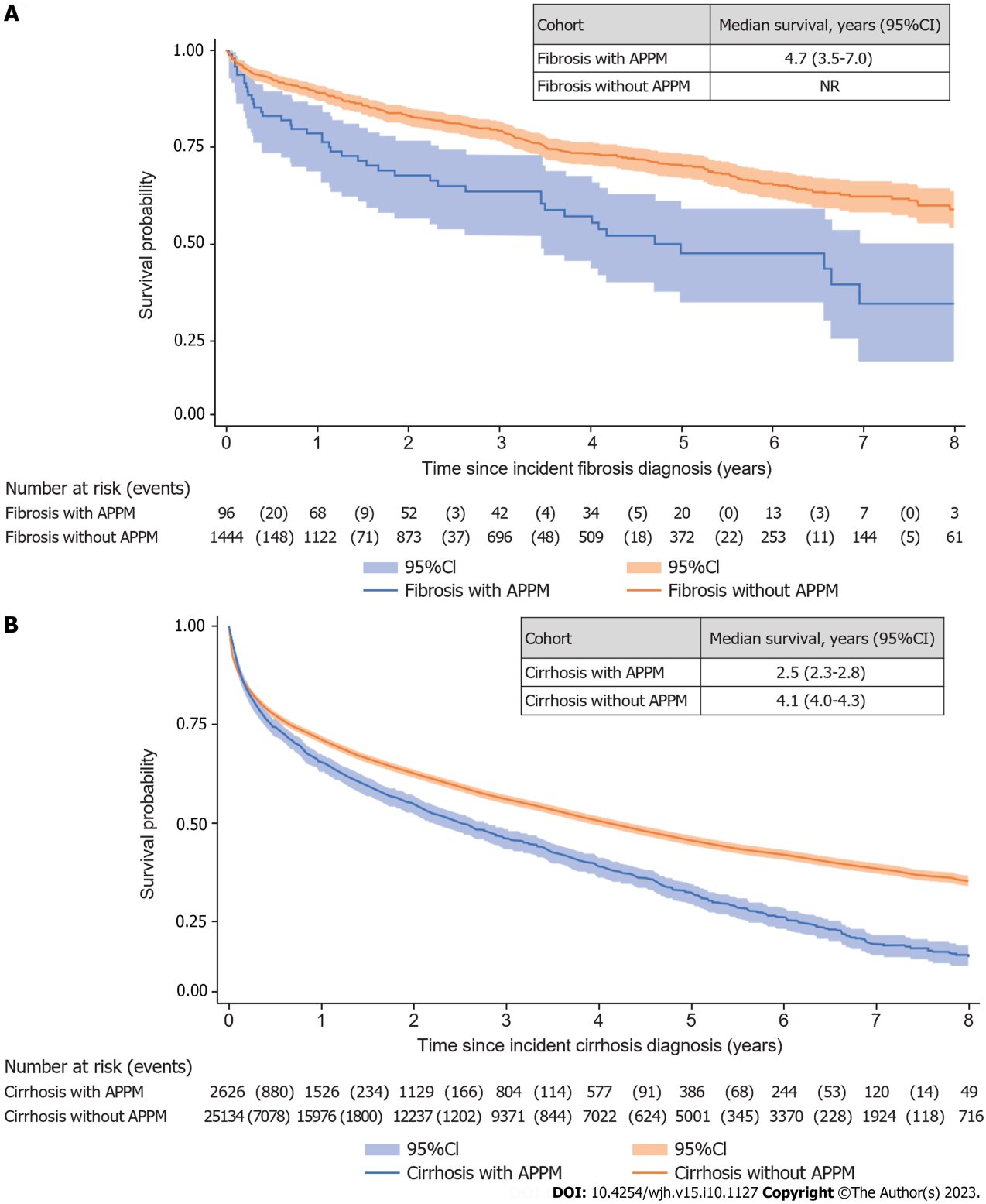
Number at risk (events)

Cirrhosis with APPM	2341	(383)	1221	(112)	853	(61)	596	(53)	412	(44)	259	(28)	152	(13)	71	(9)	28
Cirrhosis without APPM	23466	(1512)	14630	(416)	11052	(249)	8404	(177)	6245	(103)	4445	(72)	2968	(35)	1686	(17)	613

95%CI — Cirrhosis with APPM; 95%CI — Cirrhosis without APPM

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Figure 3 Event-free survival for disease progression events in patients with cirrhosis with and without alterations in plasma protein metabolism. A: Ascites; B: Hepatic failure; C: Esophageal and gastric varices; D: Hepatic encephalopathy. APPM: Alterations in plasma protein metabolism; CI: Confidence interval; NR: Not reached.



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Figure 4 Survival in patients with fibrosis and cirrhosis. A: Fibrosis; B: Cirrhosis. APPM: Alterations in plasma protein metabolism; CI: Confidence interval; NR: Not reached.

and 31.7%), which might indicate that the APPM cohort included a substantial number of patients without AATD. The E88.0 code we used to identify patients with APPM cannot be equated with AATD as the code includes a broad range of acquired and inherited APPM disorders, such as plasminogen deficiency. The European Commission Expert Group on Rare Diseases currently recommends the Orphanet nomenclature of rare diseases (ORPHA) codes to identify rare disorders[19]. The adoption of ORPHA codes is expected to facilitate the transition to ICD-11 codes, which include an expanded set of rare disorder codes compared with ICD-10[19]. In addition, developments of the ICD coding system, such as the addition of the E88.0A code for AATD, could improve the identification of patients with AATD in future administrative insurance claims analyses. In a recent registry-based cohort study of the prevalence, incidence and

mortality associated with AATD in Denmark using the E88.0A code, a sensitivity analysis demonstrated a predominance of AATD in the E88.0 category for APPM and a near complete shift to the more specific E88.0A code for AATD between 2000 and 2018[20]. The adoption of diagnosis codes specific to patients with AATD may facilitate earlier diagnosis and improved patient management, which may, in turn, contribute to slowing disease progression and decreasing the burden of disease in these patients with a rare, chronic disease.

The limitations of this study are typical of those seen in other insurance claims-based analyses. As noted previously, we were unable to determine the proportion of patients included in the study who had AATD due to the limitations of the ICD-10-GM coding system. In addition, as AATD is a highly underdiagnosed disease[21], we cannot exclude the possibility that some cases may have been included in the control cohort. The general lack of laboratory test results, direct clinical measures and biomarkers in the database confounded our ability to analyze the AATD genotype distribution. Only 96 patients in the APPM cohort and 1444 in the control cohort were recorded as having fibrosis, which was lower than anticipated, likely owing to underdiagnosis (patients are often asymptomatic in the early stages of fibrosis) and/or underreporting[22]. Furthermore, a substantial number of patients with fibrosis developed cirrhosis and liver decompensation events, which suggests that these patients were more likely to be at an advanced stage of fibrosis. Therefore, the development and implementation of structured early screening programs may be useful to increase the early detection of fibrosis in the general population[23]. Fibrosis stage data were not available in the database, which together with the low number of patients in this category constitutes a significant limitation as survival prognosis is thought to be highly linked to fibrosis stage[24]. In addition, regional differences in morbidity and mortality may exist, and our data may not be representative of geographic regions outside of Germany. However, in Germany, approximately 73.3 million people were insured by an SHI fund in 2020, which equates to approximately 88% of the general population. Owing to the uniform structure of SHI funds in all regions of Germany, we consider the data to be representative of the German population[25]. Lastly, as this was a retrospective insurance claims-based study that collected data using ICD-10-GM codes, we were unable to assess body weight/body mass index, which are known risk factors for early progression to advanced liver disease[26], and unable to determine the exact procedures involved in the diagnosis of liver disease.

CONCLUSION

Among patients with liver disease in Germany, those with APPM experience substantial burden and a higher rate of liver disease progression than patients without APPM. To enable accurate diagnosis and inform disease management, it is important to have specific diagnostic codes that differentiate between genetic liver disease and liver manifestations from other causes.

ARTICLE HIGHLIGHTS

Research background

Alpha-1 antitrypsin deficiency (AATD) is a rare genetic disease that can result in the development of liver and/or lung disease, and is a leading cause of inherited alterations in plasma protein metabolism (APPM).

Research motivation

Currently, there is a lack of information on the natural history and epidemiology of AATD.

Research objectives

To understand the prevalence, burden and progression of liver disease in patients with APPM, which includes patients diagnosed with AATD, in Germany.

Research methods

A retrospective analysis of anonymized, patient-level, insurance claims data from a German health insurance provider (AOK PLUS) was conducted. The APPM cohort comprised patients with APPM (01/01/2010-30/09/2020) and incident liver disease (01/01/2012-30/09/2020) and the control cohort comprised patients without APPM but with incident liver disease. Outcomes were incidence/prevalence of liver disease in patients with APPM, demographics/baseline characteristics, disease progression, progression-free survival, mortality, and diagnostic procedures.

Research results

Overall, 2680 and 26299 patients were included in the APPM [fibrosis (96); cirrhosis (2584)] and control [fibrosis (1444); cirrhosis (24855)] cohorts, respectively. The annual incidence and prevalence of APPM and liver disease was 10-15/100000 and 36-51/100000, respectively. Median survival was shorter in the APPM cohort (2.6 years) than in the control cohort (4.3 years). In patients in the APPM cohort with fibrosis and cirrhosis, respectively, median survival was 4.7 years and 2.5 years. More patients in the APPM cohort (92.0%) experienced liver disease progression than in the control cohort (67.2%). Median progression-free survival was shorter in the APPM cohort [0.9 years (95%CI: 0.7-1.1)] compared with the control cohort [3.7 years (95%CI: 3.6-3.8); $P < 0.001$]. In patients with cirrhosis, event-free survival for ascites, hepatic encephalopathy, hepatic failure, and esophageal/gastric varices was longer in the control cohort than in the APPM cohort

($P < 0.001$). In patients with fibrosis, event-free survival for ascites, cirrhosis, hepatic failure, and esophageal/gastric varices was longer in the control cohort than in the APPM cohort ($P < 0.001$). The most common diagnostic procedures within 12 mo after the first diagnosis of liver disease in the APPM cohort were imaging procedures (66.3%) and laboratory tests (51.0%).

Research conclusions

In Germany, patients with APPM and liver disease experience substantial burden and a higher rate of and earlier liver disease progression than patients without APPM.

Research perspectives

The adoption of diagnosis codes specific to AATD should enable differentiation of this disease from other APPM disorders and facilitate earlier diagnosis and patient management. This should contribute to slowing disease progression and decreasing the burden of disease in patients with this rare, chronic disease.

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FOOTNOTES

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Data sharing statement: The data that support the findings of this study are available from AOK PLUS. Restrictions apply to the availability of these data, which were used under license for this study.

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ORIGINAL ARTICLE

Observational Study

Prevalence and risk factors of lymphatic dysfunction in cirrhosis patients with refractory ascites: An often unconsidered mechanism

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Abstract

BACKGROUND

The lymphatic system is crucial in maintaining the body fluid homeostasis. A dysfunctional lymphatic system may contribute to the refractoriness of ascites and edema in cirrhosis patients. Therefore, assessment of lymphatic dysfunction in cirrhosis patients with refractory ascites (RA) can be crucial as it would call for using different strategies for fluid mobilization.

AIM

To assessing the magnitude, spectrum, and clinical associations of lymphatic dysfunction in liver cirrhosis patients with RA.

METHODS

This observational study included 155 consecutive cirrhosis patients with RA. The presence of clinical signs of lymphedema, such as peau d'orange appearance and positive Stemmer sign, intestinal lymphangiectasia (IL) on duodenal biopsy seen as dilated vessels in the lamina propria with strong D2-40 immunohistochemistry, and chylous ascites were used to diagnose the overt lymphatic dysfunctions.

RESULTS

A total of 69 (44.5%) patients out of 155 had evidence of lymphatic dysfunction.

Peripheral lymphedema, found in 52 (33.5%) patients, was the most common manifestation, followed by IL in 42 (27.0%) patients, and chylous ascites in 2 (1.9%) patients. Compared to patients without lymphedema, those with lymphedema had higher mean age, median model for end-stage liver disease scores, mean body mass index, mean ascitic fluid triglyceride levels, and proportion of patients with hypoproteinemia (serum total protein < 5 g/dL) and lymphocytopenia (< 15% of total leukocyte count). Patients with IL also had a higher prevalence of lymphocytopenia and hypoproteinemia (28.6% *vs.* 9.1%, $P = 0.004$). Seven (13%) patients with lymphedema had lower limb cellulitis compared to none in those without it. On multivariate regression analysis, factors independently associated with lymphatic dysfunction included obesity [odds ratio (OR): 4.2, 95% confidence intervals (95%CI): 1.1–15.2, $P = 0.027$], lymphocytopenia [OR: 6.2, 95%CI: 2.9–13.2, $P < 0.001$], and hypoproteinemia [OR: 3.7, 95%CI: 1.5–8.82, $P = 0.003$].

CONCLUSION

Lymphatic dysfunction is common in cirrhosis patients with RA. Significant indicators of its presence include hypoproteinemia and lymphocytopenia, which are likely due to the loss of lymphatic fluid from the circulation. Future efforts to mobilize fluid in these patients should focus on methods to improve lymphatic drainage.

Key Words: Cirrhosis; Lymphedema; Lymphangicetasia; Refractory ascites; Chylous ascites; Lymphocytopenia

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Core Tip: Lymphatic dysfunction is often underappreciated in advanced cirrhosis patients. Our study evaluated the magnitude, spectrum, and associations of lymphatic dysfunction in cirrhosis patients with refractory ascites (RA). Nearly half (44.5%) of the studied population ($n = 155$) revealed evidence of overt lymphatic dysfunction in the forms of peripheral lymphedema (33.5%), intestinal lymphangiectasia (27.0%), and chylous ascites (1.9%). Obesity, hypoproteinemia, and lymphocytopenia were independently associated with lymphatic dysfunction in said patients. From a therapeutic standpoint, it can be extremely important to evaluate lymphatic dysfunction in cirrhosis patients with RA since it would call for using different strategies for fluid mobilization.

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INTRODUCTION

The lymphatic system is crucial in maintaining the body fluid homeostasis[1]. By recirculating surplus tissue fluid back into the bloodstream, the lymphatic system prevents tissues from becoming edematous. In patients with cirrhosis and portal hypertension (PHT), the production of lymph from the liver and intestines is significantly increased[2,3]. Increased production of lymph promotes lymphangiogenesis, which in turn tends to improve the functional capacity of the lymphatics. However, as cirrhosis progresses, these compensatory mechanisms become overwhelmed, leading to the development of ascites and edema[2-4]. Subsequently, functional impairment of the lymphatic system also sets in, resulting in further worsening of the fluid accumulation[4,5]. The consequent lymphatic flow stagnation and leakage lay the ground for lymphedema development, which is the deposition of protein-rich lymph fluid within the tissues[6]. The gut lymphatics are important for maintaining the abdominal fluid balance. Studies have shown that patients with cirrhosis have much higher abdominal lymph production (up to 30-fold) and lymph flow in the thoracic duct (8-9 L/day) [7,8]. Moreover, a persistently high lymphatic pressure associated with PHT has the potential to cause intestinal lymphangiectasia (IL) and chylous ascites (CA) in cirrhosis patients[9-11]. Rupture of IL with subsequent loss of lymph can result in hypoproteinemia, lymphocytopenia, and malabsorption of fat[4,12].

Lymphatic dysfunction occurs in patients with cirrhosis, although the amount of published data on this subject is extremely limited[2-5]. There have been only a few reports of CA and IL in these patients[10,11]. Lymphedema has not even been considered in cirrhosis patients with persistent peripheral edema. The structural and functional changes in gut lymphatics, which are vital in splanchnic lymph drainage, remain an unexplored area in cirrhosis patients. Despite being a significant factor in maintaining fluid homeostasis, the lymphatic system is commonly ignored when assessing the pathophysiology of refractory ascites (RA) in cirrhosis patients. RA, which represents an extreme form of fluid accumulation, eventually develops in about 10% of cirrhosis patients with advanced decompensation[13].

Over the past two decades, a better understanding of the lymphatic vascular system has emerged; however, little is known about how lymphatic dysfunction contributes to the pathophysiology of advanced cirrhosis. Given the significant role the lymphatic system plays in maintaining the balance of body fluids, it is reasonable to assume that a dysfunctional lymphatic system may contribute to the refractoriness of ascites and edema in cirrhosis patients. Therefore, assessing

lymphatic dysfunction in such patients can provide a novel approach to tissue decongestion. Case studies have shown dietary changes to be effective in controlling ascites and improving liver stiffness in cirrhosis patients with IL[10,14].

In a recent study, treatment with recombinant vascular endothelial growth factor (VEGF) C, a lymphatic-specific growth factor, resulted in decreased portal pressure, enhanced lymphatic drainage, and decreased ascites in cirrhotic rats [15]. However, techniques for assessing lymphatic structure and function in cirrhosis are limited. Lymphography and lymphoscintigraphy lack sufficient accuracy and are not readily available. Lymphedema, IL, and CA are significant surrogate markers of lymphatic dysfunction that need to be investigated in patients with cirrhosis. Hence, this study assessed the magnitude, spectrum, and clinical associations of lymphatic dysfunction in patients with liver cirrhosis and RA using surrogate markers.

MATERIALS AND METHODS

This observational study was conducted in the Department of Gastroenterology, All India Institute of Medical Sciences, Patna, a tertiary care medical center in India. The protocol was approved by the institute's research board, and investigations were conducted according to the Declaration of Helsinki principles. Consecutive adult liver cirrhosis patients between 18 years and 75 years admitted with RA from December 2021 to March 2023 were screened according to the inclusion criteria. RA was diagnosed as ascites that could not be mobilized or the early recurrence of which could not be prevented because of a lack of response to a maximum dose of diuretic treatment or because patients developed complications that precluded the use of an effective dose of diuretics[16]. Liver cirrhosis was diagnosed by clinical features, imaging characteristics, and endoscopic findings. Patients with low serum ascites albumin gradient (< 1.1) ascites, congestive heart failure, primary or metastatic abdominal malignancy, history of radiation therapy, concomitant tuberculosis, history of abdominal surgery, and filariasis were excluded from the study. Cirrhosis patients with severe sepsis, advanced hepatic encephalopathy, and respiratory failure were also excluded from the study.

Demographic and clinical data, including the degree of ascites and duration of RA, were noted at baseline. Estimates of dry weight were made for the corrected body mass index calculation by deducting 15% of the actual weight due to grade-3 ascites and an additional 5% because of peripheral edema. Per the Asian standard, body mass index > 25 kg/m^2 was considered obese. Routine investigations, including hemograms, liver function tests, kidney function tests, international normalized ratio, fasting blood sugar, and ascitic fluid analysis, including ascitic fluid triglyceride estimation, were performed in all patients, who also underwent a complete etiological workup. The severity of cirrhosis was assessed by Child-Pugh classification and model for end-stage liver disease (MELD) scores. Standard medical therapy, including etiology-specific treatment, was given to all patients.

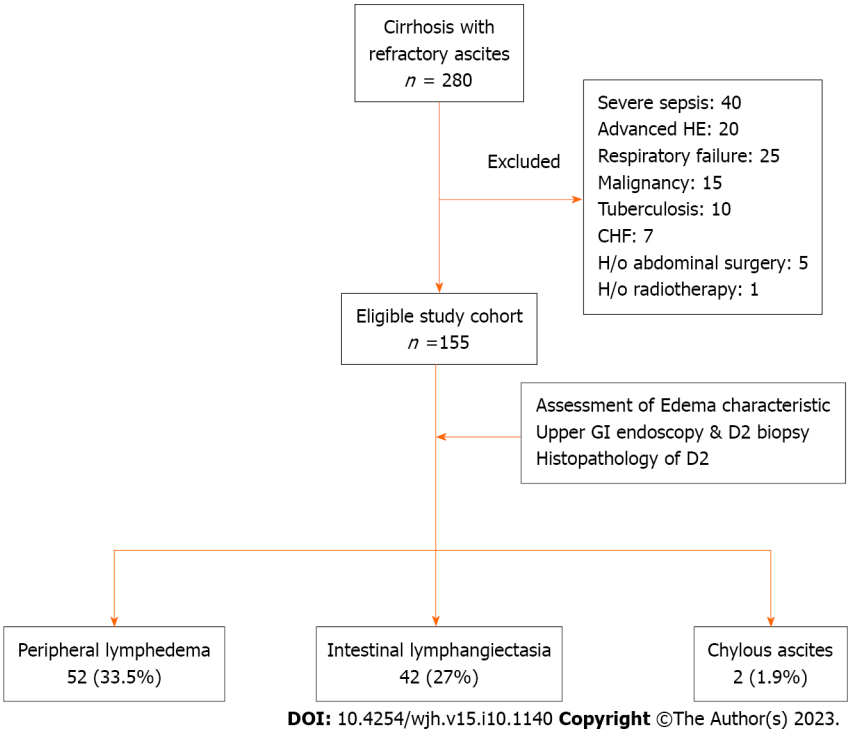
Assessment of lymphatic dysfunction

The lymphatic dysfunction was ascertained by the presence of one or more of the following surrogate markers: (1) Features of peripheral lymphedema, as evident by physical characteristics such as painless leg edema, pitting or non-pitting, showing orange peel (peau d'orange) appearance, and positive Stemmer sign; (2) Presence of IL on endoscopy and/or histopathological examination of duodenal biopsy specimens; and (3) Presence of CA, as indicated by milky white ascites with elevated triglyceride level > 110 mg/dL.

The diagnosis of IL on endoscopy was based on the presence of swollen mucosa with scattered white spots suggestive of dilated lacteals. Portal hypertensive duodenopathy (PHD) was considered in the presence of swollen duodenal mucosa with varying degrees of erythema, erosions, friability, and telangiectasia. Irrespective of endoscopic evidence of PHD, biopsies were obtained from the second part of the duodenum (D2), distal to the ampulla of Vater, using standard endoscopic biopsy forceps in all patients. The biopsies obtained were submitted in a vial containing diluted formalin for fixation. Histological examination (hematoxylin and eosin) was performed by an expert pathologist. A markedly dilated vessel in the lamina propria, which on immunohistochemistry showed strong D2-40 positivity, confirmed the presence of IL. Hypoproteinemia was considered when total serum protein was < 5 g/dL, with a decrease in both albumin and globulin. Lymphocytopenia was considered when the proportion of blood lymphocytes was < 15% of the total leukocyte count.

Statistical analysis

Because the magnitude and impact of lymphatic dysfunction in cirrhosis are not clearly defined, we conducted this observational study as an exploratory research project with a cross-sectional design intended to include a minimum of 100 eligible patients. Continuous variables, depending on the normalcy of distribution, were expressed as mean ± standard deviation or median (range). Categorical data were represented as proportion. To compare the normal covariates between patients with and without lymphatic dysfunction, independent sample *t*-test or Mann-Whitney *U* test was used when applicable. Comparisons in categorical variables were performed using χ^2 or Fisher's test. Multivariate regression analysis (MVA) was used to determine independent associates of lymphatic dysfunction. The relevant variables in the univariate analysis with $P < 0.10$ were considered for MVA. However, highly correlated variables were excluded to avoid multicollinearity in regression analysis. The odds ratio (OR) and 95% confidence interval (95%CI) for all significant variables in MVA were reported. Data were analyzed using SPSS software version 23.0 (SPSS, Chicago, IL, United States), wherein $P < 0.05$ was taken as significant.



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Figure 1 Flow-diagram showing selection, evaluation, and categorization of study population based on lymphatic dysfunction. A total of 280 cirrhosis patients with refractory ascites were screened, and eventually 155 patients were found to be eligible based on the inclusion and exclusion criteria. Subsequent to evaluation, 69 (44.5%) patients were found to have evidence of lymphatic dysfunction in the form of lymphedema, intestinal lymphangiectasia, and chylous ascites, either alone or in combination. CHF: Congestive heart failure; D2: Second part of duodenum; GI: Gastrointestinal; HE: Hepatic encephalopathy; H/o: History of.

RESULTS

A total of 280 cirrhosis patients with RA were screened during the study, but 125 of them were found to be ineligible based on the inclusion and exclusion criteria (Figure 1). The study cohort ultimately consisted of 155 cirrhosis patients with RA.

Cohort characteristics

The mean age of the patients was 49.0 ± 13.1 years with a predominance of male subjects (77.0%). The predominant etiology of cirrhosis involved alcohol (37.4%), followed by non-alcoholic steatohepatitis (35.4%). The median Child-Pugh and MELD scores were 10.3 and 18.1, respectively. Twenty-seven (17.4%) patients were diabetic, 16 (10.3%) patients were obese, and 9 (5.8%) patients had functional chronic kidney disease. All patients had grade-3 ascites, and the median duration of RA was 5 (3-48) mo. On endoscopy, large esophageal varices were found in 60 (38.0%) patients, and severe portal hypertensive gastropathy (PHG) was noted in 35 (22.6%) patients. Additionally, 31 (20.0%) patients had evidence of PHD. The presence of PHD was independent of the severity of PHG as 70.0% of patients with PHD had mild PHG. Other baseline characteristics are specified in Table 1.

Evaluation for lymphatic dysfunction

Edema characteristics: The physical appearance of peripheral edema revealed two distinct patterns (Figure 2). In 52 (33.5%) patients, edema was severe, pitting or non-pitting, with skin texture showing exaggerated dorsal skin creases, hyperkeratosis, and peau d'orange appearance. Moreover, these patients had a positive Stemmer sign, suggesting the presence of lymphedema. In the remaining 103 (66.5%) patients, edema was of the pitting type, skin texture was smooth, and the Stemmer sign was negative.

Endoscopic and histopathological evidence of IL: All patients tolerated the endoscopic procedure with D2 biopsy irrespective of coagulopathy and thrombocytopenia. Eight (5.1%) patients had endoscopic evidence of IL as a whitish enlarged villi on swollen mucosa (Figure 3). On histopathological examination of D2 biopsy specimens, 42 (27.0%) patients revealed markedly dilated vessels in the lamina propria, which on immunohistochemistry with D2-40 confirmed the presence of IL (Figure 4). Thus, 34 (22%) patients revealed IL on histopathological examination without endoscopic evidence of the same.

CA: The median level of triglyceride in ascitic fluid was 19.5 (0–224.0) mg/dL. Based on a physical examination of ascetic fluid and triglyceride levels, 2 patients (1.29%) were found to have CA.

Table 1 Clinical and laboratory characteristics of study population at baseline, *n* (%)

Parameters	*n* = 155
Age in yr, mean ± SD	49.0 ± 13.1
Male	119 (77.0)
Etiology of cirrhosis	
Alcohol	58 (37.4)
Hepatitis B	23 (14.8)
Hepatitis C	7 (4.5)
NASH	55 (35.4)
Others	14 (9.0)
Duration of RA in mo, median (range)	5 (-48)
MELD scores, median (range)	18.1 ± 8.6, 17(6-42)
Child-Pugh score, mean ± SD	10.3 ± 1.8
Diabetes mellitus	27 (17.4)
Hypertension	12 (7.7)
Corrected body mass index in kg/m^2, mean ± SD	20.25 ± 3.40
Obesity	16 (10.3)
Chronic kidney disease	9 (5.8)
Serum total bilirubin in mg/dL, median (range)	2.7 (0.3-28.5)
Serum AST, median (range) U/L	60 (18-672)
Serum ALT, median (range) U/L	32 (4-250)
Serum total protein, mean ± SD mg/dL	5.8 ± 0.7
Serum albumin, mean ± SD mg/dL	2.5 ± 0.5
Hypoproteinemia	42 (27.0%)
INR, median (range)	1.6 (0.4-6.7)
Serum sodium, mean ± SD meq/L	130.0 ± 6.5
Serum potassium, mean ± SD meq/L	4.07 ± 2.40
Serum urea in mg/dL, median (range)	34 (12-191)
Creatinine in mg/dL, median (range)	0.9 (0.3-3.7)
Hemoglobin in g/dL, mean ± SD	8.8 (4.2-65.0)
Total leukocyte count as /cmm, median (range)	6130 (1750-34300)
Lymphocytopenia	72 (46.5)
Esophageal varices	
None	3 (1.9)
Small grade	92 (59.4)
Large grade	60 (38.0)
PHG	
Mild	118 (76.0)
Severe	35 (22.6)
PHD	31 (20.0)
Ascitic fluid analysis	
Cell count as /μL, median (range)	100 (0-11200)
Protein median in gm/dL, median (range)	1.1 (0.1-3.5)

ADA in U/L, median (range)	10 (1-33)
Triglycerides in mg/dL, median (range)	19.5 (0-224.0)
SAAG, median (range)	2.0 (1.2-4.0)

ADA: Adenosine deaminase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; cmm: Cubic millimeter; INR: International normalized ratio; MELD: Model for end-stage liver disease; NASH: Non-alcoholic steatohepatitis; PHG: Portal hypertensive gastropathy; PHD: Portal hypertensive duodenopathy; RA: Refractory ascites; SAAG: Serum ascites albumin gradient.



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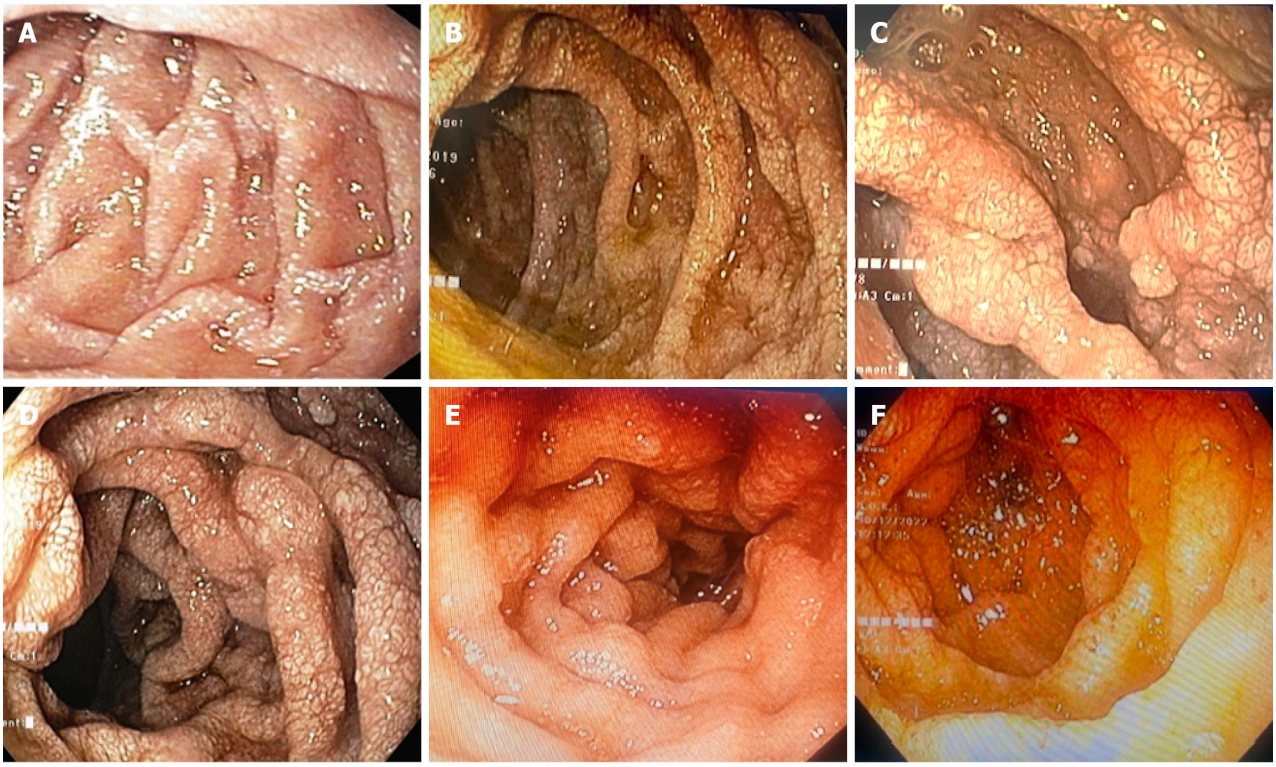
Figure 2 Representative images showing two distinct patterns of peripheral edema among cirrhosis patients with refractory ascites. A and B: Physical appearance of peripheral edema revealed two distinct patterns. In the majority of patients, edema was pitting type and skin texture was smooth (A), which is a finding consistent with edema due to the hydrostatic-oncotic pressure imbalance. In about one-third of patients, edema was severe, mostly non-pitting type with skin texture showing exaggerated dorsal skin creases, hyperkeratosis, and peau d'orange appearance (B), suggesting lymphedema. Some patients with lymphedema also had evidence of cellulitis.

Thus, a total of 69 (44.5%) cirrhosis patients with RA had evidence of lymphatic dysfunction in the forms of lymphedema, IL, and CA either alone or in combination. Lymphedema, present in 52 (33.5%) patients, was the most common manifestation of lymphatic dysfunction. Twenty-six patients had only lymphedema, 18 patients had only IL, 24 patients had both lymphedema and IL, and 2 patients had lymphedema as well as IL and CA.

Clinical characteristics and association of lymphatic dysfunction

Clinically, lymphedema was the most relevant marker of lymphatic dysfunction in the study population. Compared to patients without lymphedema, those with lymphedema were older (mean age of 53.3 years *vs* 47.7 years, $P = 0.007$), had more severe liver disease (median MELD scores of 21 *vs* 14, $P < 0.001$), and had a greater proportion of obese cirrhosis (21.0% *vs* 5.8%, $P = 0.012$). Patients with lymphedema had a higher prevalence of PHD (32.7% *vs* 13.6%, $P = 0.001$) and histopathological evidence of IL (46.2% *vs* 17.5%, $P < 0.001$) than those without it. Furthermore, the mean ascitic fluid triglyceride levels ($P = 0.006$), the proportion of lymphocytopenia (73.0% *vs* 33.0%, $P < 0.001$), and hypoproteinemia (50.0% *vs* 15.5%, $P < 0.001$) were significantly higher in patients with lymphedema than those without it (Supplementary Table 1). Lower limb cellulitis was noted in 7 (13.0%) patients with lymphedema *vs* none in patients where lymphedema was absent.

Patients with IL had a higher prevalence of lymphedema than those without IL (57.1% *vs* 23.6%, $P < 0.001$). The frequency of lymphocytopenia and hypoproteinemia was higher in patients with IL than those without it (28.6% *vs* 9.1%, $P = 0.004$). However, IL was not associated with age, severity of liver cirrhosis, or metabolic comorbidities. With regard to the only 2 patients with CA, the levels of triglyceride in their ascitic fluid were observed at 137 mg/dL and 224 mg/dL. Both patients were male, diabetic, and had high Child-Pugh scores (13 and 12). Both had evidence of IL on endoscopy and D2 biopsy.



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Figure 3 Endoscopic evaluation of study population. A-F: Representative endoscopic images of duodenum of study population showing portal hypertensive duodenopathy with evidence of intestinal lymphangiectasia visualized as scattered white spots suggestive of dilated lacteals.

Comparison of clinical and laboratory characteristics of patients with and without lymphatic dysfunctions

The mean age (52.0 ± 13.8 *vs* 47.8 ± 12.3, $P < 0.04$) and proportion of obese cirrhosis (16.0% *vs* 5.8%) were higher in patients with lymphatic dysfunction than those without it. The median duration of RA (6 mo *vs* 4 mo, $P = 0.02$), median MELD score (18 *vs* 14, $P = 0.003$), and mean Child-Pugh score (10.0 ± 1.9 *vs* 9.9±1.6) were similarly higher in patients with lymphatic dysfunction (Table 2). Among the patients with lymphatic dysfunction, lymphopenia was noted in 49 (79.0%) patients, hypoproteinemia in 29 (42.0%) patients, and a combined hypoproteinemia plus lymphopenia in 21 (30.0%) patients. The values of serum bilirubin, serum aspartate aminotransferase, serum alanine aminotransferase, and international normalized ratio were higher, while serum sodium was lower in patients with lymphatic dysfunction compared to those without it.

Independent associations of lymphatic dysfunction

On MVA (Table 3), factors independently associated with lymphatic dysfunction included obesity (OR: 4.2, 95%CI: 1.1–15.2, $P = 0.027$), lymphocytopenia (OR: 6.2, 95%CI: 2.9–13.2, $P < 0.001$), and hypoproteinemia (OR: 3.7, 95%CI: 1.5–8.8, $P = 0.003$). When independent predictors of only lymphedema were assessed by MVA, age (OR: 1.06, $P = 0.002$) and Child-Pugh scores (OR: 1.82, $P = 0.005$) were found to be significant, apart from obesity (OR: 6.3, $P = 0.012$), lymphocytopenia (OR: 3.5, $P = 0.01$), and hypoproteinemia (OR: 7.1, $P = 0.001$) (Table 4).

DISCUSSION

Our study was the first to assess the characteristics of lymphatic dysfunction in liver cirrhosis patients who have RA, an extreme form of fluid accumulation. We found evidence of lymphatic dysfunction in nearly half (44.5%) of the patients. The spectrum of dysfunctions included peripheral lymphedema (33.5%), IL (27.0%), and CA (1.3%). Obesity, lymphocytopenia, and hypoproteinemia independently predicted the presence of lymphatic dysfunction in such patients. Additionally, higher mean ages and Child-Pugh scores were independently associated with peripheral lymphedema, the most common manifestation of lymphatic dysfunctions.

Portal pressure in cirrhosis patients positively correlated with lymphatic flow[17,18]. As cirrhosis progresses, functional deficiencies in the lymphatic system emerge, causing flow stagnation and leakage of lymph from the ectatic lymphatic system[4,5]. These changes lay the ground for the lymphedema development. Our study detected evidence of lymphedema in one-third of cirrhosis patients with RA. Lymphedema should be common in patients with advanced cirrhosis, given the lymphatic failure that often follows cirrhosis, yet there is a dearth of research on it in the existing literature. It is difficult to distinguish early lymphedema from edema due to the change in plasma hydrostatic-oncotic pressure balance. The presence of physical signs, such as a peau d'orange appearance and the positive Stemmer sign, may

Table 2 Comparison of clinical and laboratory characteristics of patients with and without clinically evident lymphatic dysfunction (Univariate analysis)

Parameters	LD present, *n* = 69	LD absent, *n* = 86	*P* value
Age in yr, mean ± SD	52.0 ± 13.8	47.8 ± 12.3	0.040
Male, *n* (%)	56 (81.2%)	63 (73.3%)	0.320
Etiology of cirrhosis, *n* (%)			0.050
Alcohol	22 (31.8%)	36 (41.8%)	
Hepatitis	14 (20.2%)	9 (10.4%)	
Hepatitis C	01 (1.4%)	6 (7.0%)	
NASH	28 (40.5%)	27 (31.3%)	
Others	04 (5.7%)	10 (11.6%)	
Duration of RA in mo, median (range)	06 (5-48)	4 (3-36)	0.020
MELD scores, median (range)	18 (7-42)	14 (6-38)	0.003
Child-Pugh score, mean ± SD	10 ± 1.9	9.9 ± 1.6	0.001
Diabetes mellitus, *n* (%)	15 (21.7%)	12 (14.0%)	0.280
Hypertension, *n* (%)	06 (8.7%)	6 (7.0%)	0.760
Corrected BMI in kg/m^2, mean ± SD	22.2 ± 2.3	20.8 ± 4.6	0.060
Obesity, *n* (%)	11 (16.0%)	5 (5.8%)	0.030
Chronic kidney disease, *n* (%)	4 (5.8%)	5 (5.8%)	1.000
Serum total bilirubin in mg/dL, median (range)	3.6 (0.3-28.5)	2.1 (0.4-26.4)	0.040
Serum AST in U/L, median (range)	67 (18-672)	56 (22-238)	0.010
Serum ALT in U/L, median (range)	34 (4-250)	30 (7-202)	0.020
Serum total protein in mg/dL, mean ± SD	5.6 ± 0.6	6.0 ± 0.8	0.001
Severe hypoproteinemia, *n* (%)	29 (42.0%)	13 (15.1%)	< 0.001
Albumin in mg/dL, mean ± SD	2.4 ± 0.5	2.6 ± 0.5	0.005
INR, median (range)	1.8 (0.8-3.8)	1.5 (0.4-6.7)	0.007
Serum sodium in meq/L, mean ± SD	128.0 ± 7.2	131.0 ± 5.5	0.001
Serum potassium in meq/L, mean ± SD	4.2 ± 1.2	3.9 ± 2.4	0.130
Serum urea, median (range) mg/dL	42 (12-191)	31 (12-188)	0.060
Creatinine in mg/dL, median (range)	1.0 (0.4-2.9)	0.9 (0.3-3.7)	0.160
Hemoglobin in g/dL, mean ± SD	8.6 (5.3-12.3)	8.8 (4.2-15)	0.360
Total leukocyte count as /cmm, median (range)	7000 (1750-34300)	5550 (2050-12680)	0.960
Lymphocytopenia, *n* (%)	49 (79.0%)	23 (26.7%)	< 0.001
Combined lymphocytopenia and hypoproteinemia, *n* (%)	21 (30.4%)	2 (2.3%)	< 0.001
Esophageal varices, *n* (%)			0.610
None	02 (2.9%)	1 (1.2%)	
Small grade	40 (58.0%)	52 (60.5%)	
Large grade	27 (39.1%)	33 (38.4%)	
PHG, *n* (%)			0.025
Mild	46 (66.7%)	72 (84.0%)	
Severe	21 (30.4%)	14 (16.0%)	
PHD, *n* (%)	22 (31.8%)	9 (10.4%)	

Ascitic fluid analysis			
Cell count as /μL, median (range)	150 (0-3500)	100 (0-1120)	0.970
Protein in gm/dL, median (range)	0.9 (0.1-2.5)	1.1 (0.6-3.5)	0.370
ADA in U/L, median (range)	8.5 (1.0-33.0)	11.0 (2.0-30.0)	0.200
Triglycerides in mg/dL, median (range)	23 (2-224)	18 (0-64)	0.070
SAAG, median (range)	1.8 (1.2-4.0)	2.1 (1.2-3.5)	0.390
Lower limb cellulitis	7 (10.0%)	0	0.003

ADA: Adenosine deaminase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BMI: Body mass index; cmm: Cubic millimeter; INR: International normalized ratio; LD: Lymphatic dysfunction; MELD: Model for end-stage liver disease; NASH: Non-alcoholic steatohepatitis; PHG: Portal hypertensive gastropathy; PHD: Portal hypertensive duodenopathy; RA: Refractory ascites; SAAG: Serum ascites albumin gradient.

Table 3 Multivariate regression analysis for determining independent associations of lymphatic dysfunction in cirrhosis patients with refractory ascites

Parameters	*P* value	OR	95%CI
Obesity	0.027	4.2	1.1-15.2
Lymphocytopenia	< 0.001	6.2	2.9-13.2
Hypoproteinemia	0.003	3.7	1.5-8.8

95%CI: 95% Confidence interval; OR: Odds ratio.

Table 4 Multivariate analysis for determining independent associations of lymphedema

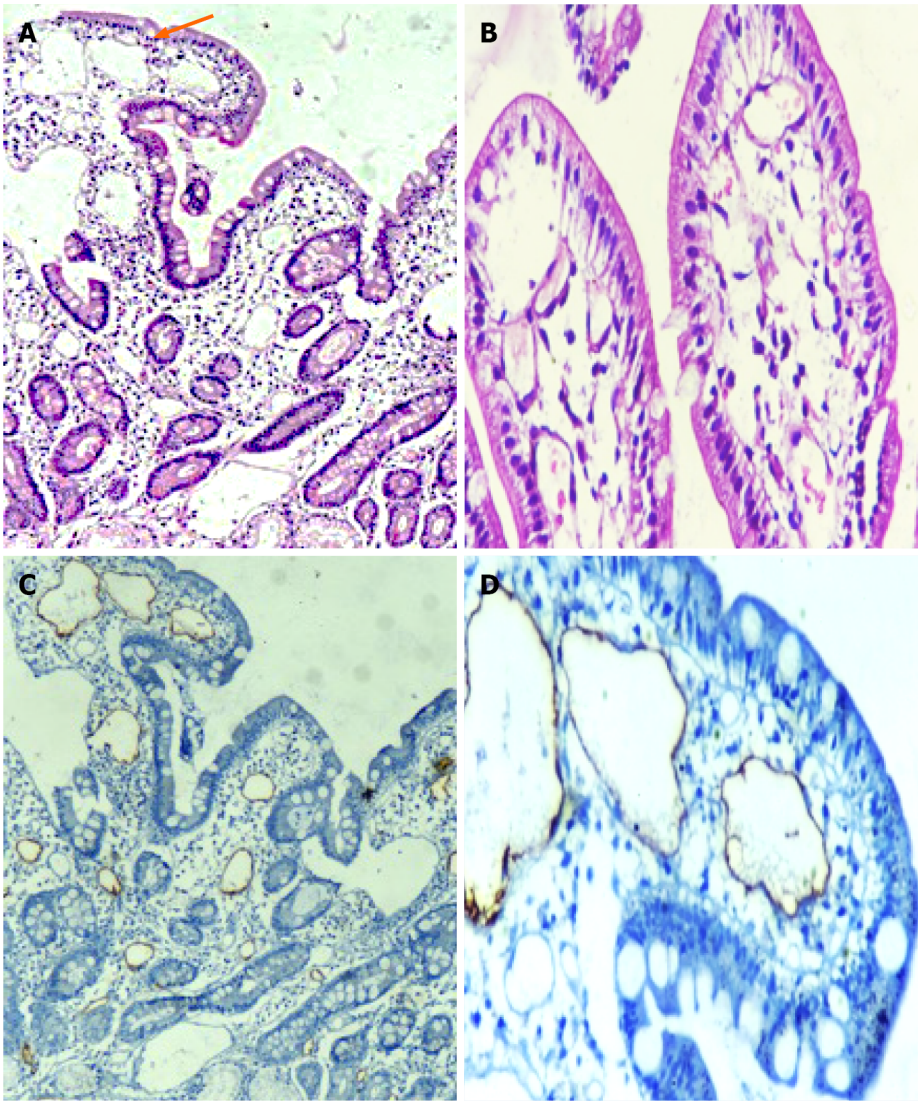
Parameters	*P* value	OR	95%CI
Age	0.002	1.06	1.02-1.10
Child-Pugh score	0.005	1.82	1.19-2.79
Obesity	0.012	6.33	1.49-26.90
Hypoproteinemia	0.001	7.10	2.44-20.70
Lymphocytopenia	0.011	3.55	1.34-9.38

95%CI: 95% Confidence interval; OR: Odds ratio.

reflect the advanced stage of lymphedema. Patients with lymphedema are more susceptible to cellulitis due to their hyperkeratotic surface, deep fissures, stagnant lymph, and weakened immunity. In our study, 13% of lymphedema patients had lower limb cellulitis, compared to none in those who did not have lymphedema. In fact, lymphedema is considered the most important risk factor for cellulitis[19].

In our study, 31 (20.0%) patients revealed evidence of PHD on endoscopy, though only 8 (5.1%) patients had macroscopic evidence of IL. PHD in cirrhosis has been reported at 8.4% by Menchén *et al*[20], 14% by Misra *et al*[21], and 51% by Barakat *et al*[22] in earlier studies. No study has so far reported the endoscopic prevalence of IL in cirrhosis patients. Notably, 27.0% of the study subjects had evidence of IL only on histological examination of D2 biopsy specimens. Very few studies have examined histopathological changes in the duodenum of cirrhosis patients[15,21,22]. Barakat *et al*[22] reported marked capillary congestion and capillary angiogenesis in duodenal mucosa of cirrhosis patients. The changes were mostly marked in the subepithelial location, which on the immunohistochemical stain was CD34 positive. Notably, CD34 is a pan-endothelial marker that can also be positive in the lymphatic endothelium[23]. As a selective marker of lymphatic endothelium (D2-40) was not used in that study, it is possible that IL could have been misinterpreted as capillary congestion and angiogenesis. In a recent prospective study, IL was found to be significantly higher in patients with decompensated cirrhosis than in compensated cirrhosis, and the density of IL on duodenal biopsy was associated with systemic inflammation and 3-mo mortality[24]. It is believed that IL in cirrhosis results from a persistent rise in lymphatic pressure secondary to PHT. However, it is unclear why IL does not manifest in all cirrhotic patients despite sustained PHT.

Because intestinal lymph contains proteins, chylomicrons, and lymphocytes, rupture of IL with subsequent leakage of lymph into the intestine can lead to hypoproteinemia and lymphocytopenia[4,5]. Regardless of the presence of IL, study



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Figure 4 Histological examination of second part of duodenum biopsy specimens. A-D: Representative histopathological images (hematoxylin and eosin) of duodenum showing markedly dilated vessels in the lamina propria (A and B), which on immunohistochemistry showing strong D2-40 positivity (C and D), confirming the presence of intestinal lymphangiectasia.

participants with lymphatic dysfunction displayed lymphocytopenia in 79.0% of cases and hypoproteinemia in 42.0% of the cases, indicating that lymphatic dysfunction at non-enteric sites can also result in loss of lymphocytes and protein from circulation. CA, which was observed in 2 patients, is a rare complication caused by rupture of subserosal lymphatic vessels secondary to a sustained high portal pressure[25]. As intestinal lymph contains triglyceride-rich fat droplets (chylomicrons), CA appear milky in color. Notably, the rupture of hepatic lymphatics does not produce CA as it is devoid of fat droplets. Although < 1% of cirrhosis patients develop CA, cirrhosis has been attributed to 11% of atraumatic CA cases[11,26,27].

Our study revealed several risk factors and indicators of lymphatic dysfunction, including older age, higher MELD and Child-Pugh scores, obesity, lymphocytopenia, and hypoproteinemia, which may point to lymphatic dysfunction in a given patient. It is well recognized that aging alters the structure and function of the lymphatic system. Important aging-related lymphatic alterations include impaired contractile function, decreased nitric oxide lymphatic collectors, and loss of endothelial glycocalyx[28]. Similarly, recent evidence suggests that obesity can significantly impair lymphatic function and increase risk of lymphedema, whereas losing weight can enhance lymphatic functioning[29]. Moreover, lymphatic dysfunction is also involved in the pathogenesis of obesity and obesity-related chronic inflammation[30].

The pathophysiological process underlying lymphatic dysfunction in patients with cirrhosis needs to be explored at the molecular level. In addition to old age and obesity, other variables affecting lymphatic function in patients with cirrhosis include diabetes, dyslipidemia, neurohormonal alterations, and chronic inflammation[4,31,32]. Intestinal lymphatic function may be impacted by intestinal dysmotility and intestinal dysbiosis typically found in advanced cirrhosis[33,34]. Ribera *et al*[5] discovered that excess nitric oxide production by lymphatic endothelial cells was responsible for poor lymphatic drainage in cirrhotic rats with ascites. Interestingly, when these rats were given a nitric oxide synthase inhibitor, lymphatic drainage was improved and the ascitic volume was much reduced, suggesting an influential role for nitric oxide in the dysfunction of the lymphatic system.

From a therapeutic standpoint, the lymphatic dysfunction in cirrhosis patients with RA are worth evaluating because it would call for using different strategies for fluid mobilization. Some clinical and experimental studies have found improved ascites with dietary changes, VEGF C, and nitric oxide synthase inhibitors in cirrhosis subjects with evident lymphatic dysfunction[8,14,15]. Thus, future efforts to mobilize fluid in these patients might focus on methods to improve lymphatic drainage. Unfortunately, there is no recommendation on how to diagnose and evaluate lymphatic functions in patients with cirrhosis. There are many imaging techniques, such as lymphoscintigraphy and magnetic resonance lymphography, but they are frequently constrained by poor resolution, a lack of standardization, the need for invasive procedures, the danger of radiation exposure, and a lack of accessibility[4]. Low attenuation rims surrounding the portal veins and the intrahepatic vena cava on the computed tomography scan correspond to the lymph congestion secondary to impaired lymphatic drainage[35]. However, the clinical implications of these findings in cirrhotic patients need to be determined. In light of these limitations, surrogate markers of lymphatic dysfunction based on physical examination, routine blood investigations, and endoscopy can be an important leap forward.

Our study data was novel, relevant, and generalizable. It sheds light on a well-known but understudied area that calls for further research to determine the role of lymphatics in the complication of liver cirrhosis. It would be interesting to further investigate whether use of prolymphangiogenic substances like VEGF C and D can enhance lymphatic functioning and fluid mobilization in patients with advanced cirrhosis. It would also be worthwhile investigating whether the placement of a transjugular intrahepatic portosystemic shunt aids in improving lymphatic functions in cirrhosis patients, given the involvement of PHT in lymphatic stasis and leakage.

There were some limitations in our study. First, our study was an association study, so causal inference cannot be drawn. We also did not use any lymphangiographic methods to demonstrate lymphatic stasis or leakage. Further, the diagnosis of lymphedema based on physical characteristics has inherent limitations and is at risk of subjective bias and misclassification error in borderline cases. Hence, further studies are recommended to address such gaps.

CONCLUSION

In conclusion, patients with advanced liver cirrhosis frequently exhibit signs of overt lymphatic dysfunction. Given the crucial role of the lymphatic system in volume management, its failure may be at the root of many complications of cirrhosis, including RA. Therefore, addressing the lymphatic system in patients with liver cirrhosis may offer a novel strategy in decongesting tissue and improving outcomes.

ARTICLE HIGHLIGHTS

Research background

Lymphatic dysfunction occurs in patients with liver cirrhosis, although the published data on this subject is extremely limited. Given the significant role the lymphatic system plays in maintaining the balance of body fluids, it is reasonable to assume that a dysfunctional lymphatic system may contribute to the refractoriness of ascites and edema in cirrhosis patients.

Research motivation

From a therapeutic standpoint, it can be extremely important to evaluate lymphatic dysfunction in cirrhosis patients with refractory ascites (RA) since it would call for using different strategies for fluid mobilization.

Research objectives

The objectives of this study were to assess the magnitude, spectrum, and clinical associations of lymphatic dysfunction in cirrhosis patients with RA using surrogate markers such as lymphedema, intestinal lymphangiectasia (IL), and chylous ascites (CA).

Research methods

This observational study was conducted as an exploratory project with a cross-sectional design and included 155 consecutive cirrhosis patients with RA. The presence of clinical signs of lymphedema, IL on duodenal biopsy, and CA were used as surrogate markers of lymphatic dysfunction.

Research results

The study found evidence of lymphatic dysfunction in nearly half of the cirrhosis patients with RA. The spectrum of dysfunction included peripheral lymphedema in 33.5%, intestinal IL in 27.0%, and CA in 1.3%. Obesity, lymphocytopenia, and hypoproteinemia were independently associated with the presence of lymphatic dysfunction in such patients.

Research conclusions

Lymphatic dysfunction is common in cirrhosis patients with RA. Hypoproteinemia and lymphocytopenia are significant indicators of its presence.

Research perspectives

Evaluation of lymphatic dysfunction in cirrhosis patients with RA can serve as a guide for future research into novel approaches for tissue decongestion.

FOOTNOTES

Author contributions: Arya R and Kumar R designed the manuscript, collected data, and wrote the manuscript; Kumar T contributed to the histopathological examination and data collection; Kumar S, Anand U, Priyadarshi RN, and Maji T collected data and provided critical input for the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institute research board (Reference No: AIIMS/Pat/IRC/2020/PGTh/Jan 21-22).

Informed consent statement: Informed written consent was obtained from all study participants.

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SYSTEMATIC REVIEWS

Exercise training as an intervention for frailty in cirrhotic patients on the liver transplant waiting list: A systematic review

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Abstract

BACKGROUND

The existing literature suggests that exercise for cirrhotic patients is safe and favours significant improvement to their physical capacity. However, exercise training for this population and how to deliver activities, especially in severe stages of the disease and while waiting for a liver transplant (LT), remain undefined.

AIM

To review the existing exercise prescriptions for cirrhotic patients on the waiting list for LT, their results for frailty evolution and their effect on clinical outcomes.

METHODS

A systematic review was performed following the Preferred Reporting Review and Meta-Analysis guidelines and searching the PubMed, MEDLINE, and Scopus databases. The keyword "liver transplant" was used in combination with the free terms "frailty" and "exercise" for the literature review. Clinical studies that evaluated the effect of a regular training program, independent of supervision or the duration or intensity of physical exercise, in cirrhotic patients on the waiting list for LT were reviewed. The data on safe physical activity prescriptions following Frequency, Intensity, Time, and Type recommendations were extracted and summarised.

RESULTS

Nine articles met the inclusion criteria for this review. Various instruments for

frailty assessment were used, frequently in combination. Five studies prescribed physical activity for patients, one in-person and four to be performed remotely and unsupervised. The remaining four studies only used a self-report instrument to assess the level of physical activity. None reported adverse events related to exercise training. The exercise frequency mainly varied from daily to a minimum of twice per week. The intensity depended on frailty and included increasing levels of activity. The type of exercise was predominantly a combination of aerobic and resistance training. The duration of exercise varied from 4 to 12 wk. Three articles evaluated the effect of the exercise program on clinical outcomes, reporting a reduction in 90-d readmission rates post-transplant and improved frailty scores, as well as improved survival of cirrhotic patients waiting for LT.

CONCLUSION

Routine frailty assessment is essential for this population. Although more robust evidence is required, the prescription of exercise is safe and can improve patients' functional capacity, improving pre- and post-LT outcomes.

Key Words: End-stage liver disease; Liver transplant; Frailty; Exercise; Rehabilitation; Sarcopenia

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Core Tip: Frailty negatively affects the outcomes of patients waiting for liver transplants (LTs). However, the tools used to assess frailty and functional performance vary amongst the existing studies, limiting the estimation of the real and accurate prevalence of the condition and the effectiveness of proposed treatments. So far, existing studies suggest that exercise may improve cirrhotic patients' functional capacity and frailty while they are on the waiting list for LTs. In addition, although evidence is scarce, studies affirm that exercise training improves pre- and post-LT outcomes.

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INTRODUCTION

Frailty is a multidimensional clinical syndrome that encompasses physical, psychological, social, and environmental components[1,2]. The physical component relates to sarcopenia, reduced physical function, reduced aerobic function, and physical disability[2]. Frailty is defined as a clinical state of decreased physiological reserves and increased vulnerability to health stressors, which predisposes individuals to adverse clinical outcomes[1]. It develops in elderly patients and earlier in patients with debilitating chronic diseases. In patients with end-stage liver disease (ESLD) who are candidates for liver transplant (LT), frailty is associated with decreased strength, physical deconditioning, and worse clinical outcomes with a higher probability of death and dependence after LT[2-4].

Approximately 18%-43% of cirrhotic patients are expected to be considered frail[5,6]. These figures vary largely according to the definition used and the population studied. Although there are some similarities, the mechanisms of frailty in ESLD patients are distinct from those in the geriatric population. The latter entails the original concept of frailty and the multidimensional derangement of physiological systems and is also called global frailty. In ESLD patients, the aetiology of the condition is complex but predominantly driven by liver impairment, such as synthetic protein dysfunction, ammonia-associated muscle toxicity, and encephalopathy-related physical inactivity[7-9]. Therefore, it manifests mainly as physical frailty and, consequently, the tools that have gained broad acceptance in liver clinics and research settings tend to focus unidimensionally on assessing the loss of muscle function[7]. Thus, frailty in ESLD patients is closely related to sarcopenia, which involves the loss of muscle mass and is considered a poor prognostic trait[10,11]. Although both conditions are linked and share common clinical significance, they can occur in isolation.

Sarcopenia assessment involves tests to quantify muscle mass; for example, cross-sectional imaging *via* computed tomography or magnetic resonance imaging, bioelectrical impedance analysis, dual-energy X-ray absorptiometry, or ultrasound, and a deep discussion of these is beyond the scope of this review[2,10]. To assess physical frailty in ESLD patients, three tests have been used: The Fried Frailty Index[1], the Clinical Frailty Scale (CFS)[12], and the Liver Frailty Index (LFI)[13]. Other tests that are also typically used in the care of these patients to assess their physical function and the relationship between muscle strength and function are the Short Physical Performance Battery[14], the Six-Minute Walk Test (6MWT)[15], Gait Speed[16], Hand Grip Strength[17] tests. In an inpatient setting, generic instruments can be used to measure global frailty, including the Frailty Index, the Hospital Frailty Risk Score[18], Activities of Daily Living [19,20], and Karnofsky Performance Status[21]. Regardless of the instrument used for the assessment, frailty in cirrhotic patients is associated with worse pre- and post-transplant outcomes[22-25].

The identification of interventions that could modify or reverse this condition is, therefore, crucial. Although exercise training is widely recommended for people with other chronic diseases[26], evidence for this treatment lags well behind for the population of cirrhotic patients on a waiting list for LT. Perhaps this is due to concerns about exercise causing increased portal pressure[27] or the lack of a standardised exercise protocol. Exercise programs for this population remain uncommon, and debate about the optimal method to deliver exercise (in-person supervised exercise or home-based programs) continues. Regular physical activity and exercise may favour better outcomes by adding muscle mass, as sarcopenia is associated with poor prognostic outcomes[11], and improving these individuals' functional capacity - *i.e.*, reducing their frailty. Therefore, this review aims to investigate the exercise programmes and prescriptions currently used for cirrhotic patients on the waiting list for LT, their results for frailty pre- and post-intervention and their effect on clinical outcomes.

MATERIALS AND METHODS

This systematic review was performed according to the Preferred Reporting Systematic Review and Meta-Analysis protocol[28]. No review protocol was registered before this review began.

Literature search strategy and data source

Articles were identified with the keyword "liver transplant" in combination with the free terms "frailty" and "exercise". The PubMed, MEDLINE and Scopus databases were searched for this review. There was no limitation according to publication date, and the search ended in January 2023.

Method of study screening and selection

Two authors (Loschi TM and Baccan MDTA) screened the articles independently, and there were no disagreements about the studies selected. In the first step, studies with titles that were not related to the theme were excluded. The second step involved reading the full abstracts and excluding articles that did not address important topics for this review. Finally, the full text of the remaining articles was evaluated for eligibility and articles that met the criteria were included in this review. The flow chart for the literature selection process is shown in Figure 1.

Eligibility criteria

The inclusion criteria were: (1) Articles that assessed physical frailty in cirrhotic patients on waiting lists for LT and the effect of physical exercise in this setting using any measurement instrument; and (2) Articles written in English and published. Physical exercise was considered a regular training program regardless of supervision, duration, or intensity. The exclusion criteria were: (1) A study population that was not candidates for LT; (2) Review articles; (3) Editorials and pre-projects; or (4) Articles not written in English.

Data extraction and outcome measures

Details about the exercise program, method of exercise delivery (supervised or unsupervised), and results regarding frailty pre- and post-intervention and the evaluated clinical outcomes were retrieved from each manuscript and analysed. The main outcomes evaluated were the assessment instrument for physical frailty, whether any pre-established criteria for inclusion in the transplant list related to frailty assessment were included; the frequency, intensity, type, and duration of exercise performed; and the primary and secondary outcomes evaluated after the physical exercise program.

Assessment of the risk of bias

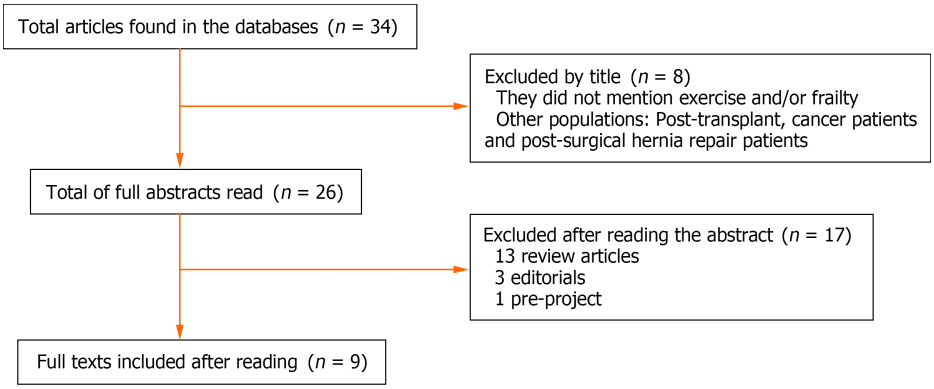
The quality of the included studies was assessed based on the National Institutes of Health Study Quality Assessment Tools. Due to the variable study designs, any identified risk of bias was discussed in the study. No simplifications or assumptions were made.

Literature quality assessment

The Newcastle-Ottawa Scale (NOS) was used to assess the quality of the studies included in this review[29]. The tool applies a "star system" to evaluate three broad perspectives: The selection of the study groups, the comparability of the groups, and the ascertainment of exposure or assessment of the outcome of interest for case-control and cohort studies, respectively. The high-quality features of the numbered items evaluating each of the three domains are identified with a star. A study can be awarded a maximum of one star for each item across a total of four for the selection category, three for the outcome/exposure category, and one for comparability. Therefore, each article can score up to 9 points. Scores of 7 to 9, 4 to 6, and < 4 points indicate high, moderate, and low methodological quality, respectively[29].

RESULTS

The search strategy yielded nine articles that met the eligibility criteria and were, therefore, included in this systematic review.



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Figure 1 Flowchart of the systematic review on exercise therapy for frail cirrhotic patients on the waiting list for liver transplant. After searching the literature, texts whose titles did not fit this review and non-clinical studies were excluded. The nine articles that met the inclusion criteria were read and included in this review.

Frailty assessment or objective measurement of functional performance

The included studies used various instruments to assess frailty; most of them used a combination of instruments. The tools used were LFI (frailty for chronic liver disease), dynamometry (objective measure of handgrip strength), the Duke Activity Status Index (self-reported estimate of functional capacity), 6MWT (functional capacity), Gait Speed (functional capacity), the Rosow-Breslau scale (self-reported assessment of independence for activities), the Incremental Shuttle Walking Test (functional capacity), step count, the Fried Frailty Scale (objective score for assessing frailty), and Cardiopulmonary Exercise Testing (exercise capacity).

Exercise prescription

Of the nine studies included in this review, only one used in-person supervised exercise[30], four prescribed physical activity to be performed remotely and unsupervised[31-34], and the remaining did not specify exercise but recorded participants' self-reported levels of physical activity[35-38].

From the articles that prescribed exercise, we extracted data on the prescription of safe physical activity for this population. The acronym Frequency, Intensity, Time, and Type (FITT) was used to guide us in achieving this aim. We evaluated whether the exercise was supervised or not and whether the activity was performed in person or remotely.

The exercise frequency was 1-5 times per week for the study that prescribed in-person exercise. The studies that adopted remote exercises prescribed daily to a minimum of twice per week exercise, usually for 30 min per session. The intensity of training depended on the existence of frailty and included increasing levels of activity. The intensity progression was mainly based on professional evaluation. The type of exercise was predominantly a combination of aerobic and resistance training, although one study opted for aerobic exercise alone. The duration of exercise varied from 4 to 12 wk, and most studies included reassessment during the program. A detailed description of the exercise prescriptions is provided in Table 1.

Effect of exercise training programs on frailty status

Only one article of the nine used frailty to indicate the decision to include a cirrhotic patient on the LT waiting list[30]. In that study, patients with acceptable functional mobility were considered fit for inclusion on the list. The parameters were: a 6MWT of > 250 m, handgrip strength of > 30 kg for men and > 14 kg for women, and a Duke Activity Status Index of > 4 METS. Patients who were not listed were closely monitored, and goals related to the 6MWT were established to optimise functional mobility.

Post-transplant (length of hospital stay; hospital readmission rate) and pre-transplant (survival rate; quality of life assessment) endpoints were investigated as study outcomes. Physical activity and frailty improvement were consistently associated with favourable outcomes. Detailed information on the results is presented in Table 2**.** In addition, we used the NOS to assess article quality. The NOS scores were between 4 and 9, indicating moderate to high quality for the included articles (Table 2).

Effect of exercise training programs on clinical outcomes

Three articles evaluated the effects of exercise programs on clinical outcomes[30,31,34]. Al-Judaibi *et al*[30] in a retrospective single-centre study that analysed 458 LT patients, showed a trend towards shorter hospital stays (14 *vs* 17 d) and a reduction in 90-d readmission rates (17.9% *vs* 20%) in patients who underwent comprehensive exercise training programs before LT. Lin *et al*[31] analysed the effects of a prehabilitation strategy that involved home-based exercise in a study that included 517 patients and reported that a median improvement of 0.3 points in frail patients' LFI scores was associated with improved survival. Adherence to a physical therapy program was independently associated with increased survival. One year later, the same group reported more hospital admissions and high mortality rates among waiting list patients in the lowest quartile of the daily step count (*i.e.*, < 1200 steps per day). When adjusted by the Model for End-stage Liver Disease (MELD)-Na and the use of a physical training-dedicated smartphone application (Exercise

Table 1 Exercise prescription for cirrhotic patients on the waiting list for liver transplant

Ref.	Frequency	Intensity	Type	Time	Supervised?	In-person?
Al-Judaibi *et al*[30], 2019	In-person group: 1 to 5 times/wk. Remote group: 2 to 3 times/wk	-	In-person group: Aerobic and resistance training	-	Yes, remote group was supervised by videoconference	Yes
Lin *et al* [31], 2021	30 min a day, 5 times/wk	Frail and pre-frail: Aerobic: Encouraged to walk; purchasing a stationary bicycle or a pedal boat was suggested if the patient was at risk for walking. Resistance: Initial prescription at the time of evaluation with the physiotherapist. Weights or elastic bands can be used. Initially, 1 series of 10 repetitions, increase by 5 to 10 repetitions until reaching 30. Only then progress the load	Aerobic and resistance training	150 min of exercise per week	No	No, only if complications prevented the performance of the unsupervised exercise
Williams *et al*[32], 2019	Resistance: 20 min 2 times/wk. Aerobic: 10 min of brisk walking 3 times/d	Resisted: BORG between 12 and 14. Three levels: Low, moderate, and high. Aerobic: Increasing the number of steps	Aerobic and resistance training	Total of 12 wk, with reassessment at 6 and 12 wk and telephone contact 1 time/wk until 6 wk	No	No
Chen *et al* [33], 2020	Daily	Increase of 500 steps/d at each evaluation	Walking	Total: 8 wk, with a baseline assessment and every 2 wk	No	No
Lin *et al* [34], 2022	-	The group that used the app: The app's algorithm established the intensity after an initial assessment by a professional	-	Baseline assessment and 4 wk after	No	No

The data were extracted and reported based on the Frequency, Intensity, Time, and Type recommendation (exercise frequency, intensity, time, and type of activity) and whether the program was supervised, in-person or remote.

and Liver FITness, or EL-FIT), both hospital admissions and death were significantly associated with the lowest quartile [hazard ratio (HR) = 1.9, confidence interval (CI): 1.09-3.30 and HR = 3.42, CI: 1.23-9.68], 6MWT (HR = 0.63, CI: 0.47-0.83 and HR = 0.66, CI: 0.44-0.99 per 100 m), and Gait Speed Test (HR = 0.29, CI: 0.11-0.72 and HR = 0.21, CI: 0.05-0.84). Notably, there was a 5% reduction in the risk of admission and a 12% reduction in the risk of death for every additional 500 steps taken per day regardless of MELD-Na score and exercise program use[34]. None of the articles reported adverse events related to exercise training in this population.

DISCUSSION

Frailty is a clinical condition that negatively affects the outcomes of cirrhotic patients on waiting lists for LT[22-24]. Although the benefits of exercise training are well-established for other chronic conditions, it has not been deeply investigated in this population, mainly due to concerns for their safety[27] and the lack of a standard recommendation. In this review, we assessed the existing literature investigating the effect of exercise training on patients on the waiting list for LT, particularly the prescription of exercise. The current, extremely limited literature suggests benefits from exercise training programmes for clinical outcomes; however, frailty assessment tools lack standardisation, and the evidence for exercise therapy prescription is currently in its early stages.

In a large multicentric retrospective study in the United States that encompassed 1044 patients, frailty (defined as LFI ≥ 4.5) was associated with a 1.82-fold increase in the adjusted risk of mortality on the waiting list compared to non-frail patients[39]. Another North American study found that a 0.1 unit increase in the LFI at three months was associated with a 2-fold increased risk of death or delisting (95%CI: 1.35-3.09) regardless of baseline LFI score or MELD-Na value[22]. When applied to patients listed for LT, the 6-minute walk distance showed a moderate inverse correlation with the MELD score (r = -0.61). In addition, a 6-min walk distance of < 250 m was significantly associated with an increased risk of death on the waiting list (P = 0.0001), and every 100-m decrease in performance predicted a 2-fold increase in mortality[24]. Studies that applied other instruments to measure frailty, such as the Fried Frailty Index[23], the CFS score[5], the Gait Speed test[16], and the 6MWT[24], also found associations between high frailty and increased mortality.

Nowadays, LT specialists accept the benefits of regular physical activity and exercise to improve the functional capacity of cirrhotic patients on waiting lists for LT. The studies summarised in review articles in the literature suggested that exercise is safe for cirrhotic patients and favours a significant improvement in peak exercise capacity (VO_2), a significant increase in the distance walked in the 6MWT, improved muscle strength and function, decreased fatigue, a reduced hepatic portal venous gradient, increased lean mass, and improved quality of life[40-42]. Nevertheless, exercise

Table 2 Impact of the exercise training programs on clinical outcomes, their relationship to adverse events, and adherence to the program

Ref.	Outcomes analysed	Adverse events	Adherence	NOS quality assessment
Al-Judaibi *et al*[30], 2019	Patients who participated in the rehabilitation program showed a tendency towards a decrease in length of stay and a reduction in readmission rates in patients undergoing liver transplantation. Although not statistically significant, the observed trend towards early discharge after liver transplantation was observed in patients in the intervention group			Selection: 4*. Comparability: 0*. Outcome: 3*
Lin *et al*[31], 2021	LFI: Patients more adherent to the prehabilitation program have better results. Frailty is associated with mortality. An improvement of 0.3 in LFI is potentially associated with improved survival. 6MWT: Tendency of improvement with the pre-rehabilitation program, especially in the most adherent patients, after visit 4. Frailty is associated with mortality. Gait speed: There was no change with pre-rehabilitation. Frailty is associated with mortality. Frailty is more prevalent in females, higher BMI (as assessed by the 6MWT and GST), cirrhosis from alcohol or NASH, HB values, albumin, and bulky appearance, do not correlate with MELD. Patients with COPD and CVD are frailer by 3 frailty assessment metrics		Reposted by the patient at follow-up assessment: non-adherent < 20%, partially adherent: 20%-79%, adherent: ≥ 80%. Members: 38%. Partially adherent: 51% and non-adherent: 11%. Adherence to physical therapy was independently associated with increased survival	Selection: 2*. Comparability: 0*. Outcome: 2*
Dunn *et al* [35], 2016	Karnofsky scale and Rossow-Breslau indicated habitual physical activity performance close to normal. Comparing with objective data of counting daily steps, it showed that 75.9% of waking time was in sedentary activity. There was a significant association between the percentage of sedentary behaviour and deaths on the waiting list			Selection: 4*. Comparability: 0*. Outcome: 3*
Williams *et al*[32], 2019	SPPB, ISWT: Improved at 6 wk and no improvement at 12 wk. Step count: Improvement in the index at 12 wk. Quality of life improves in the 12th wk, mainly regarding mobility	No events	Adherence up to 6 wk: 82% step target and 90% resistance exercises. Already at 12 wk, they dropped to 53% and 78%, respectively	Selection: 4*. Comparability: 0*. Outcome: 3*
Ney *et al* [36], 2017	IPAQ: 47%, 38% and 15% of patients had low, moderate and high activity levels, respectively. The main barrier perceived for not performing physical exercise was fatigue			Selection: 2*. Comparability: 0*. Outcome: 2*
Lai *et al*[37], 2016	Fried Frailty Index > 3 frail. 6MWT: Frail with less walking distance. Frail: Less sit and stand up and lower isometric knee extension strength-tendency of critically ill patients to be more fragile			Selection: 2*. Comparability: 0*. Outcome: 2*
Chen *et al* [33], 2020	The control group had more patients who walked less than 2500 steps/d. 6MWT: Greater distance walked in the group with home exercises. CPET: No difference between groups. Computed tomography: Increased psoas muscle mass in the group with home exercises. Quality of life: No differences between groups	No events		Selection: 4*. Comparability: 2*. Outcome: 3*
Oikonomou *et al*[38], 2022	Significant correlation between LFI and physical activity level. LFI: Best in active (active: 3.75, sedentary: 4.42). No frail in the active group. Six frails in the sedentary group. Greater distance in active compared to sedentary (458.2 × 324.7). Peak VO_2 and the highest AT in the active			Selection: 3*. Comparability: 0*. Outcome: 2*
Lin *et al*[34], 2022	They were considered fragile if LFI was 4.5 or greater, 6MWT was less than 250 m, or GST was less than 0.8 m/s. Patients who walk less than 1200 steps/d have a higher LFI. Every additional 500 steps taken per day reduces the risk of hospitalisation by 5% and the risk of death by 12%. Patients in the group using an exercise app took more daily steps. Daily step count was moderately correlated with frailty metrics, and frail patients walked less than their less frail peers by any metric			

NOS: Newcastle-Ottawa Scale; LFI: Liver Frailty Index; 6MWT: Six-minute walking test; BMI: Body mass index; GST: Gait speed test; NASH: Non-alcoholic steatohepatitis; HB: Haemoglobin; MELD: Model for End-Stage Liver Disease; COPD: Chronic obstructive pulmonary disease; CVD: Cardiovascular disease; SPPB: Short Physical Performance Battery; ISWT: Incremental shuttle walk test; IPAQ: International Physical Activity Questionnaire; CPET: Cardiopulmonary exercise testing; VO_2: Oxygen uptake; AT: Anaerobic threshold.

prescriptions for this population remain poorly defined, likely because frailty assessment is predominantly based on subjective clinician assessment. These professionals focus mainly on managing the underlying disease aetiology and liver-related complications. Moreover, practical tools for clinicians to translate their knowledge into a safe and effective exercise prescription are limited. In a review article, experts suggest using the simple and time-efficient CFS and LFI scores alongside routine clinical patient evaluation to identify the patients at the highest risk for physical frailty and recommend them for prehabilitation with specialists, dieticians, and physiotherapists[2].

Therefore, one of the factors affecting the use of physical activity in this population is the physician-centred care model, which frequently delays or prevents access to professionals who prescribe exercise, such as physiotherapists and physical educators. In Brazil, few transplant centres have specialist teams to assess frailty and decreased physical performance, which can reduce the perception of the condition and, consequently, the prescription of exercise and the use of a monitoring program for these patients. In developed countries, the situation is similar. For example, a Canadian study investigating barriers to lifestyle modification in cirrhotic patients reported that only 1 in 7 centres offer their patients regular exercise programming[36]. A professional, detailed functional assessment and patient-specific exercise program that is adapted at regular follow-up sessions are desirable for optimal results. Other barriers to the adoption of prehabilitation exercise training programs are logistical - related to the distance from the transplant centre or caregiver availability due to the need to maintain a constant and continuous exercise regimen for approximately 8-12 wk - or related to the care team's fear of exacerbating or promoting an ESLD complication[42]. In addition, cost is an essential variable as that not all insurance covers more than a few sessions of physiotherapy.

While studies with cirrhotic patients generally focused on physical frailty, the tools currently used to measure frailty and functional performance varied. In addition, the method of exercise delivery encompassed in-person or remote and supervised or unsupervised approaches. Independent of the method employed, the existing studies show favourable results from exercise training in terms of improved functional capacity and frailty[30,31,34]. This finding suggests the need to encourage healthy and active lifestyles for cirrhotic patients; physicians should advise them to stay active and adhere to exercise training. This need is even more crucial in transplant centres with a predominantly physician-centred care model because adherence to these programs depends strongly on physicians raising patients' awareness of the importance of this strategy. Instruments that take objective measures, such as the LFI[13], the Short Physical Performance Battery[43], the Gait Speed test and the 6MWT[44], evaluate the strength and resistance of the lower limbs, the strength of the upper limbs, and balance. According to the literature, these are relevant predictive tools in this setting and appropriate drivers for prescribing exercise therapy.

Thus far, we know that exercise prescriptions should adhere to the FITT recommendations. However, specifications for each parameter have not been completely defined for this population. In recent review articles, the authors proposed relevant, useful orientations based mostly on expert opinions[7,45]. An exercise prescription must contain aerobic training (*e.g.*, walking), resistance exercises for the lower and upper extremities (*e.g.*, functional - stairs - or progressive weight training) and flexibility or balance training (*e.g.*, stretching and balance exercises) and use the talk test to ensure adequate intensity throughout the training[7,42].

Cirrhotic patients are more prone to remaining sedentary more frequently. Dunn *et al*[35] showed, by analysing the level of physical activity exhibited by cirrhotic patients on the transplant waiting list, that they engaged in some of the lowest reported activity of all chronic disease patients, similar to that of patients with advanced chronic lung disease or renal failure. On average, they spent 75.9% of their waking hours in sedentary activities and only 18.9% in moderate-vigorous activity[35]. In addition, Dunn *et al*[35] stated that sedentariness was associated with patient death while on the list.

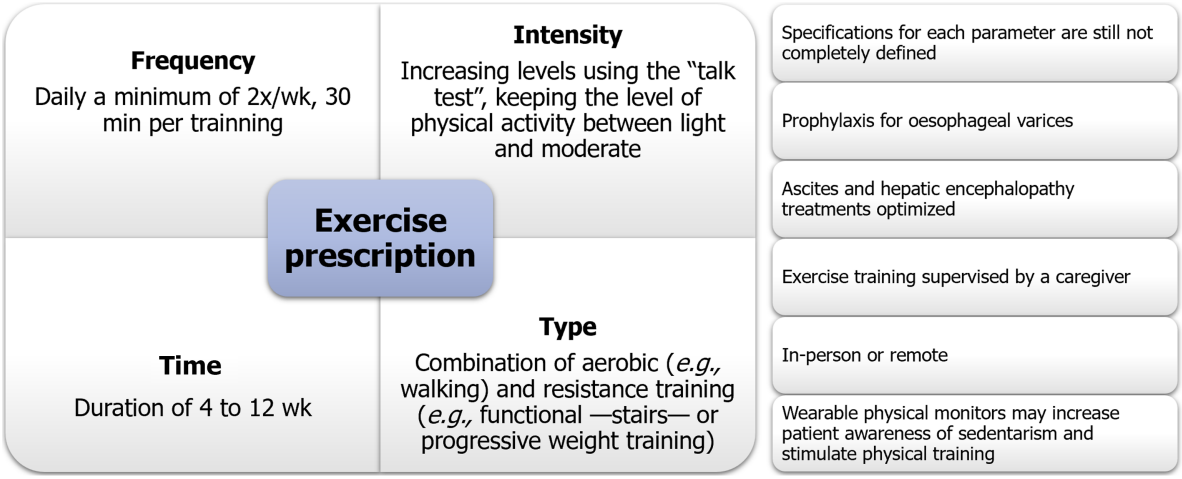
Technology may contribute to monitoring these critical patients' physical activity, increasing their compliance and response to interventions. Wearable physical monitors may provide a more realistic estimation of sedentary cirrhotic patients' activity levels as patient self-assessments and provider physical activity assessments do not reliably indicate the actual level of physical exercise[35]. In addition, the use of these gadgets may increase patients' awareness of sedentary physical activities and even stimulate their engagement in physical training, as Lin *et al*[34] showed.

None of the reviewed articles reported adverse events related to exercise training in this population, suggesting that this approach is safe. Pre-exercise safety may be assessed through disease-related safety issues, screening for cardiopulmonary diseases, and evaluating the effects of other comorbidities[40]. Uniformly, the reviewed studies excluded patients who lacked adequate primary or secondary prophylaxis for oesophageal varices. Other worrying conditions include prohibitive cardiopulmonary diseases, recent alcohol consumption, haemoglobin levels of < 11 g/dL, and incapacitating mental or physical disabilities[40]. Ascites and hepatic encephalopathy treatments should be optimised, and the exercise training should be supervised by a caregiver[7].

Regarding the format for delivering exercise, only one of the articles in this review applied an in-person approach for patients who lived near the rehabilitation centre[30]. Others adopted remote exercise training to facilitate patient engagement and showed benefits of frailty improvement[31,34]. Thus, the evidence suggests that both approaches are safe and effective, allowing the transplant centre and/or the patient to choose the modality that most fits their agenda. In a non-cirrhotic context, a randomised clinical trial of sarcopenic elders suggested that early exercise (supervised exercise followed by home-based exercise) and nutritional intervention aided in restoring lower extremity muscle mass but not physical function[46].

Although the results that these studies reported were not consistent for each criterion of the FITT principles individually, keeping the level of physical activity light-to-moderate, *i.e.*, using exercise that is not so strenuous that the patient cannot talk while performing it ("talk test"), offers a good starting point regardless of the chosen delivery modality. A summary of the exercise recommendations for cirrhotic patients waiting for LT based on the evidence in the literature is presented in Figure 2. A literature review from North America on the use of expert opinion to guide professionals in the prescription of exercise for patients with chronic liver disease recommends moderate-intensity aerobic exercise daily to achieve the goal of walking 150 min a week, which corroborates the findings of this systematic review. Experts also suggest resistance exercises with stretching focused on large muscle groups on alternate days at least twice a week with progressive intensity[40].

This review has some limitations to define. Primarily, due to the limited number of existing studies on this subject, the risk of bias and heterogeneity assessments were not strict, and we opted not to follow with a meta-analysis. Therefore, whilst an acceptable level of design was set, design-based quality checklists were not used. Given the weaknesses of the



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Figure 2 Summary of exercise prescription for cirrhotic patients on the waiting list for liver transplantation.

current evidence and the heterogeneity of the reviewed studies, the above recommendations should be considered carefully. In addition, this review focused on transplant candidates; therefore, extrapolation to other stages of chronic liver diseases or in-hospital patients due to decompensation must be done carefully. Exercise training in this population is intended to maintain physical function during a period of illness and prepare patients for a major surgery that requires hospitalisation and gradual rehabilitation. In addition, no studies thus far have stratified their exercise recommendations based on the severity of liver disease. Future studies should apply well-established frail assessment tools and the existing exercise recommendations, seek to achieve a clinically significant endpoint, and employ a randomly assigned control group.

CONCLUSION

Although further studies are still needed to guide the prescription of exercise and validate the use of regular physical activity to prevent and treat physical frailty in cirrhotic patients who are on the waiting list for LT, the routine assessment of frailty and practice of regular physical exercise, either in-person or remote, of low to moderate intensity is safe and can improve patients' functional capacity and pre- and post-LT outcomes.

ARTICLE HIGHLIGHTS

Research background

In patients with end-stage liver disease candidates for liver transplant (LT), frailty is associated with worse clinical outcomes and a higher probability of death. Therefore, the identification of interventions which could modify or reverse this condition is of paramount importance. Although exercise training is widely recommended for individuals with other chronic diseases, evidence still lags well behind for this population.

Research motivation

Exercise programs for cirrhotic patients are still not so frequent, and there is still debate about the optimal method to deliver exercises to them (in-person supervised exercise or home-based programs).

Research objectives

To investigate the existing exercise programmes and prescriptions for cirrhotic patients on the waiting list for liver transplantation, their results concerning frailty pre- and post-intervention and their impact on clinical outcomes.

Research methods

We searched the PubMed, MEDLINE and Scopus databases using the keyword and free terms "liver transplant", "frailty", and "exercise". The research findings, their contributions to the research in this field, and the problems that remain to be solved should be described in detail. The results were subsequently analysed for the instrument for physical frailty assessment, whether there were any pre-established criteria for inclusion in the transplant list related to frailty assessment, frequency, intensity, type and time of exercise performed, and primary and secondary outcomes evaluated after the physical exercise program.

Research results

We identified nine research articles that were included in this review. The instruments for frailty assessment varied amongst them, and five studies prescribed physical activity to patients, one in-person and four to be performed remotely and unsupervised. None reported adverse events related to exercise training. Three articles evaluated the impact of the exercise program on clinical outcomes, reporting a reduction in 90-d readmission rates post-transplant and improvement of frailty scores followed by improved survival of cirrhotic patients waiting for a transplant.

Research conclusions

We found that the routine assessment of frailty and practice of regular physical exercise, either in-person or remote, of low to moderate intensity is safe and capable of improving the patient's functional capacity and favour positive pre- and post-LT outcomes.

Research perspectives

Although further studies are still required to guide exercise prescription and validate the practice of regular physical activity for cirrhotic patients on the waiting list for LT, it may improve their outcomes.

FOOTNOTES

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CASE REPORT

Inflammatory pseudotumors in the liver associated with influenza: A case report

Ankoor Patel, Alexander Chen, Alexander T Lalos

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Abstract

BACKGROUND

Inflammatory pseudotumor (IPT) is a rare and benign lesion that mimics malignancy and can develop in any part of the body. The pathophysiology and etiology of these quasineoplastic lesions remain unclear.

CASE SUMMARY

We report a case of a 65-year-old male who presented with fevers, night sweats, and unintentional weight loss following an influenza infection and was found to have multiple hepatic IPT's following an extensive work up.

CONCLUSION

Our case highlights the importance of considering hepatic IPT's in the differential in a patient who presents with symptoms and imaging findings mimicking malignancy shortly following a viral infection.

Key Words: Inflammatory pseudotumor; Influenza; Malignancy; Liver mass; Case report

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Core Tip: Inflammatory pseudotumor (IPT) is a rare and benign lesion that can develop in any part of the body. Although the pathophysiology remain unclear, it is thought to develop in the setting of infection, inflammation, autoimmunity, trauma, *etc.* In this case report, we are the first to highlight the development of IPT in the liver in a patient following a recent influenza infection. Our case report emphasizes the importance of including IPT in the differential in a patient who presents with symptoms and radiologic findings concerning for malignancy shortly after a viral infection, such as Influenza.

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INTRODUCTION

Inflammatory pseudotumor (IPT) is a rare, mostly benign inflammatory solid tumor containing spindle cells, myofibroblasts, plasma cells, and histiocytes. Although IPT can occur in any age, it typically affects children and young adults. The pathophysiology and etiology of IPT is not fully understood and the diagnosis continues to remain one of exclusion. IPTs often mimic malignancy and can develop in various organs, including the gastrointestinal (GI) tract (*e.g.*, liver). Case reports of IPT involving the GI tract are scarce.

Several hypotheses suggest systemic inflammatory conditions, infection, autoimmune conditions, and trauma/surgical inflammation to be causes of IPT. One case series reported three patients with hepatic IPTs that occurred following either biliary drainage and stent placement or hepatic abscess[1]. Viral infections may contribute to the development of IPTs[2]. A case report of IPT following coronavirus disease 2019 vaccination has been reported[3]. However, there are no cases reporting the development of IPT in the GI tract following influenza. We report a case of a 65-year-old male who we believe developed hepatic IPTs following influenza.

CASE PRESENTATION

Chief complaints

A 65 year old male was referred to our hospital after multiple liver masses were detected on computed tomography (CT) of the abdomen and pelvis.

History of present illness

In March 2023, a 65-year-old male with a history of microcytic anemia due to erosive gastritis, diverticulosis, and diabetes mellitus presented for evaluation of multiple liver masses. He reported high-grade fevers, night sweats, confusion, and unintentional weight loss of 40 pounds weight in a 3-wk timespan that occurred five months prior to presentation in our hepatology clinic.

History of past illness

Patient with a past medical history of microcytic anemia due to erosive gastritis, diverticulosis, and diabetes mellitus.

Personal and family history

Personal and family history is noncontributory.

Physical examination

During the general examination, no abnormalities were detected.

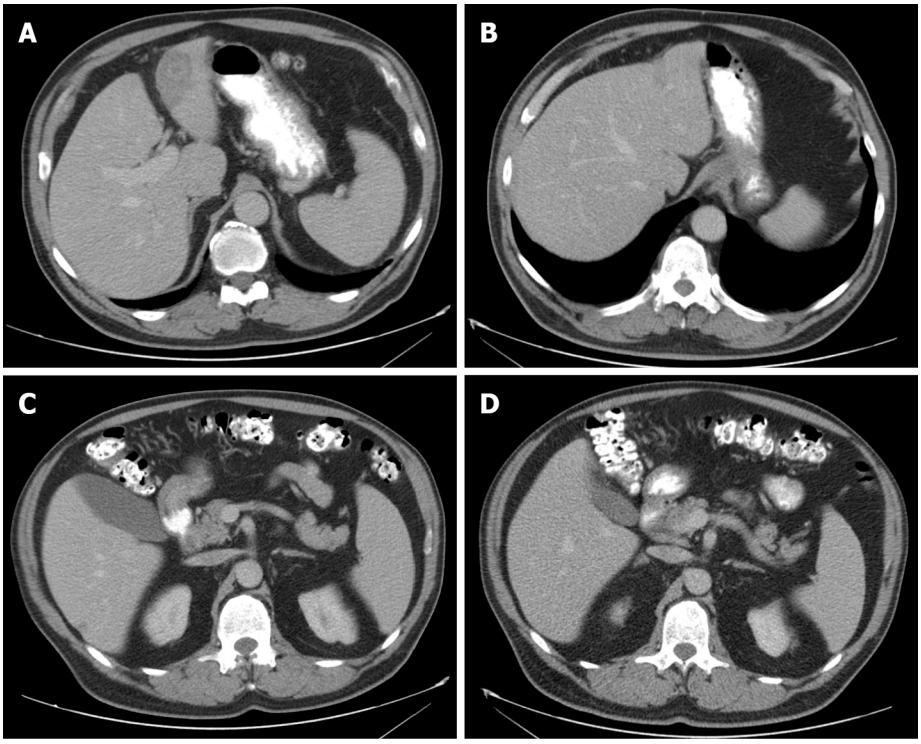
Laboratory examinations

Bloodwork at that time revealed a microcytic anemia of hemoglobin 10.2 g/dL and elevated C-reactive protein (CRP) and erythrocyte sedimentation rate of 191.0 mg/L and > 130 mm/h, respectively. He was initially diagnosed with influenza A *via* PCR of nasal swab.

Alpha-fetoprotein, hepatitis B (core and surface antibody) and C, antinuclear antibody, mitochondrial antibody, actin (smooth muscle) antibody and carcinoembryonic antigen were all negative. IgG antibody titers to hepatitis B surface antibody were low/non-immune. Cancer antigen 19-9 was measured at 42 U/mL (reference range: 0-35 U/mL).

Imaging examinations

A CT scan was performed, which showed heterogeneous left hepatic lobe masses measuring 5.0 cm × 3.0 cm, 4.0 cm × 3.1 cm and 4.1 cm × 3.7 cm as well as a hypodense right hepatic lobe mass measuring 1.1 cm × 1.1 cm (Figure 1). Adenopathy of the periportal, paraceliac, and multiple mediastinal lymph nodes were noted. The patient was referred to oncology for



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Figure 1 Computed tomography of the Abdomen and Pelvis illustrating the interval changes of the hepatic inflammatory pseudotumor in a span of 3 mo. A: A 5.0 cm × 3.0 cm mildly hypodense hepatic mass on computed tomography (CT) A/P dual phase liver; B: A 1.9 cm × 1.3 cm mildly hypodense hepatic mass (previously 5.0 cm × 3.0 cm) on CT A/P dual phase liver; C: A 1.1 cm × 1.1 cm right hepatic lobe mass on CT A/P dual phase liver; D: Resolution of the 1.1 cm × 1.1 cm right hepatic lobe mass (visualized in C).

presumed metastatic malignancy and underwent a positron emission tomography (PET) scan which showed small focal areas of uptake. However, the overall uptake of the left lobe hepatic masses appeared similar to the remainder of the liver (Figure 2).

Biopsy of the liver masses showed inflammatory cells but no malignant cells, and subsequent core biopsies revealed replacement of multiacinar liver parenchyma by fibrous and myxoid stroma with dense lymphoplasmacellular infiltrate (Figure 3). Trichrome stain highlighted dense fibrosis, CK7 immunostain showed preserved bile ducts, and smooth muscle actin immunostain showed extensive reactivity in the fibrous stroma. Immunostains for IgG4 and ALK1 were negative.

The patient was subsequently referred to hepatology and underwent a repeat CT scan, which showed a decrease in size of the left hepatic lobe masses and resolution of the right hepatic lobe mass. Previously enlarged lymph nodes either decreased in size or remained stable except for a 1.1 cm × 0.9 cm soft tissue density anterior to the left hepatic lobe which was suspicious for a new lymph node. An esophagogastroduodenoscopy and colonoscopy were performed to investigate his microcytic anemia and were significant for erosive gastritis.

FINAL DIAGNOSIS

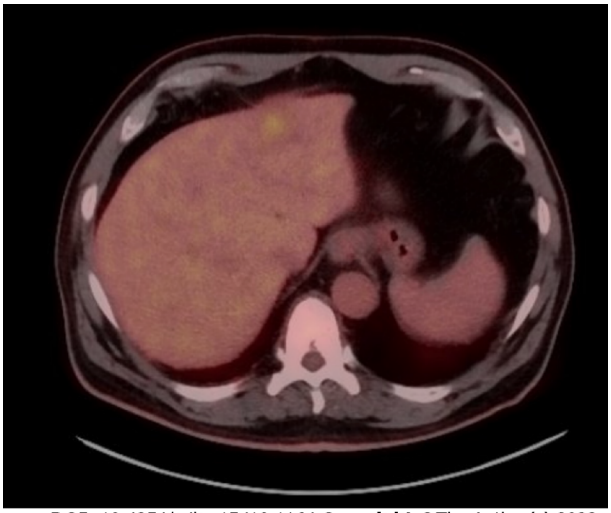
Based on radiologic and histologic examination of the liver masses, the patient was diagnosed with inflammatory pseudotumors of the liver.

TREATMENT

The treatment of hepatic IPTs in our patient was conservative management with supportive care and serial imaging to ensure resolution.

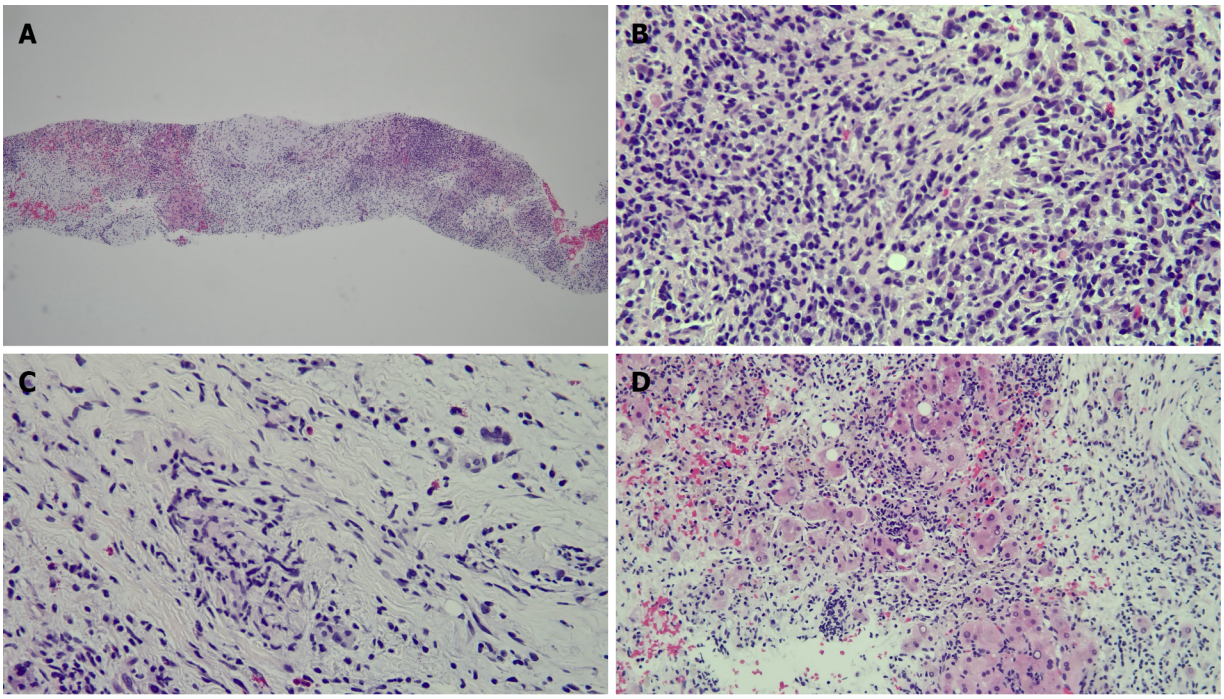
OUTCOME AND FOLLOW-UP

Serial CT imaging showed significant improvement in lymphadenopathy, significant decrease in size of the left hepatic lobe masses, and resolution of the right hepatic lobe mass. Normalization of inflammatory marker (CRP). Patient has



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Figure 2 Whole body fluorodeoxyglucose-positron emission tomography/computed tomography illustrating overall uptake of left lobe hepatic masses similar to the remainder of the liver.



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Figure 3 Ultrasound-guided fine-needle aspiration of the liver mass visualized on computed tomography. A: Core biopsy of the liver mass; B: Core biopsy showing lymphoplasmacytic infiltrate and absence of malignant cells; C: Core biopsy with replacement of multiacinar liver parenchyma by fibrous and myxoid stroma with dense lymphoplasmacellular infiltrate; D: Core biopsy visualizing benign hepatocytes.

returned to his baseline health and weight.

DISCUSSION

IPT is a benign, rare disease process which mimics malignancy on both clinical and imaging findings. It can present as a solitary mass or multiple masses composed of polymorphous inflammatory cell infiltrate. Although it can occur anywhere in the body and at any age, it is more typically found in the lungs and occurs more commonly in children and young adults. Hepatic IPT is a rare phenomenon first described by Pack and Baker[4] in 1953 and is most frequently seen in men[5,6].

Although the pathophysiology and etiology of hepatic IPT remains unclear, infection and autoimmune disorders are thought to be a few of the causes. Various infectious agents have been hypothesized to play a role in the development of IPTs, such as Mycoplasma and Nocardia in lung, Epstein-Barr virus in the spleen and lymph nodes, mycobacteria in spindle cell tumors, and Actinomycetes in liver pseudotumors[5]. Recently, a patient with human immunodeficiency virus was diagnosed with biopsy-proven, herpes simplex virus-positive pseudotumor in the gastroesophageal junction [7]. Otherwise, IPT's have been associated with autoimmune conditions such as IgG4-related disease. Interleukin 1, a cytokine produced by monocytes and macrophages, contributes to the local and systemic effects of IPT. Although treatment of GI IPT's with surgical resection is usually curative, other reports have shown response to steroids, nonsteroidal anti-inflammatory drugs, and thalidomide. The reported recurrence rate of GI IPT's is between 18% and 40% [8]. If there is recurrence following initial therapy, surgical resection is highly advised. Spontaneous regression and malignant transformation have also been rarely reported.

While diagnosis and differentiation of IPT from other etiologies requires histologic examination of the tissue, radiographic characteristics may be helpful in ascertaining whether there is potential of the tumor to be malignant[9]. CT and magnetic resonance imaging findings are variable which is attributed to the variability in histologic composition[10]. Similarly, contrast enhanced ultrasound was unable to solely differentiate IPT from other hepatic malignancies[11,12]. However, several imaging characteristics have been studied and may help guide diagnostic workup[13,14].

We believe our case is likely a consequence of the inflammatory state caused by influenza. The temporal association with contracting influenza may be coincidental but the patient does not have any other risk factors including recent infections, malignancy, immunosuppression, trauma, or autoimmune conditions. Our diagnosis of IPT is supported by the lack of uptake on PET-CT, the absence of malignant cells on biopsy, the presence of mixed inflammatory infiltrate and fibrosis on cytopathology, and the resolution with anti-viral treatment directed against influenza.

CONCLUSION

Due to the rarity of hepatic IPT's and their features mirroring that of malignancy, our case highlights the importance of including pseudotumor in the differential diagnoses of a new liver mass(es) associated with regional lymphadenopathy following a viral infection, such as Influenza. To date, there have been no reported cases of IPT following influenza and therefore would add to current gaps in knowledge when evaluating patients with masses concerning for malignancy.

FOOTNOTES

Author contributions: Patel A, Chen A, and Lalos A had substantial contributions to the conception and study design, analysis and interpretation of the data, creating figure/tables, drafting manuscript, final approval of manuscript.

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EDITORIAL

Editorial: Metabolomics in chronic hepatitis C: Decoding fibrosis grading and underlying pathways

Jorge Quarleri, M Victoria Delpino

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Abstract

In the management of the growing population of hepatitis C virus-infected patients, a significant clinical challenge exists in determining the most effective methods for assessing liver impairment. The prognosis and treatment of chronic hepatitis C depend, in part, on the evaluation of histological activity, specifically cell necrosis and inflammation, and the extent of liver fibrosis. These parameters are traditionally obtained through a liver biopsy. However, liver biopsy presents both invasiveness and potential sampling errors, primarily due to inadequate biopsy size. To circumvent these issues, several non-invasive markers have been proposed as alternatives for diagnosing liver damage. Different imaging techniques and blood parameters as single markers or combined with clinical information are included. This Editorial discusses the identification of a set of six distinctive lipid metabolites in every fibrosis grade that appear to show a pronounced propensity to create clusters among patients who share the same fibrosis grade, thereby demonstrating enhanced efficacy in distinguishing between the different grades.

Key Words: Hepatitis C virus; Chronic hepatitis C; Liver fibrosis; Biomarker; Liquid biopsy

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Core Tip: Accurate diagnosis of liver damage in chronic hepatitis C is pivotal for decision-making. Liver biopsy, the traditional "gold standard" for assessing tissue damage, offers valuable insights but is invasive, with potential complications and sampling errors. Non-invasive methods have made progress in the last decade, but challenges remain. Various non-invasive techniques are in development, including serum biomarker assays and advanced imaging. They often struggle to distinguish intermediate fibrosis stages and are affected by hepatic and extrahepatic factors. This Editorial discusses which identified potential biomarkers in plasma samples linked to each fibrosis grade and hepatitis C virus-induced pathogenesis.

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INTRODUCTION

The natural evolution of chronic hepatitis C (CHC) involves a continuous inflammatory response triggered by recurring liver injuries. This is subsequently accompanied by the activation of hepatic stellate cells, the accumulation of fibrillar collagen within the extracellular matrix (ECM), and the gradual development of fibrosis. These sequential events can potentially lead to ECM degradation, which in turn may result in vascular and architectural modifications, ultimately culminating in the occurrence of cirrhosis or hepatocellular carcinoma (HCC)[1].

Timely diagnosis and intervention are pivotal in preventing the progression to liver cirrhosis and HCC, particularly in light of the advent of direct-acting antiviral therapy, which has revolutionized the treatment of CHC. Nevertheless, effectively reducing the morbidity and mortality associated with this condition necessitates a more comprehensive understanding of liver involvement, improved prognostication, and rigorous monitoring[2]. In this context, accurate determination of the degree of liver fibrosis assumes paramount significance in the clinical management of HCC, as it not only informs treatment decisions but also aids in predicting patient outcomes. However, this endeavor is fraught with challenges, as the methods employed for fibrosis staging encompass both histological assessment through liver biopsies and various imaging modalities. The Metavir classification system, which employs a 0-4 scale, is commonly utilized for staging the various grades of fibrosis in biopsied liver tissue[3]. While liver biopsy remains the acknowledged "gold standard" for diagnosing and staging liver fibrosis, its invasiveness and associated discomfort, coupled with the risk of complications, subject to sampling errors and subjectivity between observers make it a less-than-ideal option[4-6].

Conventional imaging modalities (ultrasonography, computed tomography, and magnetic resonance imaging) are valuable but their sensitivity is limited when it comes to detecting moderate or advanced fibrosis[7]. Besides, advanced acoustic technologies (hepatic elastography) enhance the precision of imaging approaches but the cost of the equipment is a limitation, among others[8].

In the present issue of the JWH, the Ferrasi *et al*[9] study aims to investigate the plasma metabolome using mass spectrometry on samples obtained from individuals with CHC and varying degrees of fibrosis with the goal of identifying prospective biomarkers for categorizing these fibrotic conditions.

The potential clinical utility of these markers presents a compelling avenue for not only staging liver fibrosis but also evaluating the rate and progression of liver fibrogenesis. This assessment, in turn, translates into valuable prognostic insights and serves as a tool for assessing treatment response and monitoring the effectiveness of antifibrotic medications. Nevertheless, the available data regarding their performance in defining the stage of liver fibrosis is variable, and their routine availability may be limited in certain hospital settings[10]. These markers encompass various glycoproteins (such as hyaluronan and laminin), members of the collagen family (including procollagen III, type IV collagen, and type IV collagen 7s domain), collagenases and their inhibitors (metalloproteinases and tissue inhibitors of metalloproteinases), along with numerous cytokines implicated in the fibrogenic process, notably transforming growth factor-β1. These markers have been individually and collectively assessed to gauge the severity and progression of hepatic fibrosis and to monitor changes associated with viral treatment[2] or, even HCC[11-18].

The metabolome comprises the entirety of metabolites that are internally generated within a particular physiological state and can be considered as the ultimate outcome of gene expression. This approach enhances the biomarker identification in human plasma as an invaluable tool in clinical practice and research. They facilitate early detection, accurate diagnosis, personalized treatment, and improved patient outcomes, ultimately contributing to more effective healthcare and better public health.

For every stage of fibrosis, the researchers identified a distinct metabolite profile, and the significance of each molecule varies based on the fibrosis stage, potentially intensifying or diminishing over the course of the disease. Hence, the employment of metabolomics techniques in liquid biopsies exhibits potential as diagnostic, prognostic, and therapeutic monitoring tools.

The pro-viral implications of lipid metabolic reprogramming during virus infection encompass four distinct functions. Firstly, lipids play crucial roles in virus entry and trafficking, serving as attachment factors, internalization receptors, or transportation shuttles during the initial stages of viral entry. Secondly, lipids contribute to virus replication and assembly by providing subcellular spaces essential for key events in the viral life cycle. Thirdly, lipids are indispensable for the generation of energy and essential nutrients required for viral replication. Lastly, lipids serve as pivotal

components in viral envelopment and fulfill diverse functions in the process of virus egress[19]. The study from Ferrasi *et al*[9] analyzes the link between hepatitis C virus (HCV)-induced lipid metabolism abnormalities with the fibrosis grade score, at first with an emphasis on those involved in cholesterol biosynthesis[9].

In the case of grade F1, certain biomarkers that appeared to be more associated with HCV infection rather than fibrosis progression were noticed when compared to individuals with more advanced fibrosis stages. Consequently, the initial molecule detected in grade F1 belonged to the sterol category, featuring distinct characteristics related to cholesterol ester, already recognized as a critical component of HCV lipoviral particles[20]. Furthermore, a diacylglycerol was also identified in grade F1, and its elevated levels were associated with a less advanced state of fibrosis, specifically.

When considering lipid metabolism and the accrual of lipids, it became feasible to pinpoint the presence of the sphingolipid class in the intermediate-grade F2, specifically represented by ceramide. Their accumulation potentially leads to steatosis, which, in turn, may contribute to the progression of liver fibrosis[21]. Furthermore, the authors identified in F2 grade a molecule from the eicosanoid class. This particular molecule is a bioactive lipid that serves as a potent mediator of inflammation in infectious diseases and HCC.

In the case of intermediate-grade F3 and advanced-grade F4, another lipid class (glycerophospholipids) was identified, with the specific biomarkers recognized as phosphoethanolamines. Besides, in F3 grade samples the authors identified the farnesylcysteine, a prenol lipid, as a plausible biomarker for assessing the risk of tumor development, that was previously linked to liver carcinogenesis.

As mentioned above, several studies indicate the potential disruption of fatty acid lipid metabolic pathways during HCV infection. This disruption leads to the accumulation of acyl-coenzyme A (CoA) and intermediary products in fatty acid metabolism belonging to the CoA class. Among them, the authors identified the cis, cis-3,6-dodecadienoyl-CoA among those patients exhibiting F1 grade, while in those with F3 the S-2-octenoyl CoA was found.

Finally, in the advanced grade (F4), a metabolite associated with CoA was detected, along with malonyl carnitine. The presence of malonyl carnitine is noteworthy as it is intricately tied to disease progression and the development of HCC, primarily due to the dysregulation of energy-supplying metabolic pathways.

In addition to the lipid-based biomarkers, the authors identified other plausible markers such as polypeptide angiotensin III [Ang III, also called Ang-(2-8), is generated from Ang II by aminopeptidase A] in grade F1 which may augment collagen production, methyladenosine in F2 and (S)-2,3,4,5-tetrahydropiperidine-2-carboxylate in F3 grade.

CONCLUSION

In conclusion, despite a limited number of samples, Ferrasi *et al*[9] analysis found potential biomarkers specific to each grade of liver fibrosis. These biomarkers showed a propensity to group patients with similar fibrosis grades, although there were instances of overlap such as those proposed for grades F2 and F3. The score plot analysis showed greater efficiency in discriminating between the extreme grades (F1 and F4). This study represents an advancement in the quest for non-invasive serum markers that reflect the progression of liver damage.

FOOTNOTES

Author contributions: Quarleri J and Delpino MV contributed equally to this work. Both authors have read and approved the final manuscript.

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REVIEW

Budd-Chiari syndrome in children: Challenges and outcome

Arghya Samanta, Moinak Sen Sarma, Rajanikant Yadav

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Abstract

Budd-Chiari syndrome (BCS) is an uncommon disease of the liver, characterised by obstruction of the hepatic venous outflow tract. The etiological spectrum of BCS as well as venous obstruction pattern show wide geographical and demographic variations across the globe. Compared to adults with BCS, children have primary BCS as the predominant etiology, earlier clinical presentation, and hence better treatment outcome. Underlying prothrombotic conditions play a key role in the etiopathogenesis of BCS, though work-up for the same is often unyielding in children. Use of next-generation sequencing in addition to conventional tests for thrombophilia leads to better diagnostic yield. In recent years, advances in radiological endovascular intervention techniques have revolutionized the treatment and outcome of BCS. Various non-invasive markers of fibrosis like liver and splenic stiffness measurement are being increasingly used to assess treatment response. Elastography techniques provide a novel non-invasive tool for measuring liver and splenic stiffness. This article reviews the diagnostic and therapeutic advances and challenges in children with BCS.

Key Words: Budd-Chiari syndrome; Radiological endovascular intervention; Transjugular intrahepatic porto-systemic shunt; Direct intrahepatic porto-systemic shunt; Liver stiffness; Splenic stiffness; Shear-wave elastography

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Core Tip: Budd-Chiari syndrome (BCS) is a rare disease of the liver, characterised by obstruction of hepatic venous outflow tract. The effectiveness of radiological endovascular interventions in alleviating clinical symptoms as well as hepatic congestion has been shown both in adults and children. However, unlike in adults, established treatment guidelines have not been developed in children with BCS. Long-term follow-up studies including the prevalence of hepatopulmonary syndrome and hepatocellular carcinoma in this patient population are lacking in children. The role of liver and splenic stiffness measurement by elastography techniques is poorly studied in patients with BCS.

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INTRODUCTION

Budd-Chiari syndrome (BCS) is a rare vascular disease of the liver with a promising outcome if treated optimally on time. It occurs due to obstruction in the hepatic venous outflow anywhere from the hepatic vein (HV) to the entry of the inferior vena cava (IVC) to the heart[1]. Involvement of at least two HV leads to an increase in hepatic sinusoidal pressure and congestion resulting in symptoms of BCS[2]. Global epidemiological data on BCS is scarce[3]. The incidence of BCS reported in the published literature ranges from 0.2 to 4.1 cases per million population per year, with an estimated prevalence of 2.4–7.7 per million population in Asian countries[4,5] and of 1.4–4.0 per million population in Western countries[6-9]. Population-based study on the epidemiology of BCS in children is lacking. Large case series on pediatric BCS are available[10-15]. Chronic BCS constitutes 3%-7% of cases of portal hypertension (PHTN) in the pediatric population[10,11]. So far, interventional and long-term outcome studies in children are limited[12-15]. Over the years there has been remarkable progress in understanding the evolution of this disease. This review aims to discuss the various aspects of BCS, including recent updates on diagnostic and treatment modalities for BCS in children as well as the challenges in them. The review will particularly focus on chronic BCS which accounts for the majority of cases in children.

VARIATIONS IN THE ETIOLOGICAL SPECTRUM OF BCS

Primary BCS is an obstruction of the hepatic venous outflow tract that occurs due to an endoluminal venous lesion (thrombosis or web) resulting from an unidentifiable cause or an inherent prothrombotic condition. Secondary BCS results from obstruction from an invasive lesion (malignant tumor or a parasitic mass) or extrinsic compression by space-occupying lesions (abscesses, cysts, and benign or malignant solid tumors)[1]. The above inflammatory or neoplastic conditions can also result in a secondary prothrombotic state which further adds to thrombosis in the HV. Secondary causes of BCS are common in adults as compared to children[16]. Global variations in the type of BCS may reflect different predisposing factors in different countries. Studies from Western countries have shown HV to be the most common site of obstruction in BCS[17,18]. There seems to be a shift in the pattern of obstruction in studies from Asian countries. Earlier, isolated IVC obstruction was the most type of BCS in Asian patients. Recent studies from China[19,20] as well as India[16,21,22] have noted that a combined IVC and HV obstruction (40%-75%) is the most common type of BCS. A similar pattern has also been documented in Indian children with BCS[12-15]. Over the years, the changes in the spectrum have been better documented due to the advancements in radiology such as high-resolution Doppler ultrasonography (DUS) and non-invasive venography by computed tomography (CT), and magnetic resonance imaging.

BCS AND THROMBOPHILIA

Primary BCS is often regarded as a result of a unique constellation of prothrombotic conditions. The inherited prothrombotic conditions associated with BCS are protein C deficiency, protein S deficiency, antithrombin III deficiency, factor V Leiden mutation, prothrombin gene mutation, and hyper-homocysteinemia with methyltetrahydrofolate reductase mutation[23]. Acquired prothrombotic conditions like antiphospholipid syndrome (APLS), paroxysmal nocturnal hemoglobinuria (PNH), sickle cell disease, and myeloproliferative disorders (MPD) also predispose to BCS[24,25]. Systemic disorders like inflammatory bowel disease, Behcet's disease, and other inflammatory intraabdominal lesions can cause BCS in adults but rarely in children[26]. Recent studies reported an identifiable etiology in 80-84% of cases in adults[27-30]. Underlying myeloproliferative disorders (45%–51%) and exposure to oral contraceptives (50%) are the commonest etiologies[27,28]. In over 25% of cases, more than one thrombophilic state may be present[29,30]. From the available pediatric literature, we understand that the prothrombotic workup in children is often unyielding, inconclusive, or ambiguous. Table 1 summarizes the findings of prothrombotic workup of prior pediatric studies on BCS[11-14,31-35]. As shown, most of the pediatric publications are from India including authors' own experience. One of the major

Table 1 Summary of various pediatric studies (national) of thrombophilia profile in children with Budd-Chiari syndrome

Ref.	Country	No. of cases	Age group	Disease under evaluation	Proportion of cases with prothrombotic work-up	Yield of prothrombotic work-up, *n* (%)	Conditions detected
Nagral *et al*[12]	India	16	4 yr (2-11 yr)	Primary BCS	15/16	4/15 (27)	Protein C deficiency 2; APLA syndrome 1; Antithrombin III deficiency 1
Kathuria *et al*[13]	India	45	10 yr (2-16 yr)	Primary BCS	12/45 children	8/12 (67)	PNH 1; APLA 4; Protein C deficiency 5; Protein S deficiency 3; Hyperhomocystinemia 2 (60% multiple prothrombotic conditions)
Sharma *et al*[14]	India	32	9 yr (5-15.5 yr)	Primary BCS	Not available		
Alam *et al* [11]	India	13	9 yr (5-13.5 yr)	Primary BCS	13/13	10/13 (77)	MTHFR mutation 5; Factor V mutation 1; Celiac disease 1
Malik *et al* [31]	India	11	10 yr (3-16 yr)	Primary BCS	11/11	4/11 (37)	APLA 2; PNH 1; JAK2 1
Shukla *et al*[32]	India	36	1-10 yr	Primary BCS	36/36	15/36 (41.7)	JAK 2V617F 0; Protein C deficiency 2; Protein S deficiency 2; Antithrombin III deficiency 3; APLA 4; Factor V Leiden 4; High homocysteine level 0; Multiple conditions 0
Shukla *et al*[32]	India	43	10-19 yr	Primary BCS	43/43	16/43 (37)	JAK 2V617F 5; Protein C deficiency 1; Protein S deficiency 1; Antithrombin III deficiency 3; APLA 4; Factor V Leiden 1; High homocysteine level 4; Multiple conditions 4
Zhou *et al* [33]	China	35	22 yr (10-25 yr)	Primary BCS	35/35	22/35 (63)	Hyperhomocyteinemia14; APLA 5; JAK2 V617F 1; IBD 1; Behcet disease 1
Dobre *et al*[34]	England	7	7 yr (4-14 yr)	Primary BCS	7/7	7/7 (100)	JAK2 V617F 2; Protein C deficiency 3; Antithrombin III deficiency 2; PNH 1; Factor V Leiden 1; APLA syndrome 2
Revil-Vilk *et al*[35]	Canada	171 BCS -31 cases	3.5 yr (2-13 yr)	Venous thrombosis of different sites	171/171	23/171 (13.4)	Factor V Leiden 8; Prothrombin gene mutation 4; Protein S deficiency 2; Protein S deficiency 1; High lipoprotein a 8

PNH: Paroxysmal nocturnal haemoglobinuria; APLA: Anti phospholipid antibody; IBD: Inflammatory bowel disease; JAK: Janus kinase; MTHFR: Methyl tetrahydro folate reductase.

drawbacks is the lack of in-depth testing for prothrombotic conditions. Shukla *et al*[32] found thrombophilia in 42% of children and 37% of adolescents. The commonest etiologies were Factor V Leiden in children and JAK 2V617F mutation in adolescents. Although thrombophilia may be present in 68%-75% of children, the cause-and-effect relationship is not established[12,32]. Relative and multiple deficiencies of proteins C and S, antithrombin III deficiency, and hyperhomocysteinemia (9%-15%) may be a result of an advanced liver disease resulting in poor synthetic function of the liver rather than a true thrombophilia state[29]. In a systematic review of adults with BCS, MPD was found to be more associated with HV block (16%-62%) than IVC block (4%-5%)[36].

Oral anticoagulants may alter proteins C and S, activated protein C levels, and lupus anticoagulant levels. Hence these tests are best performed before starting thromboprophylaxis. Further, the presence of hypersplenism and hemodilution in cirrhosis with portal hypertension masks the peripheral blood findings of a concomitant MPD[29]. Genetic testing is the gold standard for documentation of thrombophilia. In a study of 80 adults with non-cirrhotic splanchnic vein thrombosis, next-generation sequencing was able to identify JAK2 mutations, previously undetected by conventional techniques in one-third of cases[37].

It is also suggested that the prevalence of various thrombophilic disorders is different between younger children and adolescents. In a study from India, the JAK2V617F was found to be common in adolescents but not in children[32]. Recently it has been shown that adolescent venous thromboembolism is multifactorial in the majority with more than two risk factors at diagnosis[38]. Warfarin is the most commonly used oral anticoagulant in BCS patients, especially after radiological intervention. As it has a narrow therapeutic window, the dose needs to be titrated with regular monitoring of international normalized ratio (INR) (target: 2-3). Vitamin K epoxide reductase complex subunit C1 (VKORC1) and cytochrome P450 2C9 (CYP2C9) are the two major genes involved in the metabolism of warfarin and they determine the dose requirement of warfarin[39]. CYP2C9 metabolizes the more potent S-enantiomer of warfarin while VKORC1 is the target protein for warfarin. Multiple single nucleotide polymorphisms have been described in these genes, of which the most important ones are CYP2C9*2, CYP2C9*3, and VKORC1 heterozygous haplotype GA and homozygous AA[40]. Thus, the patient's genotype is a major determinant of not only the dose requirement but also the risk of anticoagulation-

related complications. In a study from India in adults with BCS, patients with the presence of mutations in VKORC1 or CYP2C9 were associated with an increased risk of bleeding[41]. More intensive monitoring while on warfarin is recommended for these patients. Some guidelines have now started recommending incorporating the results of genetic testing for clinical use while on warfarin therapy[42].

Identification of thrombophilia in a child has implications in terms of the need for lifelong oral anticoagulation, increased risk of other venous thromboembolic events, lifestyle modifications like avoiding oral contraceptive pills, risk of complications like leukemia in myeloproliferative disorders, and implications for other family members. In addition, there is a need to educate asymptomatic family members regarding the risk factors and lifestyle modifications.

CLINICAL FEATURES

Clinical manifestations can be diverse, ranging from acute liver failure to completely asymptomatic patients, making BCS a possible differential diagnosis in many acute and chronic liver diseases. Most patients with BCS have a chronic presentation, whereas only a small number of patients present with a fulminant type of BCS[12-14]. The clinical and radiological features of children with BCS in various pediatric studies are summarized in Table 2. The usual age at presentation in children is 10 (range 1.5–17) years but BCS has been reported in children as young as 4.5 mo[11,12,32]. The commonest symptom is rapidly reaccumulating ascites (83%–90%) with dilated tortuous abdominal and back veins (60%-70%)[11-14,31]. Up to 15%-20% of patients can be completely asymptomatic, hence a high index of suspicion should be kept, especially when there is no clear detectable etiology for chronic liver disease and/or a prothrombotic condition exists[43]. In such scenarios, proper imaging by an experienced radiologist is warranted[44]. The presentation of BCS depends on the extent and rapidity of hepatic venous outflow obstruction and the development of decompressing venous collaterals. With this concept, BCS can be classified as fulminant, acute, subacute, or chronic[45,46]. However, a pathological examination of the liver illustrates a dissociation between the rapidity of the clinical presentation and the acuteness of the histological damage. Up to 50% of patients clinically classified as acute have histological features of chronicity (*e.g.*, fibrosis or cirrhosis)[46]. The prognostic value of this clinical classification in predicting mortality has not been prospectively evaluated[47]. Over time, several prognostic indices have been designed to predict mortality and response to therapeutic interventions[15,18,46,47]. These scoring systems incorporate clinical and laboratory features to stratify patients, although their use for the management of an individual patient is debatable[48].

DIFFERENCE BETWEEN ADULTS AND CHILDREN WITH BCS

BCS can occur at any age group but it most commonly affects young adults. In adults, secondary causes of BCS are much more frequently seen than children in whom primary causes are predominant. Regarding the underlying etiology, thrombophilic conditions are reported in more than 80% of adults with BCS. In Asian children, the etiology is largely idiopathic. Thrombophilia work-up is often under-reported and the results are variable. Acquired prothrombotic conditions like MPD, APLS, PNH, sickle cell disease, and oral contraceptive pill use are predominantly seen in adults. On the contrary, inherited thrombophilias like protein C deficiency and protein S deficiency are more commonly found in children. The clinical presentation is also different for adults and children. Shukla *et al*[32] compared 43 children and 129 adult patients with BCS. They found hepatomegaly without ascites as the most common presentation in children as compared to ascites being the most common presentation in adults. The authors hypothesized that children have better angiogenesis and collateral formation, and shorter disease duration, leading to milder clinical presentation and better treatment outcome.

DIAGNOSTIC CHALLENGES

The diagnosis of BCS depends on the demonstration of HV and/or IVC obstruction. Invasive venography remains the gold standard; however, it is performed during the time of endovascular intervention procedure. DUS has therefore emerged as the primary imaging modality with a diagnostic accuracy of > 90%[12,13,15]. DUS evaluates hepatic, portal, and IVC patency, site, and length of block and liver parenchymal changes (including caudate lobe hypertrophy and intrahepatic comma-shaped collaterals). HVs may be engorged, irregular, or filled with thrombus. The "health" and residual stump of the HV are the most critical to plan an endovascular intervention. Triphasic flow may be dampened or reversed. The IVC may be narrowed or displaced by caudate hypertrophy or contain an intraluminal thrombus. Intrahepatic comma-shaped collaterals and caudate lobe hypertrophy are almost universally seen in patients with BCS. The presence of dense intrahepatic collaterals suggests chronicity. Subcapsular collaterals may bleed during a percutaneous intervention and need to be carefully documented. Non-invasive venography (CT or MRI) is required when there is a diagnostic ambiguity. Hypoenhancement of the peripheral hepatic parenchyma and relatively normal enhancement of the caudate lobe result in a mottled appearance on CT. These changes are more obvious on MRI[49]. However, if they are unyielding, invasive venography and liver biopsy should be considered.

Another challenging diagnostic dilemma in the context of BCS is hepatocellular carcinoma (HCC). Long-standing congestion and fibrosis of hepatic parenchyma in patients with chronic BCS are known to give rise to hepatic nodules. The etiology of hepatic nodules detected on radio imaging in BCS patients can be benign regenerative nodules and HCC.

Table 2 Clinical presentation, site of block, radiological intervention, and outcomes of major studies in children with Budd-Chiari syndrome

	Nagral *et al*[12], %	Kathuria *et al*[13], %	Sharma *et al*[14], %	Singh *et al*[15], %	Shukla *et al*[32], %
No. of patients	16	45	32	113	43
Age at presentation, median (range)	22 (4-132) mo	10.5 (2-16) yr	9 (4.5-214) mo	10 (1.5-17) yr	16.7 (10-19) yr
Symptom duration, median (range)	3 (0.5-48) mo	8.9 mo (5 d–8 yr)	-	-	12.7 (0.5-150) mo
Diagnosis by USG	63	95.6	60	-	-
Symptoms					
Ascites	81	82	96	84	91
GI bleed	25	35	8	22	23
Jaundice	12.5	20	24	12	14
Hepatomegaly	N/A	85	96	N/A	67
Splenomegaly	N/A	70	N/A	N/A	37
Abdominal vein dilation	N/A	70	70	N/A	N/A
Type of block					
Only HV	94	71	76	74	67
Only IVC	6	4	0	2	9
Both HV + IVC	0	25	24	24	24
RI, *n*	13	25	24	53	24
Angioplasty alone	4	2	7	7	-
Angioplasty + stenting	3	20	3	40	10
DIPS	-	-	-	5	-
TIPSS	6	3	14	1	14
Follow-up duration, median (IQR)	31 (12-54) mo	6.5 (0.5-86) mo	44 (5-132) mo	13.5 (1-155) mo	41 (12-168) mo
Successful RI					
Angioplasty	25	100	43	100	-
Angioplasty + stenting	100	87	67	90	90
DIPS	-	-	-	80%	-
TIPSS	80	67	72	100	80
Procedure related complications	TIPSS encephalopathy = 1; Neck hematoma = 1	Anaesthesia related death = 1; Neck Hematoma-1; Hemoperitoneum = 1	No major	TIPSS; Enceph-alopathy = 1	N/A
Stent patency rate	-	Overall 75%	-	87% at 1 yr, 82% at 5 yr, 62% at 10 yr	Overall 75%
Mortality	2: GI bleeding 1, liver failure = 1	3: Intracranial bleeding = 1, anaesthesia related = 1, liver failure = 1	5: Intracranial bleeding = 2, GI bleeding = 1, HCC = 1, liver failure = 1	3: Procedure related = 2, head trauma = 1	4: Intracranial bleeding = 1, HCC = 1, liver failure = 2
HPS	N/A	N/A	4 (12, 5%)		-
HCC	N/A	N/A	1 (3%)		1 (2.3%)

N/A: Not available; HV: Hepatic vein; IVC: Inferior vena cava; USG: Ultrasonography; GI: Gastro-intestinal; HV: Hepatic vein; IVC: Inferior vena cava; RI: Radiological intervention; DIPS: Direct intrahepatic porto-systemic shunt; TIPSS: Transjugular intrahepatic porto-systemic shunt; HPS: Hepato-pulmonary syndrome; HCC: Hepatocellular carcinoma.

It is important to distinguish between these two conditions as treatment differs significantly. HCC lesions are usually larger in size (> 2 cm) and hypervascular on DUS and CT/MR[50]. Also, for HCC, contrast enhancement in T1-weighted MR imaging can show different enhancement patterns between HCC with and without BCS[51]. Yang *et al*[51], in a comparative study between 10 adult HCC patients with associated primary BCS and 32 other HCC patients without BCS, found that significantly more lesions with BCS were hyperintense during the arterial phase and slightly hyperintense or isointense during the venous phase than lesions without BCS ($P < 0.05$ for both). Therefore, histological confirmation is required in the workup of HCC in patients with BCS.

TREATMENT OPTIONS

Therapeutic options for BCS include medical management with anticoagulation therapy, radiological interventions such as angioplasty and stenting, surgical shunting and transjugular intrahepatic portosystemic shunt (TIPSS), and lastly, liver transplantation (LT).

Medical therapy

Anticoagulation alone with oral anticoagulants is sufficient in only 10% of adult patients, especially those with mild disease, and the vast majority of them progress, requiring intervention strategies in follow-up[52-54]. Pediatric experience is limited. Sharma *et al*[14] reported a 33% response in a small cohort of Indian BCS children who were treated with warfarin alone. Data on the comparative efficacy of directly acting anticoagulants *vs* vitamin K antagonists in BCS patients is scarce[55]. A recent Austrian multicentre study on the efficacy and safety of DOAC in 22 adult BCS patients (DOAC first-line anticoagulation in 6, switched over from warfarin and LMWH in 16) showed that DOAC showed clinical response in 63% of cases while bleeding occurred in 11 (4 major bleeding, 7 minor bleeding)[55]. Confirmation of efficacy and safety by larger prospective studies is needed.

Radiological endovascular intervention

The progressive improvement in radiological endovascular intervention (REI) techniques in the past two decades has provided better procedural and clinical success for BCS treatment compared to other treatment modalities in both adults and children[14,15,56,57].

Angioplasty vs stenting

The rationale of angioplasty is to decompress the liver while restoring hepatic blood flow. Angioplasty can be done with or without stenting in BCS patients with short-segment stenosis (< 5 cm). Wang *et al*[58], in a landmark study, compared the treatment efficacy and long-term patency of angioplasty with and without stenting in 88 adult Chinese patients with BCS. They had shown that the superiority of angioplasty with stenting over angioplasty alone in the rate of re-stenosis (2% *vs* 40% over a median follow-up period of 27 mo, $P < 0.0001$)[58]. Balloon angioplasty without stenting of the obstructed veins is preferred in infants and younger children as appropriate sizes of stents are not available, thus making stenting technically difficult in this population. Also due to the relatively shorter duration of disease, the veins are likely to be much more pliant. Hence angioplasty alone may reduce and normalize the venous pressure significantly after the procedure. In older children, angioplasty alone has a technical success rate and clinical response rate of 90% but is fraught with a high risk of blockage[11-15]. Restenosis is almost always inevitable. Nagral *et al*[12], Kathuria *et al*[13], and Sharma *et al*[14] have reported higher re-occlusion rates with angioplasty alone (75%, 33%, and 57%, respectively) compared to angioplasty + stenting (0%, 13%, and 33%, respectively) and TIPSS (20%, 33%, and 28%, respectively). This highlights that angioplasty with stenting rather than angioplasty alone, should be the preferred modality of radiological intervention (RI) in children. The smaller size of the liver and caliber of the veins in children pose a challenge when choosing an appropriate size and length of the stent. An uncovered self-expandable metallic stent is preferred over fully covered stents because of the lower risk of post-procedural thrombosis. Longer stents (20–30 mm) are required for long-segment IVC blocks than HV blocks. Overall, the technical success and clinical response rate of stenting in children is excellent (> 90%) with a much less re-stenosis rate than angioplasty[11-15]. In the authors' experience, the length of the stent may appear longer as the liver decongests after the intervention. However, with the growth of the child, the liver also grows in size and the final stent size is usually appropriate for the child. Customized stents are available. It is also important to realize that once the liver is recovering and the physiology is restored, there is an expected sudden growth spurt in children with chronic liver disease, especially during puberty. With growth, the blood flow and turbulence through the recovering liver increase. Hence anticoagulation is most important in this phase till the stent undergoes complete endothelialization. Oral contraceptives in adolescence are to be prescribed with caution.

TIPSS

TIPSS is a shunt created between the portal and the systemic circulation, leading to a reduction in hepatic congestion and symptom resolution. Currently, TIPSS is performed in failed angioplasty-stenting with an available HV stump and as a bridge to LT[27]. TIPSS is technically more demanding than cirrhotic patients due to the blocked HVs. Nonetheless, various studies have established TIPSS as a safe and effective treatment for BCS in adults[22,48,59,60]. Amarapurkar *et al*[22] and Shalimar *et al*[59] have shown good technical success rates (87.5%-100%) and clinical success rates (80%-93%). Post-procedure encephalopathy occurs less frequently (3% to 5% cases) than in other cirrhotic patients, reflecting the relatively preserved hepatic function in most patients with BCS[59,60]. The main concern about TIPSS is the higher occurrence of stent dysfunction. Compared to other cirrhotic patients undergoing TIPSS, BCS patients show higher stent

dysfunction (70% *vs* 50% within 1 year), probably due to underlying prothrombotic conditions in BCS[61]. The advent of polytetrafluoroethylene covered stents has dramatically reduced the incidence of stent dysfunction (30%-70% in bare stents *vs* 10-20% in covered stents)[61,62]. The available TIPSS stents are usually expensive and are inappropriate in terms of sizes for children, thus limiting their use in the pediatric population, although reports of successful treatment of children with BCS with TIPSS are available[12,13]. TIPSS in children with BCS risks placement of the bare end of the stent beyond the portal vein and into the superior mesenteric vein if the size is too long. Hence, a "stent within a stent" is a minor modification of this technique that allows customization for the size of the liver. In this technique, a fully covered graft stent (10 mm × 3–7 cm) is placed inside the bare uncovered stent (10 mm × 8–10 cm).

Modified TIPSS/DIPS (direct intrahepatic portosystemic shunt)

Long-segment (> 5 cm) HV block is usually not amenable to angioplasty, stenting, or TIPSS. A modified TIPSS or DIPS is a procedure where a shunt is made between IVC and the right branch of the portal vein. Though technically challenging, DIPS has been shown to effectively decompress hepatic congestion and clinical resolution in patients with BCS. In a large pediatric study from the authors' center, children with chronic BCS undergoing DIPS had a procedural success and clinical response rate of 80% and 90%, respectively[15].

Surgical shunts

The principle of a surgical portosystemic shunt is to relieve the obstruction causing PHTN using a venous conduit, thereby decompressing the hepatic sinusoids. Surgical portosystemic shunts have now been almost completely abandoned because of high perioperative mortality (25%) and poor shunt patency (70%)[63-65]. Surgical portosystemic shunting can also be technically difficult when there is caudate lobe hypertrophy[66]. Most studies on surgical portosystemic shunts in adult patients with BCS failed to show any survival benefit[65,67]. In a case series of 25 Indian children with BCS, only one out of the four patients who underwent surgical shunts survived[68].

LT

LT is considered a salvage therapy in the setting of fulminant presentation, progression to end-stage liver disease, or development of HCC[27,53]. BCS accounts for approximately 1% of all pediatric LT cases[69]. Involvement of retrohepatic IVC, the proximity of thrombus near the right atrium, and an underlying prothrombotic condition causing vascular complications make LT challenging. The challenge is even greater when considering living donor liver transplantation (LDLT) since the graft does not contain the retrohepatic IVC, as in deceased-donor liver transplantation (DDLT). Therefore, HV reconstruction is more complex, especially if the IVC is also obliterated[69]. The smaller size of vessels in children complicates the situation further. Several large retrospective analyses have evaluated the benefit of DDLT in adult BCS patients with 5-year survival rates varying between 71% and 89.4%, similar to those undergoing LT for other diseases[70,71]. Due to the scarcity of deceased-donor liver grafts, LDLT has been the mainstay for BCS patients undergoing LT in most Asian countries with 5-year survival rates ranging from 75% to 81%[72-75]. Data on LT in pediatric BCS is only in the form of case reports and small case series and long-term prognosis has been reported to be good so far[76,77].

STEPWISE APPROACH OR UPFRONT REI IN CHILDREN WITH BCS: THE WAY FORWARD?

The rarity of BCS in general makes it difficult to perform randomized controlled trials in patients with BCS. Hence, most recommendations regarding treatment are based on case reports, retrospective studies, and expert opinions. Concerning the timing of the interventions, the European Association for the Study of the Liver[78] and the Asia Pacific Association for the Study of the Liver (APASL)[27] recommended a stepwise therapeutic algorithm for BCS in adults. The algorithm depends on treatment response, medical therapy with anticoagulant drugs, angioplasty, stent placement, TIPS, and LT. In contrast to the strict step-up principle, an AASLD practical guideline suggested checking for a venous obstruction amenable for angioplasty in all symptomatic patients right at the beginning and treating accordingly[79]. The step-up algorithm has also been criticized because it pays little attention to hemodynamics and its possible improvement or even relief by interventional treatment. The step-up algorithm also does not restore physiology at the onset and possibly delays the definitive procedure, leading to advanced liver disease. Guidelines are silent regarding what should be the optimal treatment approach in children with BCS. Longitudinal studies on pediatric BCS have shown that children with chronic BCS tend to decompensate early with ascites and variceal bleeding[13,14]. In those receiving medical therapy alone, it has been seen that 26% of adults die over 2 years[53,55]. Response to medical therapy has been variable in children (33-43%) and two-thirds of these children ultimately require an intervention in the long run[14]. There is also concern about the safety profile of long-term use of oral anticoagulants in children[80]. Hence, we suggest that all children with BCS (whether symptomatic or not) should undergo angioplasty and stenting as a primary treatment modality.

It is debatable whether asymptomatic or incidentally detected BCS should be subjected to REI or not, especially in children. On one hand, decompensation may rapidly set in as children do not tolerate portal hypertension for longer periods as compared to adults. On the other hand, there are no sound ethical justifications as to whether these children should be subjected to invasive procedures and maintained on lifelong anticoagulation. In a personal opinion, the authors would prefer the former in asymptomatic children given their longer life expectancy, milder liver disease, and possibly better health and compliance with the HV. Hence there is a small window of opportunity to restore normal physiology in optimal conditions.

POST-INTERVENTION COMPLICATIONS AND NEED FOR MONITORING AFTER REI

Immediate complications of REI include subcapsular hematoma, hemoperitoneum, congestive heart failure, transient hepatic encephalopathy, and pulmonary thromboembolism and are encountered in 1%–3% of procedures in experienced centers[12-16,27]. Long-term complications of bleeding secondary to anticoagulation have also been reported[12,13,22]. However, the most commonly encountered post-procedure complication is re-stenosis after REI. Singh *et al*[15], in a large cohort of Indian children with BCS, reported follow-up vascular patency rates of 87%, 82%, and 62% at 1, 5, and 10 years after intervention, respectively. In the above study, 29% of the cohort with REI (27% HV/IVC stenting; 60% modified TIPSS) had restenosis[15]. Adult studies report 17% to 41% restenosis after REI[21,22,24]. To prevent re-stenosis, heparin infusion should be started during the intervention and continued thereafter. Warfarin must be initiated within 24 h of completing the procedure. The physician should consider stopping heparin and continuation of long-term warfarin if the target INR of 2–3 is achieved. Periodic clinical examination, liver function test, and shunt patency by DUS (post-stenting: 24 h, 1 wk, 1 mo, 3 mo, and subsequently every 6 mo; post TIPS/DIPS: at 7–14 d, 3 mo, 6 mo, 9 mo, and 12 mo) is performed. Post-intervention surveillance aims to re-intervene before critical stenosis or occlusions recur.

Assessment of response to therapy

Response to therapy implies restoration of blood flow across previously blocked HV/IVC and consequent improvement in organomegaly and liver function test (LFT), no recurrence of ascites without need of diuretics, and resolution of signs of PHTN. Kathuria *et al*[13], in a study among 20 children with BCS undergoing REI, demonstrated clinical and biochemical resolution in all; however, the median follow-up duration was 6.5 mo only. Sharma *et al*[14], in another study of 20 Indian children with BCS who underwent REI, showed that HV stenting or TIPS is efficacious in improving clinical features, LFT, PHTN features, and growth parameters in 66% and 72% of cases, respectively, over a median follow-up duration of 44 (range 5-132) mo. Further long-term studies need to holistically address the natural history and timelines of resolution of organomegaly, liver stiffness, varices, liver functions, growth, pubertal maturity, and quality of life in children with BCS.

LIVER AND SPLENIC STIFFNESS MEASUREMENT IN MONITORING

Recently, liver stiffness measurement (LSM) and splenic stiffness measurement (SSM) have been extensively studied as potential non-invasive markers of hepatic and splenic fibrosis and congestion and hepatic venous pressure gradient in patients with chronic liver disease[81-84]. LSM and SSM can be measured by various imaging techniques [transient elastography (TE), shear-wave elastography (SWE), and MR elastography]. Fraquelli *et al*[82], in a study of 132 patients with chronic hepatitis B and C, showed that LSM and SSM measured by TE, were reliable in predicting significant fibrosis [odds ratio (OR) = 5.2 and 4.6, respectively] and cirrhosis (OR = 7.8 and 9.1, respectively). SSM of < 48 kPa by TE was useful in ruling out esophageal varices. In another study by the same authors in 186 chronic liver disease patients, LSM and SSM measured by SWE were equally effective and reliable in predicting significant liver fibrosis as compared to TE, with SWE having the advantage of applicability in patients with obesity or ascites[83]. The latest Baveno VII guidelines recommended that all cases of clinically advanced chronic liver disease should undergo LSM testing[85].

However, these tests are yet not standardized in children. Pediatric literature regarding LSM and SSM by elastography techniques is emerging. Chongsrisawa *et al*[86] reported significantly higher LSM in biliary atresia patients with esophageal varices than those without (37.72 ± 21.55 *vs* 10.97 ± 8.71 kPa, $P < 0.001$). Yoon *et al*[87] showed that an LSM value of > 18.4 kPa predicted clinically significant PH (CSPH) in children with CLD with a high sensitivity (87.5%) and specificity (84.0%). LSM has been used to monitor and assess treatment response in adult patients with BCS[88]. In a study to assess short- and long-term outcomes in 32 Chinese adults with BCS undergoing REI, Wang *et al*[88] measured LSM using SWE at 2 d, 3 mo, and 6 mo post-procedure. Mean LSM value before the procedure was 35.17 ± 10.60 kPa, which decreased to 15.36 ± 4.34 kPa and 15.68 ± 5.58 kPa at 3 mo and 6 mo post-procedure, respectively ($P < 0.001$). Published literature on the role of LSM in monitoring children with BCS is scarce[89]. Dohare *et al*[89] evaluated the role of LSM in 32 children undergoing REI and showed that LSM values decreased significantly after REI. A maximal decrease is seen 24 h after REI (43.7 kPa at baseline *vs* 22.5 kPa 24 h post-procedure, $P = 0.001$)[75]. Among the nine children developing re-stenosis after REI, re-stenosis was typically associated with an increase in LSM compared with the patient's prior measurement (median absolute increase 11.0 kPa; interquartile range [IQR] 6.1-24.4).

SSM reflects congestion as well as structural changes in the spleen as a direct consequence of the increased PHT[90,91]. SSM is considered a direct and more suitable surrogate marker of PHTN and performs better for the prediction of CSPH [91,92] and esophageal varices[93,94]. Sutton *et al*[94] evaluated SSM in 67 children with chronic liver disease and showed that SSM is a reliable predictor of CSPH at a value > 38.0 kPa [area under receiver operating curve (AUROC) = 0.92, sensitivity = 89%, specificity = 82%, $P < 0.01$]. Sintusek *et al*[95] studied 51 BA children and showed a higher SSM of 46.85 (IQR 25.95-54.55) kPa in patients with varices as compared to the no-varices group [median SSM-16.54 (IQR 11.75-21.75) kPa; $P < 0.001$]. SSM has been performed in adults with BCS ($n = 7$) who underwent TIPSS[96]. In this case series, SSM in combination with LSM may reflect the severity of the disease at presentation and the need for invasive treatment. SSM values also showed a significant decline after TIPSS over a median follow-up period of 1 year. Further studies are required to elucidate the role of LSM and SSM in monitoring BCS patients after REI.

PROGNOSTIC INDICES AND THEIR IMPLICATIONS

To date, many prognostic scores have been developed in patients with BCS to quantify the disease severity and prognosis (Table 3). The authors evaluated the prognostic accuracy of these indices in BCS children and found that pre-intervention PELD score with a cut–off of 4 (AUROC = 0.809, 86% sensitivity, and 75% specificity) significantly determined poor outcomes following REI. Zeitoun score independently predicted poor outcome [OR = 15.4, 95% confidence interval (CI): 1.17-203.56, P = 0.04] with a cut-off of 4.3 (AUROC = 0.923, 83% sensitivity, and 77% specificity) in the unintervened chronic BCS[15]. Hence BCS children with a Zeitoun index > 4.3 should undergo REI without any delay.

LONG-TERM COMPLICATIONS OF BCS

Hepatopulmonary syndrome

Hepatopulmonary syndrome (HPS) occurs in a substantial portion (28%) of adult patients with BCS and balloon angioplasty can reverse HPS in patients with BCS[97]. The mechanism is unknown but may be related to portal decompression. This may also explain the favorable outcomes of TIPSS creation for HPS in patients with cirrhosis and idiopathic PHTN[98]. Sharma *et al*[14] in a previous pediatric study reported the detection of HPS among five children in long-term follow-up, one in an un-intervened child and four after RI with patent stent (3 TIPSS, 1 HV angioplasty). It is not clear as to which patients will have resolution or progress to HPS as contradicting outcomes have been noted. The possible reason for developing HPS even after reduction of portal hypertension post-RI could be increased exposure of pulmonary vasculature to vasodilator mediators like increased levels of nitric oxide, endothelin-1, tumor necrosis factor-α , and endotoxemia[98].

HCC is an uncommon but dreaded long-term complication of BCS[99,100]. In a recent systematic review of adults, the prevalence of HCC in BCS is geographically varied[99]. It is documented as 2.0%–46.2% in 12 Asian studies, 40.0%–51.6% in two African studies, 11.3% in one European study, and 11.1% in one American study[99]. Irrespective of hepatitis as the underlying risk factor of HCC, the pooled prevalence of HCC was 17.6% in BCS patients (95%CI: 10.1%–26.7%), 26.5% in IVC obstruction (95%CI: 14.4%–40.7%), and 4.2% in HV obstruction (95%CI: 1.6%–7.8%). When patients with HCC and concomitant hepatitis were excluded, the pooled prevalence of HCC was 15.4% (95%CI: 6.8%–26.7%). Only 3 out of the 16 included studies evaluated the risk factors for the development of HCC in BCS patients. However, there was significant heterogeneity among these studies and the results were contradicting[99]. Further long-term prospective studies are necessary to evaluate risk factors for HCC in BCS patients. Data regarding the prevalence of HCC in children with BCS is limited. So far, only three cases of HCC in children with BCS have been reported[14,32,101]. All of them had HCC in the 2nd decade of life, liver nodules > 3 cm, and elevated alpha-fetoprotein. One patient was on anticoagulation only[101] while the other two had blocked stents[14,32]. In the authors' own unpublished experience from the authors' center, an 18-year-old boy developed HCC 6 years after blockage of DIPS. Routine surveillance for HCC is thus warranted in BCS patients, even after undergoing REI.

FUTURE DIRECTIONS

Future studies need to elucidate the underlying thrombophilic conditions and their role in the etiopathogenesis of BCS. The use of next-generation sequencing in addition to the conventional thrombophilia work-up is warranted for better understanding and higher diagnostic yield. Further studies are needed to determine the precise role of MRA in differentiating benign liver nodules with HCC in BCS patients. Liver and splenic stiffness measurement by elastography techniques (transient elastography, shear-wave elastography, and MR elastography) may serve as a useful non-invasive marker for assessing treatment response. Despite its rarity, pediatric BCS provides a unique opportunity to study the natural history, long-term complications, and treatment outcome.

CONCLUSION

BCS is a rare but potentially treatable cause of portal hypertension with an excellent prognosis after definitive treatment. Due to recent advances in interventional radiology, radiological endovascular intervention is currently the preferred primary treatment modality. Better patient and procedure selection, choice of appropriate size and type of stent, and mandatory follow-up assessment are of utmost importance for better long-term outcomes. In future, prospective and larger studies should be undertaken to study the epidemiology and establish standardized diagnostic and therapeutic management protocols for pediatric BCS.

Table 3 Budd-Chiari syndrome-specific prognostic indices

Score	Equation	Cut-off	Predicted survival rate
CTP	Serum bilirubin, serum albumin, INR, ascites, HE	-	-
MELD	9.57 × log (creatinine) + 3.78 × log (total bilirubin) + 11.2 × log (INR) + 6.43	-	-
Zeitoun index	Ascites score[a] × 0.75 + Child-Pugh score × 0.28 + age × 0.037 + creatinine × 0.0036	5.4 (range from 3.4 to 9.1)	At 5 yr; ≤ 5.4: 95%; > 5.4: 65%
New Clichy score	0.95 × ascites score + 0.35 × CTP score + 0.047 × age + 0.0045 × serum creatinine + 2.2 × type IIIb[b] −2.6	5.1 (range from 2.0 to 9.7)	At 5 yr; < 5.1: 100%; ≥ 5.1: 65%
Rotterdam BCS index	1.27 × encephalopathy + 1.04 × ascites + 0.72 × prothrombin time + 0.004 × bilirubin	Bilirubin; class I: 0–1.1; class II: 1.1–1.5; class III: ≥ 1.5 (range from 0.02 to 4.03)	At 5 yr; class I: 89%; class II: 74%; class III: 42%
TIPS-BCS index	Age (yr) × 0.08 + bilirubin (mg/dL) × 0.16 + INR × 0.63	7	1-yr OLT-free survival, ≤ 7: 95%; > 7: 12%
BCS-intervention-free survival prognostic score	Ascites (yes = 1, no = 0) × 1.675 + ln creatinine (μmol/L) × 0.613 + ln bilirubin (μmol/L) × 0.440	Interval 1: ≤ 5; interval 2: 5-6; interval 3: ≥ 6	Intervention-free survival interval 1: 78.3%, interval 2: 27.8%; interval 3: 6.8%
AIIMS-HVOTO score	1.2 × response to therapy + 0.8 × child class)	Score: < 3; 3-4; > 4	% yr survival score ≤ 3: 92%; score 3-4: 79%; score > 4: 39%

[a]Ascites score: 1, absent with free sodium intake and no diuretic agents; 2, easy to control with sodium restriction or diuretic agents; and 3, resistant to this treatment because of hyponatremia or functional renal failure.
[b]Type III' is a binary variable coded as 1 for patients with clinicopathological findings of acute injury superimposed on chronic lesions, and 0 for the other patients.
CTP: Child-Turcotte-Pugh, MELD: Model for end stage liver disease; BCS: Budd-Chiari syndrome; AIIMS: All India Institute of Medical Sciences; HVOTO: Hepatic vein outflow tract obstruction; OLT: Orthotopic liver transplantation.

FOOTNOTES

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MINIREVIEWS

Risk of hepatitis B reactivation in patients with myeloproliferative neoplasms treated with ruxolitinib

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Abstract

Classical Philadelphia-negative myeloproliferative neoplasms (MPNs), *i.e.*, polycythemia vera, essential thrombocythemia, and primary/secondary myelofibrosis, are clonal disorders of the hematopoietic stem cell in which an uncontrolled proliferation of terminally differentiated myeloid cells occurs. MPNs are characterized by mutations in driver genes, the JAK2V617F point mutation being the most commonly detected genetic alteration in these hematological malignancies. Thus, JAK inhibition has emerged as a potential therapeutic strategy in MPNs, with ruxolitinib being the first JAK inhibitor developed, approved, and prescribed in the management of these blood cancers. However, the use of ruxolitinib has been associated with a potential risk of infection, including opportunistic infections and reactivation of hepatitis B. Here, we briefly describe the association between ruxolitinib treatment in MPNs and hepatitis B reactivation.

Key Words: Ruxolitinib; Myeloproliferative neoplasms; Hepatitis B; Polycythemia vera; Myelofibrosis; JAK inhibitor

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Core Tip: The JAK inhibitor ruxolitinib has been approved for the treatment of classical Philadelphia-negative myeloproliferative neoplasms (MPNs), *i.e.*, polycythemia vera, essential thrombocythemia and primary/secondary myelofibrosis. However, its use has been associated with a potential risk of opportunistic infections, including hepatitis B reactivation. Herein, we briefly overview the association between ruxolitinib treatment in MPNs and hepatitis B reactivation.

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INTRODUCTION

Introduction to hepatitis B virus reactivation

Hepatitis B virus (HBV) infection is the most common chronic viral infection in the world. It affects more than 350 million people worldwide as chronic carriers, and more than 2 billion (30% of the world's population) people show evidence of past exposure. Additionally, HBV infection has accounted for roughly half of total liver cancer mortality in 2010[1,2]. Once contacted, the virus cannot be eliminated, even with proper and rapid antiviral treatment, but the infection is self-limiting in more than 95% of immunocompetent adults. These patients are now known as carriers 'anti-HBc positive'. They do not require specific management or monitoring unless immunosuppression is suspected[3].

If HBV persists for more than 6 mo in the body, the affected individual is considered to have chronic hepatitis B. Its incidence depends on the time of exposure: 95% of newborns, 20%-30% of children aged 1 to 5 years, and less than 5% of adults[3]. The reason for this dormant state of HBV is the presence of covalently closed circular viral DNA (cccDNA) that penetrates and persists indefinitely in hepatocyte DNA[2-4]. This cccDNA acts as a template for future viral components in the case of HBV reactivation (HBVr). Viral transmission has been greatly slowed recently by the advent of a safe and effective vaccine, available since 1981 and introduced in 2011 in routine vaccination schedules in more than 180 countries [1,5].

DEFINITION, EPIDEMIOLOGY AND MANIFESTATIONS OF HBVR

The number of cases of HBVr after treatment with immunosuppressive agents is increasing worldwide, mostly attributed to an increase in the prevalence of positive HBV serology and, at the same time, an increase in the number of clinical indications for potent immunosuppression, including solid malignancies, inflammatory bowel disease, autoimmune disorders, blood cancers, *e.g.* myeloproliferative neoplasms (MPNs), and rheumatic diseases[3].

There are, although very similar, several definitions of HBVr, proposed by several medical associations from around the globe. All of them take into account both virological and serological criteria and describe HBVr as either an exacerbation of chronic hepatitis B or a reactivation of past hepatitis B infection. The most used definition is the one proposed by the American Association for the Study of Liver Diseases, last updated in 2020, which defines HVBr according to the virological status of the patient[4,6-8].

For HBsAg-positive patients with or without detectable HBV DNA: (1) At least 2 Log (or 100-fold) increase in HBV DNA compared to the baseline level; (2) HBV DNA at least 3 Log (or 1000) IU/mL in patients with previously undetectable HBV DNA; or (3) HBV DNA at least 4 Log (or 10000) IU/mL if the baseline level is unavailable[4,6-8].

For patients with HBsAg negative and HBV DNA negative: (1) HBV DNA becomes detectable; or (2) reverse HBsAg seroconversion (reappearance of HBsAg)[4,6-8].

The natural history of HBVr depends, among others, on the underlying disease requiring immunosuppressives, host immunity and the immunosuppressive agents used. Evolution can be classified into multiple stages[4,6-8].

After the initiation of immunosuppressive therapy, viral replication resumes, leading to a gradual increase in serum HBV DNA levels. The patient is still asymptomatic and, in general, HBVr-related hepatitis, described as an increase in alanine transaminase (ALT) or aspartate transaminase (AST) to 3 times upper limit of normal (ULN), does not develop[4,6-8].

HBVr-related hepatitis

ALT or AST increases to ≥ 3 times ULN (in some cases between 5-10 times ULN). Although most patients may remain asymptomatic, a small number might experience constitutional symptoms, such as pain in the right upper quadrant and jaundice. In rare cases, hepatic injury could further progress and cause liver failure, fulminant hepatitis or even death[4,6-

8].

Spontaneous or antiviral-induced resolution

Normalization of serum ALT and AST levels, due to completion of immunosuppressive therapy, due to antiviral therapy, or due to host immunological mechanisms[4,6-8].

Acute liver failure/persistent liver injury

Found in a small number of individuals who continue to have a progressive decline in liver function, it is characterized by increased levels of bilirubin, prolonged prothrombin time, and, in very rare cases, even signs and symptoms of acute liver failure and hepatic decompensation (ascites and encephalopathy)[4,6-8].

MECHANISMS OF HBVR

As previously mentioned, after entering the hepatocytes, the viral genome is converted into plasmid-like cccDNA which can persist in liver cells in a latent state, serving as a reservoir for HBVr, in spite of active anti-HBV immune response. Compared to the hepatitis C virus (HCV) infection, complete eradication of both HBV cccDNA and integrated DNA is impossible with current antiviral treatment with nucleos(t)ide analogs. Thus, these cells constitute a reservoir of persistent HBV. Although HBVr can occur in a variety of settings, immunosuppressive therapies are the most commonly reported. A detailed description of the HBVr induction mechanisms of immunosuppressive therapies is provided in Table 1[3,4,6-12].

RISK FACTORS FOR HBVR

Host-related risk factors for HBVr include male sex, younger age and older age (the elderly are more likely to have HBsAg seroclearance but persistent levels of total HBV DNA and cccDNA in the liver) and have been associated with increased risk of HBVr. Preexisting conditions, for example, cirrhosis or MPNs, also play a role in HBVr. HBVr has been reported in patients with MPNs, lymphomas, myeloma, and acute myeloid leukemia. However, it is not yet clear whether this association is attributed to the underlying disease or to the potent immunosuppressants used in the management of these blood cancers[7-9].

Virological factors include HBsAg and HBeAg positivity (adding a 5- to 8-fold risk for HBVr), non-A HBV genotypes, elevated HBV DNA levels before starting immunosuppressive therapy, and co-infection of HBV with other viruses such as HIV and HCV[4,7,8].

Type of immunosuppression: the greatest risk of HBVr is represented by the use of B-cell depleting therapies, used in the therapeutic armamentarium of blood and solid cancers and in the setting of bone marrow or solid organ transplantation[3,4,6-12]. More details are presented in Table 1.

PREVENTION OF HBVR

Identifying infected individuals is the first and most important step for HBVr prophylaxis. According to the latest specialty guidelines, HBV infection screening must be performed in all patients who are receiving immunosuppressive treatment. Furthermore, all patients who are HBcAg positive, regardless of the status of HBsAg or the HBV DNA values, must receive prophylactic antiviral treatment. In numerous studies, prophylactic antiviral treatment has been shown to reduce the rate of HBVr, liver failure, and death in these categories of patients. Even if lamivudine was the first and for many years the most used oral antiviral agent for HBVr prophylaxis, *YMDD* gene mutations cause a high incidence of viral resistance if used for > 6 mo. This is why entecavir or tenofovir are recommended as therapies for HBVr prevention if intended for longer periods of time[4,6-8].

Duration of antiviral prophylaxis

In general, the duration of antiviral therapy varies depending on the type of immunosuppressives used. General recommendations include the use of antiviral therapy for at least 6 mo after the last dose of immunosuppressive agents is administered. However, in the case of B cell-depleting therapies (such as rituximab or obinutuzumab), it is recommended that antiviral prophylaxis be continued up to 12 mo after the last dose. Another important step is routine testing for HBV DNA and serum ALT and AST 3-6 mo after discontinuation of immunosuppressives[3,7].

Moreover, particular attention should be given to preventive measures, such as instructing patients to withdraw from alcohol consumption, as well as close monitorization of liver function tests in subjects who are prescribed pharmacological agents with a potentially hepatotoxic effect[13,14]. According to the findings of the Dionysos Study, individuals diagnosed with HBV who consume alcohol experience elevated rates of hepatic fibrosis and death[13].

Table 1 Immunosuppressive agents associated with HBVr

Immunosuppressive therapies with high risk of HBVr							
B-cell depleting therapies (rituximab and ofatumumab)		Anthracycline derivatives (doxorubicin and epirubicin)	Corticosteroids	TNF-α inhibitors (infliximab, adalimumab, certolizumab)			Anti-CD52 monoclonal antibody (alemtuzumab)
Increased HBVr risk in positive HBsAg and negative HBsAg and anti-HBc subjects by acting against the B-lymphocyte antigen CD20; The Food and Drug Administration has placed a black box warning for rituximab regarding HBVr in rituximab-treated individuals; used to treat CD20+ blood cancers (lymphomas, CLL) and IRD; B cells play a previously underestimated role in HBV immune control by producing neutralizing antibodies; rituximab associated with > 5× increase in HBVr risk (incidence 3%–55%, overall mortality rate 30%–38%)		High-risk for patients with hepatocellular carcinoma and hepatitis B undergoing TACE; used to treat lymphomas and acute leukemias, breast and ovarian cancer, and sarcoma; HBVr rate = 41% in patients with HBsAg positive	Prednisone use > 20 mg p.o. daily > 4 wk	TNF-α can activate the APOBEC antiviral pathway which causes the degradation of cccDNA in HBV-infected cells. HBVr pooled incidence in patients with resolved HBV infection = 3.0% *vs* 15.4% in HBsAg positive patients			Used for refractory CLL; causes reverse HBsAg seroconversion and reactivation-related hepatitis
Immunosuppressive agents with moderate risk of HBVr							
Less potent TNF-α inhibitors (etanercept)	Cytokine or integrin inhibitors (abatacept, ustekinumab, natalizumab, vedolizumab)	Tyrosine kinase inhibitors (imatinib, nilotinib, dasatinib)	Proteasome inhibitors: (Bortezomib)	Histone deacetylase inhibitors (HDIs) (romidepsin)		Prednisone 10-20 mg p.o. daily > 4 wk	Calcineurin inhibitors (cyclosporine or tacrolimus)
Moderate risk of HBVr in patients with HBsAg positive (1%-5%) and even lower in patients with HBsAg negative	Commonly utilized in the treatment of IBD, IRD and dermatologic conditions; inhibit local inflammatory response associated with immune-mediated diseases by blocking the localization and traffic of activated lymphocytes	Standard of treatment for all phases of CML; also used in the treatment of GIST; inhibit various kinase signaling pathways, essential for immune activation and proliferation of lymphocytes, with an important role in immune control of HBV replication; prophylactic antiviral therapy and regular monitoring of HBV DNA and liver enzymes are essential; reported HBVr rates of 26%–34.8%	Used for the treatment of MM and induction therapy for transplant- eligible patients prior to stem cell harvest; target cellular pathways that interfere with the functions of healthy B cells, which are important in HBV immune control	Used in the treatment of T-cell lymphomas; inhibit histone deacetylase, a histone-modifying enzyme that is important for epigenetic regulation of gene expression with possible deacetylation status of silent cccDNA, resulting in active HBV transcription and then HBVr		The mechanism is two-fold: The HBV genome contains a transcription regulatory element responsive to glucocorticoid that is up-regulated by corticosteroids, resulting in increased viral replication; a directly suppressive effect on cytotoxic T cells that are involved in HBV control; risk of HBVr of 10%-15.8% in HBsAg positive individuals	Suppress T cell function by inhibiting calcineurin required for signal transduction of T cell activation and inhibiting transcription of interleukin required for T cell proliferation
Immunosuppressive agents with low risk of HBVr							
Methotrexate, azathioprine or 6-mercaptopurine				Intra-articular steroid injections or prednisone < 10 mg p.o. daily			
Documented cases of HBVr are rather rare							
Novel therapies							
Immune checkpoint inhibitors such as anti-PD-L1 (nivolumab) and anti-CTLA4 (ipilimumab)	BTK inhibitor ibrutinib and PI3K delta inhibitor idelalisib	Ruxolitinib	Mogamulizumab	Brentuximab	Obinutuzumab	Hypomethylating agents: Decitabine, azacitidine	Daratumumab

HBVr rarely reporter; anti-HBV prophylaxis is recommended	B-cell receptor signaling modulators; approved by the FDA for the treatment of CLL and certain low-grade NHL; HBVr has been rarely been reported; anti-HBV prophylaxis is recommended	A novel inhibitor of JAK1 and JAK2 that has been approved for the treatment of patients with MPNs; There are reported cases of HBVr	Humanized monoclonal antibody targeting the C-C chemokine receptor 4; Used for ATLL; HBVr cases have been reported	Anti-CD30 drug conjugated antibody; used in the treatment of relapsed or refractory HL and CD30 positive T-cell lymphoma; There are reported cases of HBVr	Newer generation anti-CD20 monoclonal antibody, similar to rituximab but with greater efficacy; FDA has mandated a warning of the risk of HBVr with obinutuzumab and HBVr has been reported	Used in the treatment of AML; anti-HBV prophylaxis is recommended	Monoclonal antibody against CD38; used in the treatment of hematologic malignancies of B cells; HBVr cases have been reported

CML: Chronic myeloid leukemia; GIST: Gastrointestinal stromal tumors; ATLL: Adult T-cell leukemia/lymphoma; AML: Acute myeloid leukemia; CLL: Chronic lymphocytic leukemia; IRD: Inflammatory rheumatic diseases; TACE: transarterial chemoembolization, APOBEC: Catalytic polypeptide-like apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like; MM: Multiple myeloma; NHL: Non-Hodgkin's lymphomas; HL: Hodgkin's lymphoma; HBVr: Hepatitis B virus reactivation.

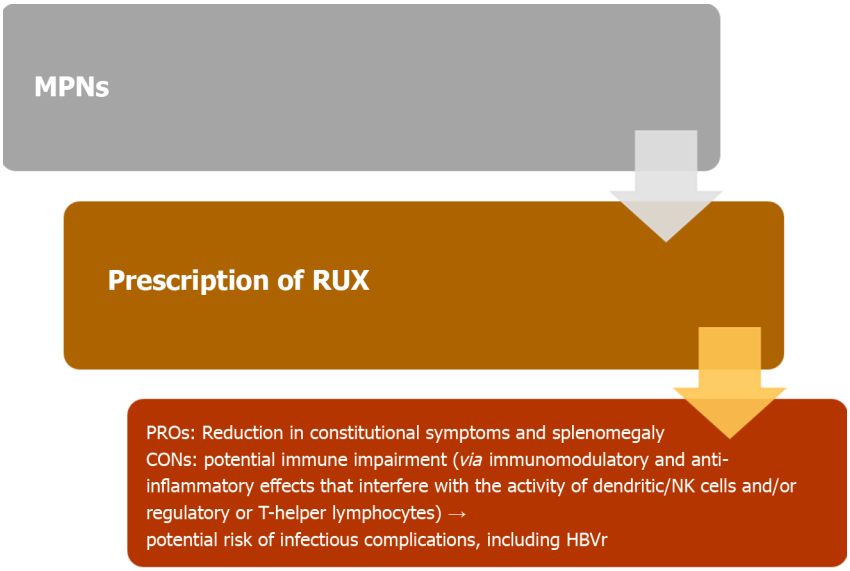
HBVR RISK IN MPNS TREATED WITH RUXOLITINIB

Ruxolitinib is a commonly used medication to treat MPNs, a group of blood disorders characterized by excessive blood cell production in the bone marrow. One of the common manifestations of MPNs is splenomegaly. Ruxolitinib acts by inhibiting Janus kinases (JAK1 and JAK2), which are enzymes involved in signaling pathways associated with cytokine receptors. By inhibiting these enzymes, ruxolitinib effectively helps control MPNs, particularly intermediate and high-risk myelofibrosis (MF) and high-risk polycythemia vera (PV). Importantly, its effect is not specific to any particular mutation. Ruxolitinib shows good oral bioavailability and reaches its maximum plasma concentration within 1-2 h after administration. Plasma half-life of this drug is approximately 3 h when administered at a maximum tolerated dose of 100 mg once a day. It is mainly metabolized through the CYP3A4 pathway, an important liver enzyme system involved in drug metabolism. Consequently, ruxolitinib has the potential for interactions with medications that induce or inhibit the CYP3A4 pathway. Ruxolitinib is primarily eliminated from the body through metabolism in urine and feces. Therefore, dosage adjustments are necessary for patients with renal or liver impairments, as these conditions can affect the clearance of the drug from the body[15].

It is important to note that this pharmacological agent exhibits immunomodulatory effects, meaning that it can modify the functioning of the immune system. As a result, ruxolitinib treatment may increase susceptibility to opportunistic infections in patients prescribed this drug. Thus, regular monitoring for signs of infection is important when subjects diagnosed with MPNs start taking this medicine[16]. In particular, this pharmacological agent exhibits immunomodulatory and anti-inflammatory actions and can interfere with or impair the innate/adaptive immune response due to its interplay with dendritic cells, regulatory/T-helper lymphocytes or natural killer cells[17,18].

In a case report by Sjoblom *et al*[19], a patient with a history of PV received initial treatment with hydroxyurea. However, due to progressive splenomegaly and fatigue, his treatment was changed to pegylated interferon. Furthermore, to more effectively manage his symptoms, ruxolitinib was introduced. The patient experienced HBVr while on ruxolitinib, which was confirmed by abnormal liver function test results, positive viremia, and newly positive surface antigen for hepatitis B (HbsAg). With the initiation of tenofovir disoproxil, the patient's liver function gradually normalized, indicating successful management of HBVr[19]. In another report by Shen *et al*[20], a patient with MF and a history of HBV infection experienced HBVr during ruxolitinib treatment. The initial elevation in transaminase levels was mistakenly attributed to drug toxicity. Subsequent detection of high plasma levels of HBV DNA confirmed the reactivation. Ruxolitinib was discontinued and antiviral therapy was started, resulting in a gradual decrease in transaminase levels[19,20]. Additionally, in another report by Passucci *et al*[21], a patient with PMF and previous HBV infection achieved resolution of splenomegaly with ruxolitinib therapy. However, HBVr occurred after the patient discontinued prophylactic lamivudine. De-escalation of ruxolitinib and the initiation of anti-HBV therapy led to a gradual decline in HBV DNA levels without signs of active hepatitis[21]. Kirito *et al*[22] highlight the importance of considering prophylactic antiviral therapy in patients with chronic HBV infection before starting treatment with ruxolitinib, as such a proactive measure can help prevent HBVr, as observed in their patient[22].

Ruxolitinib has an immunosuppressive effect, leading to an increased risk of serious infections. The immunosuppressive effect of ruxolitinib is due to its interaction with multiple pathways of the immune system, affecting both adaptive and innate immune responses. This can result in the reactivation of silent infections such as tuberculosis, HBV, and varicella-zoster virus. Therefore, proactive infection surveillance, baseline screening for latent infections, and considering prophylactic or preventive interventions for specific infections such as varicella-zoster virus and HBV virus are crucial[23]. A pilot study conducted by Crodel *et al*[24] investigated the frequency of infections in patients with MPNs. The study included multiple centers and relied on patient-reported data. The findings revealed that over 50% of MPN patients experienced one or more episodes of infection within a 12-mo period. The most frequently reported infections were upper respiratory tract infections, herpes virus infections, and gastrointestinal infections. Among the different subtypes of MPNs, subjects with MF had the highest percentage of infectious events, followed by PV and essential thrombocythemia[24]. Furthermore, Lussana *et al*[25] conducted a systematic review and meta-analysis



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Figure 1 Benefits and risks of ruxolitinib use in terms of opportunistic infections in myeloproliferative neoplasms. MPNs: Myeloproliferative neoplasms; RUX: Ruxolitinib; HBVr: Hepatitis B virus reactivation; NK cells: Natural killer cells.

examining the safety and efficacy of ruxolitinib in the treatment of MF and PV. The study specifically focused on the incidence of infections in patients receiving ruxolitinib. It was found that ruxolitinib, with its immunosuppressive effects, can affect immune functions and increase the risk of infections. Herpes zoster, pneumonia, bronchitis, and urinary tract infections were among the most frequently reported infectious complications. The aforementioned quantitative assessment emphasized the importance of carefully evaluating infection risk before initiating ruxolitinib therapy and highlighted the need to monitor and address infections in patients receiving ruxolitinib for MF and PV[25].

In a paper by Perricone *et al*[26], two case reports of HBVr in MF patients treated with ruxolitinib are discussed. The immunosuppressive effects of ruxolitinib, particularly in dendritic cells and T cells, may contribute to an increased risk of infections, including HBVr. The article emphasizes the need for vigilance among physicians when considering infectious causes when using immunosuppressive agents such as ruxolitinib[26]. In a prospective study by Gill *et al*[27], 40 patients with MPNs were included. Among the 37 subjects who were negative for HBsAg, 15 tested positive for anti-HBc antibodies, indicating occult HBV infection. Prophylactic treatment for HBV was administered to the three HBsAg positive patients. During a median follow-up of 19.2 mo, four patients (26.7%) experienced HBVr, occurring at a median of 10.5 mo after starting ruxolitinib therapy. The estimated cumulative incidence rates of HBVr at 6 and 12 mo were 7.7% and 30.8%, respectively. This investigation emphasizes the need to monitor HBVr in patients with occult HBV infection who receive ruxolitinib therapy[27]. Garcia-Horton *et al*[28] conducted a retrospective cohort study involving 1171 individuals with MPNs to evaluate the risk of HBVr in subjects treated with ruxolitinib. Among the 58 patients with prior HBV infection, 20 received ruxolitinib. Only one patient experienced HBVr during ruxolitinib therapy, and their HBV DNA levels peaked, but subsequently returned to undetectable levels without interrupting or reducing the ruxolitinib dose[28]. Duan *et al*[29] conducted a retrospective analysis to evaluate the incidence of HBVr in MPN patients treated with ruxolitinib. The study included 62 patients with a history of HBV infection, 56 with resolved infection and 6 with chronic HBV infection. Among patients with chronic HBV infection, two experienced HBVr and hepatitis flare-up after ruxolitinib therapy. None of the patients with resolved HBV infection experienced reactivation. In particular, the two patients with chronic HBV infection did not receive antiviral prophylaxis[29]. Caocci *et al*[30] presented a case report of a patient with MF who experienced HBVr during treatment with ruxolitinib. The patient had a history of HBV infection and initially received ruxolitinib for symptoms related to MF. Although there was improvement in MF symptoms, HBVr was observed through increased levels of HBV-DNA. Adjusting the dose of ruxolitinib resulted in an improvement in symptoms, but HBV-DNA levels remained fluctuating. This case report raises concerns about the management of MF patients with HBV infection receiving ruxolitinib and emphasizes the importance of careful monitoring and potential prophylactic treatment[30].

A schematic representation between the benefits and risks of ruxolitinib use in terms of opportunistic infections in MPNs is depicted in Figure 1.

CONCLUSION

In conclusion, ruxolitinib is an effective medication to manage MPNs such as MF and PV, particularly in intermediate and high-risk cases. By inhibiting JAK1 and JAK2, ruxolitinib helps control excessive blood cell production and reduce splenomegaly. However, its use carries certain risks and considerations. The interaction of ruxolitinib with the immune system can increase the susceptibility to opportunistic infections, highlighting the need for vigilant monitoring and timely

intervention. Furthermore, there is a potential for HBVr, especially in patients with a history of HBV infection. Close monitoring of liver function and proactive measures, such as prophylactic antiviral therapy, are crucial to managing these risks. In general, ruxolitinib offers therapeutic benefits for MPNs, but careful evaluation of infection risk, regular monitoring, and appropriate interventions are essential to ensure patient safety.

FOOTNOTES

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MINIREVIEWS

Function of macrophage-derived exosomes in chronic liver disease: From pathogenesis to treatment

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Abstract

Chronic liver disease (CLD) imposes a heavy burden on millions of people worldwide. Despite substantial research on the pathogenesis of CLD disorders, no optimal treatment is currently available for some diseases, such as liver cancer. Exosomes, which are extracellular vesicles, are composed of various cellular components. Exosomes have unique functions in maintaining cellular homeostasis and regulating cell communication, which are associated with the occurrence of disease. Furthermore, they have application potential in diagnosis and treatment by carrying diverse curative payloads. Hepatic macrophages, which are key innate immune cells, show extraordinary heterogeneity and polarization. Hence, macrophage-derived exosomes may play a pivotal role in the initiation and progression of various liver diseases. This review focuses on the effects of macrophage-derived exosomes on liver disease etiology and their therapeutic potential, which will provide new insights into alleviating the global pressure of CLD.

Key Words: Chronic liver disease; Macrophage; Exosomes; Function; Etiology; Treatment

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Core Tip: Chronic liver disease (CLD) affects hundreds of millions of individuals worldwide, and identifying the causes and researching viable therapies could lessen the global burden. As nanovesicles produced by cells, exosomes are able to facilitate intercellular communication and play a crucial role in a variety of systemic disorders. Immune cells such as macrophages are intimately associated with liver diseases. In this review, the importance of macrophage-derived exosomes in CLD, from pathophysiology to therapeutic potential, is highlighted.

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INTRODUCTION

Chronic liver disease (CLD) imposes a heavy burden on millions of people worldwide. A total of 1.5 billion people worldwide had suffered from CLD by 2017[1]. Approximately 2 million individuals worldwide die each year from liver disease[2]. Chronic hepatitis, cirrhosis and liver cancer are the three main CLDs. Among the ranked global causes of death, cirrhosis is 11th, while hepatocellular carcinoma (HCC) is 16th[3]. We reviewed numerous studies and found that the pathogenesis of CLD is multifactorial. Most liver diseases are associated with steatosis, oxidative stress, alcoholism, inflammation[4], the environment, the microbiome, metabolism, and genetic factors[5]. However, in addition to liver transplantation, current therapy consists only of eliminating the etiology and treating the complications of cirrhosis[6]. Therefore, the exploration of the pathogenic mechanism and identification of optimal treatment strategies are urgently needed.

Exosomes can be released into biological fluids by all cells under physiological or pathological conditions. Exosomes are produced by budding and have apparent molecular heterogeneity because of various membrane-related protein complexes. Exosomes contain components including RNAs, DNAs, lipids, proteins, amino acids, and metabolites[7]. These soluble and extracellular components can enter cells through endocytosis and plasma membrane invagination, which involve the surface proteins on exosomes[8]. Substances within exosomes stimulate recipient cells, thereby altering signal transduction pathways[9]. Therefore, by carrying these payloads, exosomes can mediate intercellular communication[10]. When exosomes are taken up by other cells, the components alter the phenotype of recipient cells and disrupt the dynamic equilibrium of cellular transformation, demonstrating their unique functions in maintaining cellular homeostasis[11,12]. Furthermore, exosomes may serve as biomarkers or therapeutic targets in the diagnosis and treatment of various diseases[9,11,13]. At present, exosomes have been widely studied in liver diseases. In alcoholic liver disease (ALD)[14], alcohol increases the generation of exosomes, which is due to autophagic damage and the destruction of autophagosomes or lysosomes. During the progression of nonalcoholic fatty liver disease (NAFLD)[15], hepatocyte-derived exosomal microRNA (miR)-192-5p can activate proinflammatory macrophages. In liver fibrosis[16], activated hepatic stellate cells (HSCs) release fibrogenic vesicles. Furthermore, engineered exosomes can be used for drug delivery. For instance, Tang *et al*[17] showed that exosomes from mesenchymal stem cells (MSCs) modified to carry small interfering RNA or an antisense oligonucleotide targeting signal transducer and activator of transcription 3 (STAT3) could directly inhibit STAT3 to treat fibrosis. Lou *et al*[18] also modified exosomes from MSCs to carry miR-199a-3p, which improved the chemotherapy sensitivity of liver cancer, proving that exosomes can be used as novel therapeutic agents by acting as nanocarriers to deliver drugs or molecules. In conclusion, exosomes can drive or inhibit disease progression and have potential utility in liver disease therapy.

Macrophages are the core cellular component of the liver and are crucial in maintaining organ homeostasis and coping with liver injury[19]. As key immune cells, they contribute to the development of hepatic disease by polarizing into diverse phenotypes in response to microenvironmental stimulation[20] or expressing their heterogeneity through the production of cytokines, cell surface markers, and transcriptomes[21]. Due to the specific physiological roles of macrophages, the involvement of macrophage-derived exosomes in the development and progression of liver disease has been extensively studied. For instance, relaxin is an antifibrotic peptide hormone that affects vasodilation, thereby alleviating fibrosis and protecting organs through its cognate G protein-coupled receptor relaxin family peptide receptor 1. A study showed that after binding to receptors expressed by hepatic macrophages, relaxin can change the phenotype of macrophages, and macrophages can release exosomes to promote relaxin-mediated HSC dormancy and alleviate hepatic fibrosis (HF)[22]. *In vitro*, interleukin-6 (IL-6) treatment upregulated exosome generation-related genes, stimulating the release of miR-223-rich exosomes from macrophages, which can transfer and reduce the expression of fibrogenic TAZ in liver cells to alleviate liver fibrosis[23]. However, macrophage-derived exosomes can also accelerate disease progression; for example, exosomes derived from macrophages treated with lipopolysaccharide (LPS) promote liver fibrosis[24], and exosomes derived from M2 macrophages can mediate HCC metastasis[25]. At present, determining how to use macrophage-derived exosomes to treat disease has become a research hotspot. Studies have shown that macrophage-derived exosomes can be used as natural nanocarriers to deliver proteins[26] or drugs[27], induce immune activation and participate in immunotherapy[28]. Overall, investigating the various physiological functions of macrophage-derived exosomes can lead to a better understanding of the pathogenesis and treatment of CLD.

This review discusses the biology and physiological functions of exosomes, focusing on exosomes derived from macrophages in the etiology of liver disease, as well as the possible use of exosomes in diagnosis, prognosis, and treatment, which will help in the search for the best therapeutic strategies for liver disease and contribute to reducing the global burden of liver disease (Figure 1).

OVERVIEW OF MACROPHAGE-DERIVED EXOSOMES

Exosomes are a subtype of extracellular vesicles secreted by all cells and are widely distributed in various body fluids. These factors mediate cell-to-cell communication and play specific roles in normal physiological functions and the occurrence of diseases. Macrophages are important immune cells in the human body, and their unique heterogeneity and phenotype result in different physiological effects. In addition, macrophage-derived exosomes participate in the occurrence of diseases in various systems.

Biology of exosomes

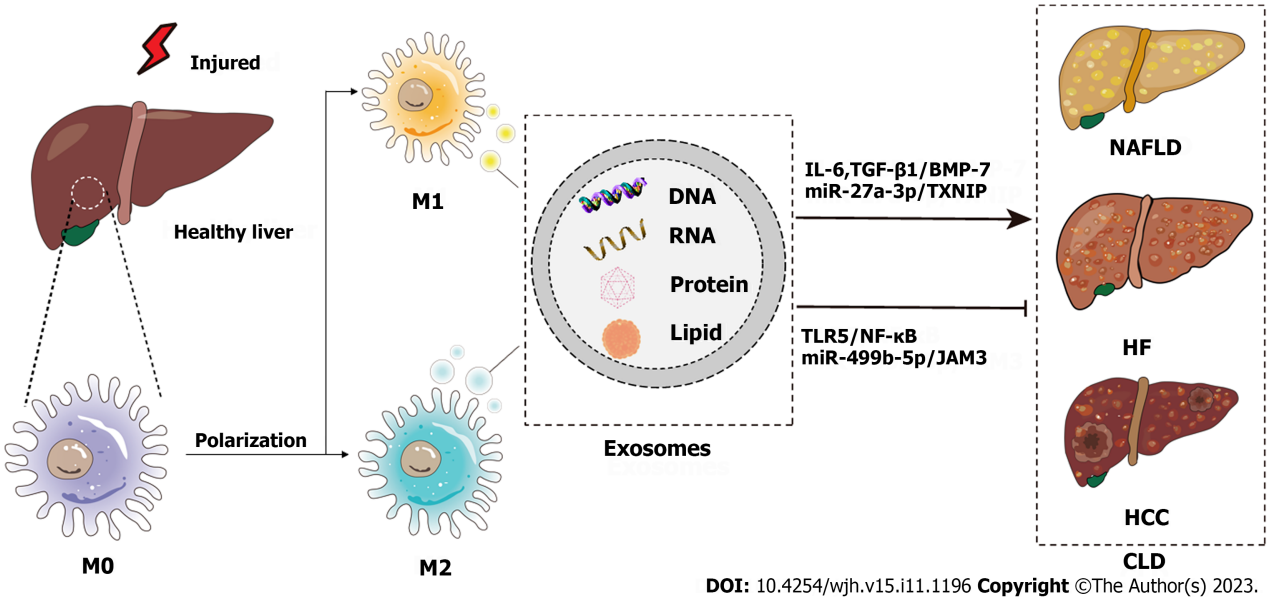
Exosomes are small nanoscale vesicles with diameters of 30-150 nm[29]. They originate in endosomes and form mature exosomes during interactions with other vesicles or organelles[8]. First, the cell invaginates the cell membrane through budding to generate clathrin bodies, which enter the cytoplasm and form early endosomes[30,31]. Late endosomes mature from early endosomes by interacting with the Golgi complex, which can form intraluminal vesicles (ILVs) by invagination of the restrictive membrane[10]. ILVs are then further endocytosed to generate multivesicular bodies (MVBs), which are known as multivesicular endosomes[8]. Ultimately, MVBs have two outcomes: Some MVBs enter the lysosomal pathway and are degraded by the lysosome, while others fuse with the cell membrane and release multiple vesicular structures into the extracellular matrix as exosomes[31]. Therefore, the biogenesis of exosomes can be divided into the following processes: Budding, envelope invagination, MVB production, and MVB release. All cells, whether normal or abnormal, can release exosomes, and cancer cells release more exosomes than other cells[9]. Exosomes can exhibit unique characteristics based on their cell origin and material composition. As a double lipid-encapsulated vesicle, exosomes contain a variety of substances, such as DNAs, RNAs, lipids, and proteins, and the composition or contents of these vesicles vary depending on the cell source[32]. Moreover, exosomes can be isolated from various types of body fluids, such as plasma[33], serum[34], and urine[35]. In summary, exosomes are tiny vesicles secreted by various cells, and their secretion mechanism is related to membrane fusion. In addition, secreted exosomes can be widely distributed throughout the body, enabling them to play a role in a variety of systems.

Function of exosomes

Because structure determines function, the function of exosomes is dependent on their complex and specific characteristics, which are determined by the cell type from which they are derived. Exosomes can be involved in the immune response, antigen presentation, cell migration, cell differentiation, tumor invasion, and other processes. The most important function of exosomes is to mediate intercellular communication[9]. Exosomes transfer cargo to recipient cells by binding to cell surface receptors, undergoing plasma membrane fusion, or through the endocytic system[36], which can activate signaling pathways, alter gene expression, or regulate the overall function of the recipient cell. For example, the decrease in hsa_circ_0074854 carried by exosomes secreted by HCC cells can inhibit M2 macrophage polarization, thus delaying the migration and invasion of HCC cells[37]. Cancer-associated fibroblasts can transfer exosomal miRNAs directly to tumor cells and enhance their cell-related functions, such as epithelial-mesenchymal transition and chemoresistance[38]. Therefore, exosomes can mediate material exchange between cells, are involved in cellular communication, and reflect the different physiological functions of cells; thus, they are often used as biomarkers of diseases or targeted delivery vectors of substances. The expression levels of some miRNAs in serum exosomes are significantly upregulated in patients with pancreatic cancer; thus, some miRNAs are considered useful markers for the early diagnosis and progression of pancreatic cancer[39]. The delivery of engineered exosomes loaded with an iron shedding inducer [erastin (ER)] and photosensitizer (rose bengal) into tumor tissue can specifically induce ferroptosis in HCC cells, which can be used as a new treatment strategy for malignant tumors[40]. In conclusion, exosomes have wide-ranging functions. Whether as carriers of substances or as signaling factors, exosomes have powerful cellular communication functions, which is an area for future basic research to determine clinical applications.

Exosomes derived from macrophages

Macrophages are immune cells that can be found in most tissues and serve numerous roles[41]. For instance, macrophages are derived from monocytes and have phagocytic functions; these cells can engulf and kill intracellular parasites, bacteria, and tumor cells, as well as aging and abnormal cells, which is critical for immune defense, immune stability, and immune surveillance. In addition, macrophages are uniquely heterogeneous. In a dynamically changing microenvironment, macrophages can exhibit two phenotypes that perform different functions: Classically activated macrophages (M1) and alternatively activated macrophages (M2)[42]. M1 macrophages can be activated by LPS alone or in combination with T-helper 1 (Th1) cytokines [such as interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor] and can produce proinflammatory cytokines, such as IL-1β, IL-6, IL-12, IL-23, tumor necrosis factor (TNF)-α, chemokine (C-X-C motif) ligand (CXCL) 1-3, CXCL8-10, chemokine (C-C motif) ligand (CCL)2-5, and CCL11. Therefore, M1 macrophages are able to mediate functions such as antigen presentation, Th1 immune reactions, proinflammatory effects, pathogen elimination, and antitumor activity[43-46]. However, M2 macrophages can be further divided into M2a, M2b, M2c, and M2d subtypes and can release complex cytokines. Specifically, when injury causes an acute

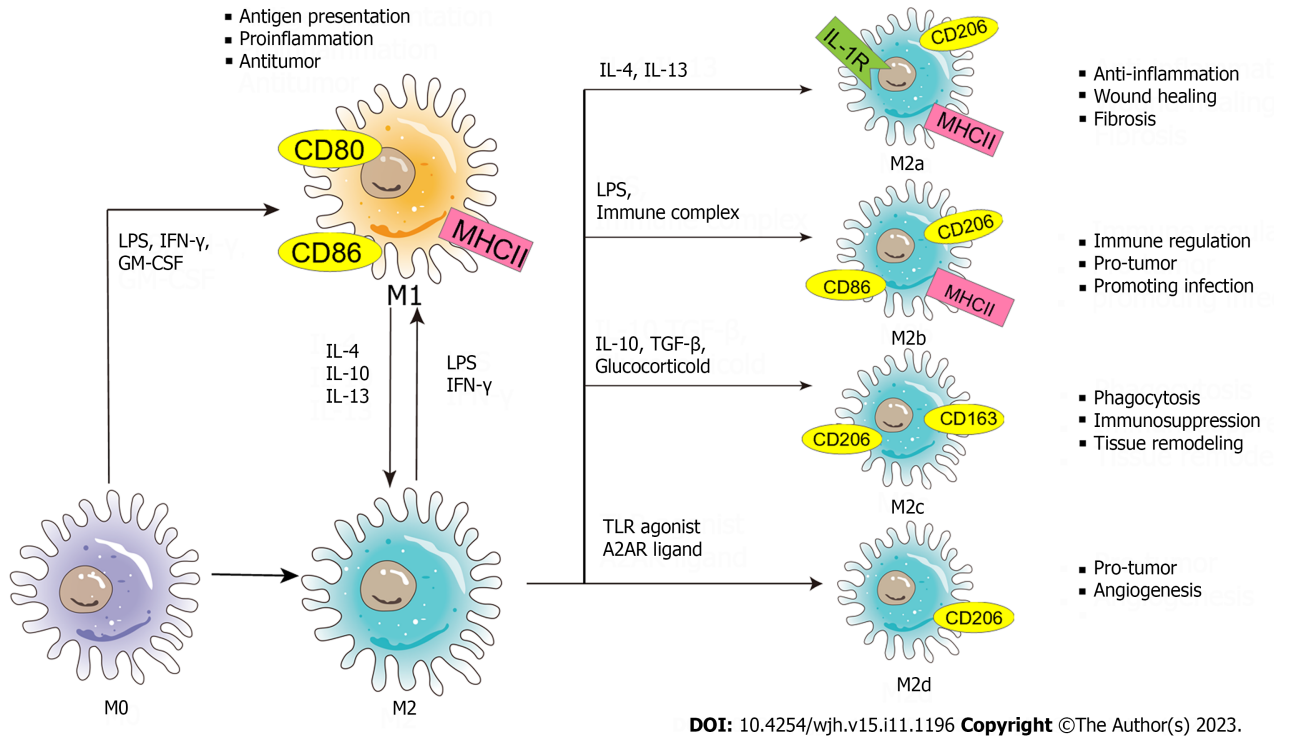


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Figure 1 Schematic diagram of the pathogenesis of chronic liver disease from the perspective of macrophage-derived exosomes. Injured livers activate macrophages to secrete exosomes that encapsulate RNAs, DNAs, lipids, proteins, *etc.*, which influence the development of chronic liver disease through various signaling pathways. CLD: Chronic liver disease; NAFLD: Nonalcoholic fatty liver disease; HF: Hepatic fibrosis; HCC: Hepatocellular carcinoma; IL-6: Interleukin-6; TGF: Transforming growth factor; BMP: Bone morphogenetic protein; TXNIP: Thioredoxin-interacting protein; TLR: Toll-like receptor.

inflammatory response or organ fibrosis, inflammatory factors such as IL-4 and IL-13 activate M2 macrophages to transform into M2a macrophages to inhibit inflammation, mediate damage repair and promote fibrosis. In response to LPS and immune complexes, M2 macrophages can be transformed into M2b macrophages, which can regulate immunity and induce the occurrence of infection and cancer. Glucocorticoids, IL-10, and transforming growth factor-beta (TGF-β) can induce the polarization of M2 macrophages to M2c macrophages, which perform functions such as phagocytosis, immunosuppression, and tissue remodeling. Moreover, M2d macrophages are induced by toll-like receptor (TLR) agonists and adenosine A2A receptor ligands to cause angiogenesis and promote cancer. M2 macrophages can produce various cytokines, including Arg1, CCL17, CCL22, IL-10, IL-1β, IL-6, TNF-α, IL-12, IL-10, TGF-β, CXCL13, and IL-10, which can mediate numerous functions, including inhibiting inflammation, wound healing, the Th2 immune response, anaphylaxis, fibrosis, immune regulation, supporting tumors, promoting infection, and angiogenesis[47-51]. Therefore, when homeostasis is disrupted, the polarization of different macrophage phenotypes occurs, which means that macrophages are remarkably plastic cells (Figure 2).

Macrophages are capable of secreting exosomes, and macrophage-derived exosomes are present in multiple systems. In addition, macrophage-derived exosomes are involved in various diseases and act as therapeutic targets and drug carriers. In recent years, research has mainly focused on the involvement of macrophage-derived exosomes in systemic diseases. For instance, in multimicrobial sepsis, lactate promotes macrophages to release exosomes containing lactated/acetylated high mobility box-1 (HMGB1), which increases endothelial cell permeability and accelerates sepsis. Therefore, reducing circulating levels of exosomal HMGB1 be a therapeutic strategy for the treatment of sepsis[52]. In head and neck squamous cell carcinoma (HNSCC), the long non-coding RNA (lncRNA) HOTTIP in exosomes secreted by M1 macrophages upregulates the TLR5/NF-κB signaling pathway *via* the competing sponges miR-19a-3p and miR-19b-3p to inhibit the progression of HNSCC[53]. In the respiratory system, Wei *et al*[54] found that the exosomes derived from M2 macrophages in patients with lung adenocarcinoma could encapsulate miR-942, and these exosomes enhanced the invasion and migration of lung adenocarcinoma cells and promoted angiogenesis by regulating FOXO1 protein to alleviate β-catenin inhibition. In addition, macrophage-derived exosomes are also valuable in the treatment of chronic pulmonary fibrosis[55] and asthma[56]. In the circulatory system, macrophage-derived exosomes accelerate atherosclerosis in patients with diabetes[57], but they can also be used for myocardial tissue repair in acute myocardial infarction[58]. Moreover, macrophage-derived exosomes can serve as carriers for drug delivery. In a mouse model of Alzheimer's disease (AD), macrophage-derived exosomes could carry silybin, allowing it to cross the blood-brain barrier and reduce astrocyte-mediated neuroinflammation and improve cognitive deficits in AD mice[59]. In conclusion, macrophage-derived exosomes are widely distributed in the body and are associated with a variety of systemic diseases, and exosomes can play an important role in the treatment of diseases by acting as therapeutic targets or drug carriers. Given the importance of macrophages in the physiology of the liver, much research has been devoted to the link between macrophage-derived exosomes and the onset of CLD. Therefore, this review focuses on the impact of macrophage-derived exosomes on the etiology and treatment of CLD.



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Figure 2 Schematic diagram of the phenotypes and functions of macrophage polarization. The nature macrophage can be activated by a variety of influencing factors (such as lipopolysaccharide, interferon-γ, granulocyte-macrophage colony-stimulating factor, *etc.*) and polarized into two phenotypes - classically activated macrophages and alternatively activated macrophages. Exosomes carry stimulatory factors to activate macrophages. Macrophages themselves secrete exosomes to form a signal transmission network between macrophages and other cells. (M0: M0 macrophage; M1: M1 macrophage; M2: M2 macrophage; M2a: M2a macrophage; M2b: M2b macrophage; M2c: M2c macrophage; M2d: M2d macrophage; LPS: Lipopolysaccharide; IFN-γ: Interferon-γ; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IL: Interleukin; TLR: Toll-like receptor; A2AR: A2A receptor; MHC: Major histocompatibility complex; TGF: Transforming growth factor.

MACROPHAGE-DERIVED EXOSOMES IN CLD

CLD has impacted tens of thousands of patients worldwide, its pathogenesis has been explored, and its therapeutic regimen has been optimized. In recent years, there has been intensive study of the function of macrophage-derived exosomes in some diseases. In this review, the use of macrophage-derived exosomes to treat NAFLD, HF, HCC, and other liver diseases is discussed in the context of pathogenesis and therapeutic potential.

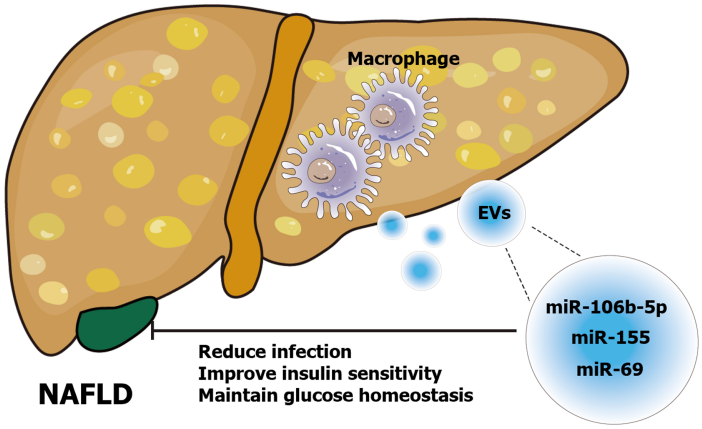
NAFLD

NAFLD is the most common CLD and is recognized as a global public health problem. The pathogenesis of NAFLD is complex[5,60]. During the pathogenesis of NAFLD, hepatic damage caused by inflammation, oxidative stress, and lipotoxicity eventually cause collagen deposition and fiber regeneration, which lead to liver fibrosis[61]. During this process, obesity, type 2 diabetes, resistance to insulin, HSC activation, the environment, genetics, and other factors accelerate the progression of liver injury. However, due to the complexity of the pathophysiology and the heterogeneity of disease phenotypes, there is currently no specific drug to treat NAFLD. Healthy lifestyle interventions and weight loss are mostly used to prevent this disease in high-risk groups, and individualized combination therapy is often used to treat patients[62]. Only a better understanding of the pathogenesis and progression of this disease can provide a more accurate treatment plan. Therefore, we focused on the role of exosomes in NAFLD and their potential use in treatments.

The study of macrophage-derived exosomes in the pathogenesis and treatment of NAFLD has made considerable and remarkable progress and has potential applications in NAFLD therapy. For example, in sepsis associated with NAFLD, exosomes released by Trem2-deficient macrophages carry a large amount of miR-106b-5p and cause abnormal mitochondrial structure and energy metabolism in hepatocytes by blocking mitofusin 2 (Mfn2), which accelerates the progression of NAFLD and increases the susceptibility of NAFLD patients to sepsis[63]. In addition, miR-155-rich exosomes secreted by adipose tissue macrophages improved insulin sensitivity and maintained glucose homeostasis in obese mice[64]. Similarly, miR-69 released from M2 macrophages had the same effect[65]. In obese individuals, macrophage-derived exosomes are increased and delivered to hepatocytes, thereby regulating obesity-related insulin resistance[66] (Figure 3). In conclusion, exosomes derived from macrophages typically carry different cargos and transfer them between liver cells, and these exosomes can not only initiate but also delay the progression of NAFLD.

HF

HF is a dynamic and highly integrated molecular, cellular, and tissue process involving most types of CLD that undergo



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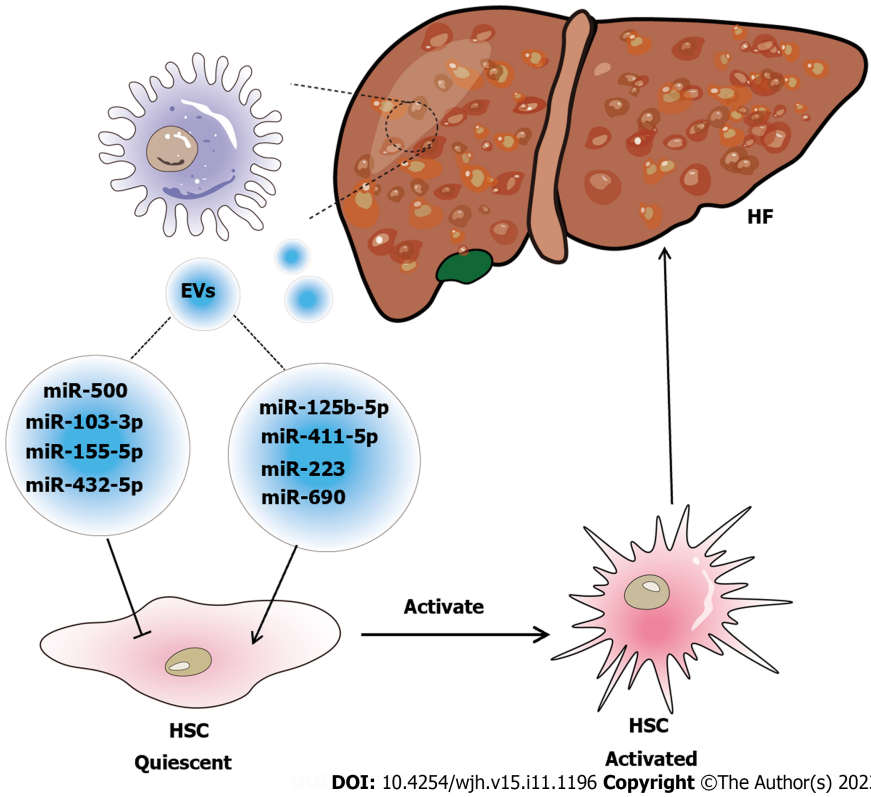
Figure 3 Schematic diagram of the role of macrophage-derived exosomes in nonalcoholic fatty liver disease. Exosomes derived from macrophages carry different microRNAs (miR-106b-5p[63], miR-155[64], miR-69[65]) to act on nonalcoholic fatty liver disease cells and alleviate the disease progression by regulating liver homeostasis. NAFLD: Nonalcoholic fatty liver disease; EVs: Extracellular vesicles; miRNA: MicroRNA.

repeated substantial liver damage and continuous activation of inflammatory responses[67]. During these processes, the activation of hepatic myofibroblasts results in the secretion of extracellular matrix proteins, including collagen, which can lead to fibrous scars and, eventually, the development of HF[68]. If not treated in time, HF will progress to cirrhosis, HCC, and eventually liver failure, but unfortunately, the only treatment that can achieve a complete cure is liver transplantation[69]. Previously, hepatic macrophages, which are specific immune cells, were shown to be important in the progression of HF. Macrophages mediate various functions in fibrotic liver homeostasis, disease progression, and injury recovery and are considered potential targets to protect against fibrosis[21]. Notably, intercellular crosstalk between hepatic macrophages and HSCs is vital for stimulating HSC activation[24]. Therefore, exosomes derived from macrophages are important for intercellular crosstalk during the pathogenesis of HF.

Recent studies have shown that exosomes derived from different sources are involved in the pathogenesis, diagnosis, and potential treatment of HF. This review will focus on research on macrophage-derived exosomes in HF. On the one hand, exosomes derived from macrophages accelerate HF. For instance, when THP-1 macrophages were treated with LPS, the exosomes secreted by these cells were changed, and the alteration in miRNA correlated with HF progression, increasing fibrotic gene expression and promoting HSC replication and activation[24]. Similarly, another study showed that exosomes secreted by LPS-treated macrophages could overexpress miR-500, which promoted the proliferation and activation of HSCs to accelerate the progression of fibrosis by inhibiting MFN2[70]. Deng *et al*[71] found that exosomes derived from LPS-treated macrophages could increase the expression levels of collagen-1 and alpha-smooth muscle actin in JS1 cells. According to recent research, LPS stimulation enhances the expression of miR-155-5p in macrophage-derived exosomes, which facilitates the activation of HSCs, resulting in oxidative stress and collagen production[72]. Autophagy contributes to the progression of liver damage in the early stages of liver fibrosis, and CCL4 can exacerbate autophagy, which causes M1 macrophages to polarize and secrete exosomes rich in miR-423a-5p to encourage HSC activation and control HF[73]. The miRNAs carried by macrophage-derived exosomes in these examples could accelerate HF, but they were also shown to be expressed at high levels in serum, which suggested that these miRNAs can be used as biomarkers for the diagnosis of fibrosis. On the other hand, exosomes derived from macrophages can be used to delay HF. Hepatic macrophages are important mediators of relaxin-mediated amelioration of HF. When the relaxin receptor on macrophages binds to relaxin, their phenotype can be changed from the profibrogenic phenotype to the pro-resolution phenotype, and the pro-resolution phenotype can secrete exosomal miR-30a-5p to inhibit the growth of HSCs. Therefore, nanoparticle-mediated delivery of miR-30a-5p can alleviate liver fibrosis[22]. In another recent study, phillygenin, an active ingredient in the Chinese medicine Forsythiae Fructus, was shown to inhibit StarD13-targeted M1 macrophage exosomal miR-125b-5p to reduce HSC activation, thereby alleviating the progression of liver fibrosis[74]. Macrophage-derived exosomes can also delay or treat the occurrence of NAFLD-HF. For instance, miR-411-5p is present in exosomes secreted by M2 macrophages and can suppress HSC activation in NAFLD[75]. In contrast, miR-223-enriched exosomes suppressed the expression of profibrotic TAZ to inhibit the development of NAFLD[23]. Kupffer cells (KCs) are a special type of macrophage in the liver that can also produce exosomes. In nonalcoholic steatohepatitis (NASH), KCs deliver endogenous miR-690 to HSCs *via* exosomes, which can help treat HF in NASH by suppressing the expression of profibrotic genes[65]. These results demonstrate that macrophage-derived exosomes can alleviate the progression of HF and interact with HSCs (Figure 4). Therefore, research on macrophage-derived exosomes in HF mainly focuses on the crosstalk between macrophages and HSCs, and the mechanisms are diverse and should be analyzed from multiple perspectives. Since the search for a better treatment strategy for HF is ongoing, future studies should focus on how to use macrophage-derived exosomes to diagnose or treat HF.

HCC

Worldwide, HCC is one of the most common causes of cancer death, and HCC is a major type of liver cancer, accounting for more than 90% of cases[76]. HCC is caused by many pathogenic factors, and its prognosis is poor. Most patients are



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Figure 4 Schematic diagram of the relationship between macrophage-derived exosomes and hepatic fibrosis. Exosome-mediated communication between macrophages and hepatic stellate cell (HSC) influences the disease progression of hepatic fibrosis (HF). Special substances carried in exosomes, such as microRNAs (miR-103-3p[24]; miR-500[70]; miR-155-5p[72]; miR-432-5p[73]; miR-125b-5p[74]; miR-411-5p[75]; miR-690[65]; miR-223[23], are signaling factors that induce the activation of HSC to form cirrhosis or inhibit the activation of HSC to alleviate HF. EVs: Extracellular vesicles; HSC: Hepatic stellate cell; HF: Hepatic fibrosis.

diagnosed with advanced HC, for which chemotherapy and immunotherapy are by far the best treatment options[77]. Therefore, the burden of HCC in the world is still severe, and it is important to continue to find new methods for early diagnosis and treatment to improve HCC prognosis. Because exosomes mediate cell communication and carry substances that can be exchanged between cells, macrophages can take up exosomes released by tumor cells in the tumor microenvironment, which affects tumor growth or metastasis by altering macrophage phenotypes. For example, Li *et al*[78] found that exosomes produced by HCC contain abundant levels of lncRNA TUC339, which alters macrophage phenotype, ultimately accelerating the rapid growth of tumors by promoting tumor immune evasion. In addition, another study showed that ER-stressed HCC cells released exosomal miR-23a-3p, which upregulated programmed cell death ligand 1 expression in macrophages and inhibited T-cell functions, thereby ameliorating tumor progression[79]. Similarly, ER-stressed HepG2 cell-derived exosomes promoted the expression of cytokines through the activation of the JAK2/STAT3 pathway, and these exosomes ultimately led to the immunosuppression of macrophages and promoted tumor growth [80]. These results indicate the close connection between exosomes secreted by hepatic carcinoma cells and macrophages, and the impact on macrophages has a robust effect on the growth of HCC. Therefore, we discuss the role of macrophage-derived exosomes in HCC, including the mechanism and application value in diagnosing or treating HCC (Figure 5 and Table 1).

Studies have shown that exosomes derived from macrophages mainly promote the growth and invasiveness of HCC. According to recent research, tumor-associated macrophage (TAM)-derived exosomal lncRNAs increase aerobic glycolysis and cell growth in HCC by controlling the miR-548s/ALDH1A3 pathway, thereby contributing to disease malignancy[81]. Liu *et al*[82] discovered that the miRNAs in exosomes secreted by macrophages were altered, and these miRNAs could reduce androgen receptor (AR) expression and translation to enhance the invasion of HCC. In addition, miR-15b was increased in the exosomes of arsenite-treated macrophages, and this factor could be delivered to HCC cells to promote HCC[83]. Exosomes produced by M2-polarized macrophages could induce TGF-β1/bone morphogenetic protein 7 pathway imbalances and promote the invasiveness of liver cancer[84]. Similarly, Li *et al*[85] reported that M2 macrophage-derived exosomes were rich in miR-27a-3p, and these exosomes could promote cancer cell stemness *via* the miR-27a-3p/thioredoxin-interacting protein pathway. Moreover, miR-660-5p-rich M2 macrophage-derived exosomes could promote liver cancer development by downregulating KLF3[86]. These results indicate that some substances loaded in exosomes can promote tumor growth, invasion, and metastasis, which can provide new insights into the pathogenesis of HCC.

In addition, these results increase enthusiasm for using exosomes in cancer treatment. For example, exosomal miR-628-5p generated by M1 macrophages prevented the m6A alteration of circFUT8, which prevented the proliferation of HCC [87]. Additionally, M1 macrophage-derived exosomal miR-326 inhibited HCC cells from proliferating, forming colonies,

Table 1 Association of the macrophage-derived exosomes with hepatocellular carcinoma

Ref.	Macrophage cell type/phenotype	HCC cell lines/model/tissue	Exosome contents	Findings
Liu *et al* [82], 2020	THP-1/M2	Human HCC cell lines: SK-HEP-1 and HepG2 cell; mouse HCC cell lines: Hepa 1-6	MiR-92a-2-5p	Promote HCC invasion *via* altering the AR/PHLPP/p-AKT/β-catenin signaling
Li *et al* [83], 2021	THP-1/M2	Human HCC cell lines: SMMC-7721	MiR-15b	Promotes the progression of HCC by blocking the LATS1-mediated Hippo pathway
Li *et al* [85], 2021	THP-1/M2	Human HCC cell lines: Huh7, 97H, HepG2, LM3 and SMMC-7721	MiR-27a-3p	Promote cancer stemness of HCC *via* the miR-27a-3p/TXNIP pathways
Tian *et al* [86], 2021	THP-1/M2	Human HCC cell lines: HepG2 and Bel-7402; human HCC tumor tissues	MiR-660-5p	Promote the development of HCC by regulating KLF3
Bai *et al* [88], 2020	THP-1/M1	Human HCC cell line BEL-7404, HepG2, SMMC-7721 and QGY-7703; Xenograft nude mouse model	MiR-326	Suppresses HCC progression *via* NF-κB signaling pathway
Pu J *et al* [89], 2021	C57BL/6 mouse bone marrow-derived macrophages/M2	Murine model of primary HCC (C57BL/6)	MiR-21-5p	Facilitate CD8+T cell exhaustion in HCC *via* the miR-21-5p/YOD1/YAP/β-catenin pathway
Wang *et al* [90], 2019	TAMs/M1	Human HCC cell lines: Huh7, HepG2 and BEL-7404; human HCC tumor tissues	MiR-125a/b	Suppressed HCC cell proliferation and stem cell properties by targeting CD90
Zhang *et al* [92], 2022	THP-1/M2	Human HCC cell lines: SMMC-7721and HepG2; Xenograft nude mouse model (BALB/C)	hsa_circ_0004658	Inhibits HCC progression *via* miR-499b-5p/JAM3

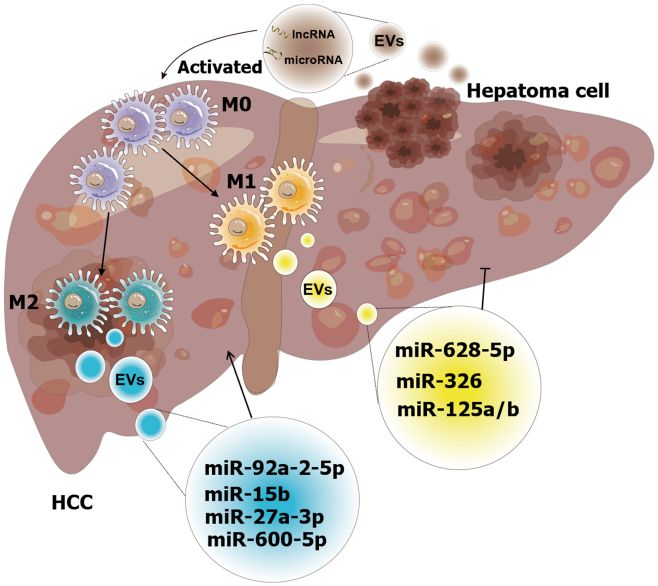
HCC: Hepatocellular carcinoma; M1: M1 macrophage; M2: M2 macrophage; TAMs: Tumour-associated macrophages; miRNA: MicroRNA; AR: Androgen receptor; LATS1: Suppressor kinase 1; TXNIP: Thioredoxin-interacting protein.

migrating, invading, and promoting apoptosis by decreasing the expression of NF-κB[88]. Exosomes released by M2 macrophages could also prevent HCC. M2 macrophage-derived exosomal miR-92a-2-5p enhanced liver cancer invasion, and preclinical research showed that inhibiting miR-92a-2-5p in macrophages could reverse the effect of coculture on AR and weaken the invasion of HCC[82]. MiR-21-5p in exosomes derived from M2 macrophages could enter HCC tissue and deplete CD8+ T cells, providing new insights into tumor immunotherapy[89]. Another team showed that the levels of miR-125a and miR-125b in exosomes secreted by TAMs could inhibit HCC stem cells[90]. Chen *et al*[91] recently showed that IL-2 was an important factor that further regulated TAM-derived exosomal miRNAs to enhance the inhibition of cancer progression. Likewise, RBPJ+/+ macrophage-derived exosomes could also suppress neoplasms[92]. Further study of the effects of these exosomes in the treatment of liver cancer would provide important value in the search for early diagnostic screening markers for patients, which will help in the development of novel schemes for clinical treatment and is critical for reducing the burden of HCC patients worldwide.

Other CLD

The range of CLDs is varied; in addition to NAFLD, HF, and HCC, CLDs also include alcoholic fatty liver disease, viral liver disease, and immune liver disease. Current research into the role of macrophage-derived exosomes in other liver diseases also deserves attention.

Viral hepatitis is a liver disease caused by infection by various hepatitis viruses. The prognosis is generally good, but due to inappropriate lifestyles and untimely treatment, some cases progress to more serious liver diseases, such as liver failure. Therefore, some studies have explored the relationship between exosomes and viral hepatitis. A study showed that macrophage-derived exosomes could spread to hepatocytes and promote IFN-α-induced hepatitis B virus (HBV) resistance, and these factors relied on the main pathways of viral invasion[93]. Antiviral molecules can also enter hepatocytes through internalized INF-α-treated macrophage-derived exosomes, thereby reducing the replication of HBV [94]. Similarly, exosomes released from Tlr3-activated macrophages are enriched in many hepatitis C virus (HCV)-resistant miRNAs, and when these exosomes are taken up by HCV hepatocytes, they can mediate anti-HCV activity by inhibiting HCV replication in cells, which suggests a potential treatment for HCV[95]. These studies show that in viral liver diseases, exosomes carrying antiviral substances are transmitted from macrophages and absorbed by diseased liver cells; thus, these exosomes are therapeutic carriers, indicating a potential novel treatment method for viral hepatitis. In ALD, the main causative factor is alcohol intake. Alcohol stimulation increases the expression of miR-155 and increases the release of macrophage-derived exosomes by reducing lysosome-associated proteins in the liver, leading to the dysregulation of lysosomal autophagy[14]. Alcohol exposure can increase the number of miR-27a-rich exosomes produced by monocytes, which can polarize primitive monocytes into M2 macrophages[96]. In another study, macrophage-derived exosomes were shown to participate in immune regulation in concanavalin A-induced hepatitis[97]. A recent study showed that Concanavalin A could promote the release of exosomes from type I macrophages and that these exosomes contained the lncRNA H19, which induced apoptosis in autoimmune liver disease cells, suggesting a new avenue for developing treatments for autoimmune liver diseases[98]. In summary, research on exosomes in liver diseases has



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Figure 5 Schematic diagram of the effect of exosomes derived from macrophages on hepatocellular carcinoma. The crosstalk of exosomes between macrophages and hepatocellular carcinoma (HCC) affects tumor progression. Both exosomes released by macrophages and HCC have unique intracellular components, including a variety of mRNAs, microRNAs, long non-coding RNAs, lipids, *etc.* These intracellular components are utilized as communicators to induce pathways that result in increased or inhibited cell proliferation, invasion, and other hallmarks of malignancy (M0: M0 macrophage; M1: M1 macrophage; M2: M2 macrophage; miR-92a-2-5p[82]; miR-15b[83]; miR-27a-3p[85]; miR-660-5p[86]; miR-628-5p[87]; miR-326[88]; miR-125a/b[90]). EVs: Extracellular vesicles; HCC: Hepatocellular carcinoma; miRNA: MicroRNA; lncRNA: Long non-coding RNA.

increased, which is conducive to exploring new treatments for CLD in the future.

CONCLUSION

The role and potential therapeutic value of exosomes in CLD have been the focus of research in recent years. Understanding the mechanism by which macrophage-derived exosomes affect liver diseases is critical for identifying their roles in liver disease pathogenesis and improving their therapeutic effects. In liver disease, macrophages can be activated and polarized. These activated macrophages secrete exosomes that carry various miRNAs and proteins, and these substances are encapsulated in exosomes and transferred from cell to cell. Macrophage-derived exosomes disrupt normal signaling between parenchymal and nonparenchymal cells in the liver, ultimately leading to liver damage and can act on specific targets to activate or inhibit signaling pathways and mediate related pathological processes. Thus, macrophage-derived exosomes are involved in the diagnosis of liver disease as biomarkers, and the signaling targets of these exosomes can also be used as potential therapeutic targets, providing more novel strategies for the diagnosis and treatment of liver disease. This article reviewed the mechanism and functions of macrophage-derived exosomes in liver diseases.

Currently, the link between macrophage-derived exosomes and liver diseases has mostly focused on a subset of liver diseases, such as NAFLD, HF, and HCC. However, the occurrence and development of CLDs are complicated processes involving multiple causes, stages, and links. In response to viral infection, alcohol intake, a high-fat diet, and drugs, liver inflammation and cellular degeneration first occur, which are accompanied by a series of adaptive events in the liver, including autophagy, aging, and the innate immune response, but these events can further aggravate liver damage, such as the activation of HSCs, leading to the accumulation of extracellular matrix and HF. Without early treatment, HF can further develop into cirrhosis, HCC, and even liver failure. Thus, some of the pathological stages of CLD overlap and further evolve. However, most current studies have only been conducted *in vivo* and *in vitro*, which limits the research object to a single liver disease while ignoring the role of macrophage-derived exosomes in the complex pathogenesis of CLD. Therefore, it is important to provide new ideas for subsequent studies so that researchers can focus on the role of macrophage-derived exosomes in different stages of CLD, which will be valuable to understanding the pathogenesis and treatment of many complex liver diseases.

Additionally, it has been found that different stimuli can cause macrophages to polarize into M1 and M2 cells and that M2 cells can then further differentiate into many subtypes. However, at present, there is still a lack of research on the mechanism by which these different isoforms release exosomes and the released exosome contents, which can be the focus of future research. In terms of clinical applications, techniques for extracting and purifying exosomes are improving, but determining how to amplify and change these extracted exosomes into a form that can be used in clinical settings requires cooperation and communication between different fields to develop a better treatment plan to reduce the global burden of CLD.

In the future, the development of exosomes is expected to shift from basic research to clinical applications. In recent years, exosomes have been recognized as potential biological treatments and drug delivery vehicles for the treatment of a variety of diseases. Compared with commonly used nanoparticles, macrophage-derived exosomes have the advantages of low immunogenicity and escape from macrophage phagocytosis. However, there are still gaps in clinical trials of macrophage-derived exosomes for the treatment of CLD. Before clinical translation, we urgently need to confirm which exosome components have profound diagnostic and therapeutic value, especially as accurate biomarkers that reflect disease status, target membrane segments, and critical cargo involved in the disease process. In addition, these systemically delivered exosomes tend to become trapped in nonspecific organs, particularly the liver, lungs, and spleen, resulting in inadequate target doses. Surface modifications for targeted delivery may provide an opportunity to enhance or expand the innate therapeutic value of exosomes. To improve the stability and delivery efficiency of natural exosomes, emerging biological nanotechnology provides a new option for precise material delivery. By designing exosome-like nanovesicles and membrane-camouflaging nanoparticles, the loading and delivery efficiency of effective substances of natural exosomes can be improved. In future clinical treatment of CLD, exosomes from macrophages can carry key effective substances and act as drug carriers after targeted modification or nanotechnology engineering and finally realize individualized targeted therapy, making great contributions to relieving the pressure of global liver diseases.

FOOTNOTES

Author contributions: Xiang SY performed the writing, prepared the figures and tables; Xiang SY and Deng KL designed the outline and coordinated the writing of the paper; Yang DX, Yang P, and Zhou YP provided review of the draft versions of the paper prior to submission of the final version; and all authors have read and approved the final manuscript.

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Abstract

BACKGROUND

Nonalcoholic fatty liver disease (NAFLD) has become the leading cause of cirrhosis and other chronic liver diseases (COCLDs).

AIM

To conduct a comprehensive and comparable updated analysis of the global, regional, and national burden of COCLDs due to NAFLD in 204 countries and territories from 1990 and 2019 by age, sex, and sociodemographic index.

METHODS

Data on COCLDs due to NAFLD were collected from the Global Burden of Diseases, Injuries, and Risk Factors Study 2019. Numbers and age-standardized prevalence, death, and disability-adjusted life years (DALYs) were estimated through a systematic analysis of modelled data from the Global Burden of Diseases, Injuries, and Risk Factors Study 2019. The estimated annual percentage change was used to determine the burden trend.

RESULTS

In 2019, the global age-standardized prevalence rate of COCLDs due to NAFLD was 15022.90 per 100000 population [95% uncertainty interval (UI): 13493.19-16764.24], which increased by 24.51% (22.63% to 26.08%) from 1990, with an estimated annual percentage change of 0.78 (95% confidence interval: 0.74-0.82). In the same year, however, the age-standardized death rate and age-standardized DALYs per 100000 population were 1.66 (95%UI: 1.20-2.17) and 43.69 (95%UI: 31.28-58.38), respectively. North Africa and the Middle East had the highest prevalence rates of COCLDs due to NAFLD. The death rate increased with age up to the 95+ age group for both sexes. Males had higher numbers of prevalence, death rate, and DALYs than females across all age groups before the 65-69 age

group. The sociodemographic index was negatively correlated with the age-standardized DALYs.

CONCLUSION

Globally, the age-standardized prevalence rate has increased during the past three decades. However, the age-standardized death rate and age-standardized DALYs decreased. There is geographical variation in the burden of COCLDs due to NAFLD. It is strongly recommended to improve the data quality of COCLDs due to NAFLD across all countries and regions to facilitate better monitoring of the burden of COCLDs due to NAFLD.

Key Words: Cirrhosis; Nonalcoholic fatty liver disease; Global burden of disease; Prevalence; Disability-adjusted life years; Death

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Core Tip: Nonalcoholic fatty liver disease is the leading cause of cirrhosis and other chronic liver diseases. The global age-standardized prevalence rate of cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease increased by 24.51% from 1990. The age-standardized death rate and age-standardized disability-adjusted life-years rate per 100000 population were 1.66 and 43.69, respectively. The highest prevalence rate was observed in North Africa and the Middle East. Males had a higher burden of prevalence, death, and disability-adjusted life-years lost than females before the 65-69 age group. Furthermore, there is a negative correlation between sociodemographic index and age-standardized death rate.

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INTRODUCTION

The incidence and prevalence of nonalcoholic fatty liver disease (NAFLD) have been rapidly increasing worldwide over the past few decades. Recent estimates suggest that approximately 25% of the world's population is affected by NAFLD, with projections indicating a potential 56% surge in the prevalence of nonalcoholic steatohepatitis (NASH) within the coming decade[1]. NAFLD encompasses a spectrum of liver damage, ranging from simple steatosis to NASH, fibrosis, cirrhosis, and even hepatocellular carcinoma[2]. It is noteworthy that NAFLD now stands as the fifth leading cause of mortality among young adults within the category of metabolic diseases. Alarming forecasts predict a staggering 158.4% increase in its death rate by the year 2050[3]. Conversely, another separate study demonstrated divergent trends, finding that the age-standardized prevalence rate (ASPR) of NAFLD increased while the age-standardized death rate (ASDR) and age-standardized disability-adjusted life-year (DALY) rate (ASDAR) decreased from 1990 to 2019[4].

Cirrhosis is the leading cause of liver-related morbidity, contributing to more than 1 million deaths annually worldwide. The mortality increase escalates markedly for individuals grappling with decompensated cirrhosis[5], and the deaths from cirrhosis increased by 47.15% globally from 1990 to 2017[6]. Beyond mortality statistics, cirrhosis imposes a significant public health burden globally, substantially compromising quality of life[7,8]. The etiologies of cirrhosis include alcoholic liver disease, hepatitis B virus, hepatitis C virus, and NAFLD[9,10]. Over the past few decades, universal hepatitis B virus vaccination initiatives, coupled with rising obesity rates and the prevalence of type 2 diabetes, have positioned NAFLD as a major etiological factor contributing to cirrhosis[11-14].

Chronic liver disease (CLD) is a disease that is characterized by decreased liver function resulting from chronic inflammation or injury to the liver, leading to fibrosis and cirrhosis that progresses for more than 6 mo[15,16]. This spectrum encompasses an array of liver pathologies, encompassing inflammation, cirrhosis, portal hypertension, and hepatorenal syndrome. Notably, the incidence of CLD is increasing yearly, and it is now the fifth leading cause of death in the United Kingdom. In Western regions, NAFLD has become the leading cause of CLD[17]. Today, the United States has nearly 4.5 million adults afflicted by cirrhosis and CLD, resulting in an overall death toll of 414731[16]. However, no studies have focused on the epidemiology of cirrhosis and other CLDs (COCLDs) due to NAFLD across the globe.

In the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2017, it was shown that the incidence of cases of liver cirrhosis caused by NASH increased by approximately 105.56%, and the age-standardized incidence rate increased by 1.35%. This study only included data from 195 countries, and data on prevalence, death, and DALYs were not provided[18]. Furthermore, no updated global studies on COCLDs due to NAFLD have been published since the 2017 estimates. Using the data from the GBD 2019, we conducted this comprehensive, updated analysis of the global, regional, and national levels of prevalence, death rate, and DALYs of COCLDs due to NAFLD with regard to age-standardized rates (ASRs) and raw numbers from 1990 to 2019, stratified by sex, age, and sociodemographic index (SDI).

MATERIALS AND METHODS

Overview

The GBD 2019 was conducted by the Institute of Health Metrics and Evaluation and analyzed approximately 369 diseases and injuries, 282 causes of death, and 84 risk factors from 204 countries/territories, 21 regions, and 7 superregions from 1990 to 2019[19]. Detailed methods for GBD 2019 regarding date inputs, analytical processes, and outputs have been described in previous publications[19,20]. Additional information on fatal and nonfatal estimates can be found at https://vizhub.healthdata.org/gbd-compare/ and http://ghdx.healthdata.org/gbd-results-tool. Our study complied with the Guidelines for Accurate and Transparent Health Estimates Reporting statement[21].

Case definition and data sources

NAFLD was defined as a range of liver conditions that mimic alcoholic liver disease but occur in people who drink little to no alcohol. It includes nonalcoholic fatty liver (characterized by fat deposition in liver cells), NASH (characterized by fat deposition and inflammation), and cirrhosis[19]. Cirrhosis is a CLD in which there is progressive destruction of functional hepatic cells and replacement with fibrosis (scarring) of the liver. In GBD 2019, COCLDs due to NAFLD were defined as COCLDs that were specifically caused by NAFLD, excluding all other potential etiologies[19]. All the GBD data used in this study are publicly available online at the Global Health Data Exchange.

Data processing and disease modelling

The Bayesian meta-regression tool DisMod-MR 2.1 was used to assess and model estimates of the burden of COCLDs due to NAFLD by pooling all the available epidemiological data. Prior settings included remission of 0 before the age of 15 years in the DisMod-MR 2.1 model. No prevalence of COCLDs due to NAFLD before the age of 15 years was assumed. The age range was restricted to ≥ 15 years and was divided into 17 5-year age groups.

The estimated annual percentage change (EAPC) values were calculated to reflect the change in ASRs over a specified period. EAPC values above or below 0 indicate that the ASR is increasing or decreasing, respectively. If the EAPC range includes 0, this means the ASR is stable during this period. ASPR, ASDR, ASDAR, and EAPC were used to quantify global trends of COCLDs due to NAFLD.

The SDI was used as a composite indicator of the development status in each country and territory. It was calculated based on lag-distributed income, the total fertility rate for individuals younger than 25 years, and average years of education in people older than 15 years. SDI ranged from 0 to 1, with a higher score indicating a higher level of development. The 204 countries and territories were categorized into five groups: low SDI, low-middle SDI, middle SDI, high-middle SDI, and high SDI.

Statistical analyses

Smoothing spline models were employed to examine the shape of the correlation curve between the burden index of COCLDs due to NAFLD and SDI according to the GBD estimates across 204 countries and 21 regions from 1990 to 2019. The 95% uncertainty intervals (UIs) were defined as the 2.5 to the 97.5 percentile of the ordered draws. R software version 3.6.3 was used for all statistical analyses and figures. A P value of < 0.05 was considered statistically significant.

RESULTS

Global level

Globally in 2019 there were 1235652879 (95%UI, 1109501987-1378481210) prevalent cases of COCLDs due to NAFLD. From 1990 to 2019, the global ASPR increased from 12065.15 (10779.06-13536.49) to 15022.90 (13493.19-16764.24) per 100000 population, with an EAPC of 0.78 (0.74-0.82) (Table 1, Figure 1). COCLDs due to NAFLD accounted for 134240 (96483-176920) deaths globally in 2019, which was a substantial increase of 76.73% (61.23%-94.75%) over that in 1990. The ASDR of COCLDs due to NAFLD decreased from 1.94 per 100000 population (1.39-2.59 per 100000) in 1990 to 1.66 per 100000 population (1.20-2.17 per 100000) in 2019, with an EAPC of 0.65 (-3.68-2.48) (Table 1, Supplementary Figure 1). In the same year, COCLDs due to NAFLD accounted for 3621471.92 (2585375.27-4862918.36) DALY cases at the global level with an ASR of 43.69 per 100000 population. The global ASDAR was reduced from 51.92 per 100000 population (37.23-69.19 per 100000) in 1990 to 43.69 per 100000 population (95%UI: 31.28-58.38) in 2019, with an EAPC of -0.73 (-1.33 to -0.13) (Table 1, Supplementary Figure 2).

SDI regions and 21 GBD region levels

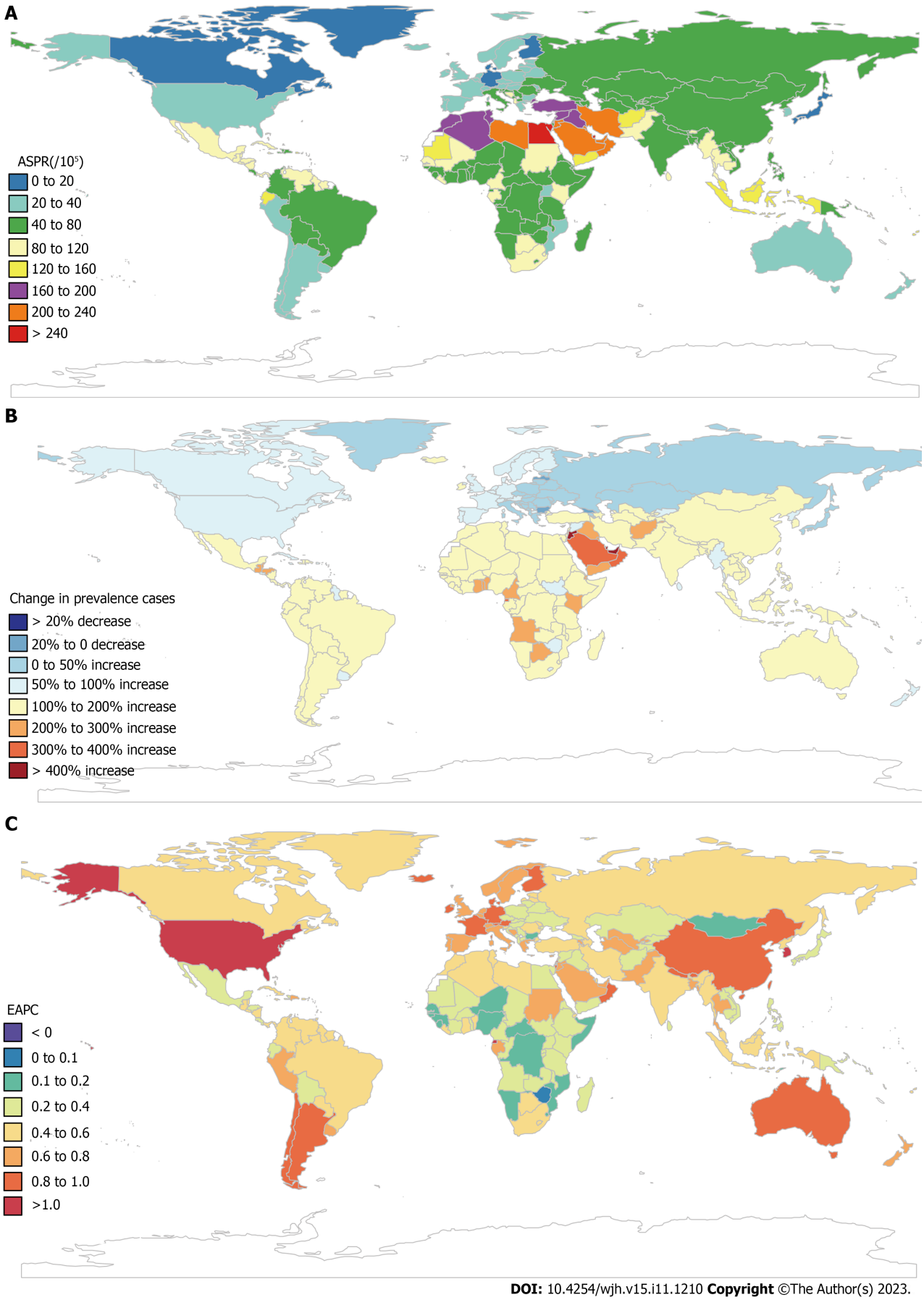
The number of prevalence, deaths, and DALYs of COCLDs due to NAFLD increased in all five SDI regions from 1990 to 2019. Among them, the greatest increases in prevalence (1.54-fold), deaths (0.96-fold), and DALY (0.77-fold) cases were observed in low-SDI, middle-SDI, and low-middle-SDI regions, respectively (Figure 2, Supplementary Figure 3). All-age prevalence rates of COCLDs due to NAFLD increased across all SDI quintiles, with the most significant increase observed in the middle-SDI region (0.65-fold). Outside of the low-SDI quintile, all-age death rates and DALYs showed an increasing trend. The ASDR and ASDAR of COCLDs due to NAFLD exhibited a decreasing trend across all five SDI quintiles. The low-SDI quintile had the greatest absolute decreases in the ASDR (EAPC = -1.04; -3.48-1.45) and the ASDAR (EAPC = -1.14; -1.63 to -0.65). The ASPR of COCLDs due to NAFLD increased in all SDI regions from 1990 to 2019, with the highest increase observed in the high-SDI region (EAPC = 1.22; 1.17-1.26) (Table 1, Supplemen-

Table 1 Global burden of cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease in 204 countries and territories from 1990 to 2019

Characteristics	Death (95%UI)			Prevalence (95%UI)			DALYs (95%UI)		
	2019	2019	1990-2019	2019	2019	1990-2019	2019	2019	1990-2019
	Death cases among COCLDs due to NAFLD	ASDR per 100000 population (95%UI)	EAPC (95%CI)	Prevalence cases among COCLDs due to NAFLD	ASPR per 100000 population (95%UI)	EAPC (95%CI)	DALY cases among COCLDs due to NAFLD	ASDAR per 100000 population (95%UI)	EAPC (95%CI)
Global	134240.56 (96483.10-176920.13)	1.66 (1.20-2.17)	-0.65 (-3.68-2.48)	1235652879.48 (1109501987.09-1378481210.81)	15022.90 (13493.19-16764.24)	0.78 (0.74-0.82)	3621471.92 (2585375.27-4862918.36)	43.69 (31.28-58.38)	-0.73 (-1.33 to -0.13)
Sex									
Male	72379.97 (51296.70-98029.91)	1.91 (1.36-2.54)	-0.62 (-3.46-2.31)	679261655.31 (610650033.21-753716195.72)	16789.38 (15136.45-18609.47)	0.77 (0.74-0.81)	2111194.29 (1490236.02-2905285.80)	52.39 (37.17-71.63)	-0.65 (-1.20 to -0.10)
Female	61860.58 (44641.50-81216.17)	1.42 (1.03-1.86)	-0.70 (-3.95-2.66)	556391224.17 (499050879.21-624989968.22)	13278.47 (11900.05-14925.95)	0.78 (0.74-0.82)	1510277.64 (1087564.54-1984959.30)	35.16 (25.35-46.24)	-0.84 (-1.49 to -0.18)
SDI									
Low SDI	12175.43 (8552.07-16892.87)	2.41 (1.65-3.38)	-1.04 (-3.48-1.45)	108176159.27 (95419333.67-122650081.72)	14278.53 (12741.85-16053.31)	0.34 (0.30-0.38)	367698.76 (255871.37-510346.73)	59.56 (41.46-82.97)	-1.14 (-1.63 to -0.65)
Low-middle SDI	28042.90 (20040.37-37459.68)	2.09 (1.52-2.77)	-0.73 (-3.47-2.08)	245742808.24 (219055120.10-276667118.34)	15232.77 (13662.46-17078.41)	0.56 (0.52-0.59)	826708.77 (584688.97-1130359.54)	54.77 (39.14-74.11)	-0.86 (-0.31 to -1.4)
Middle SDI	51854.16 (37473.84-68073.39)	2.23 (1.62-2.92)	-0.77 (-3.35-1.88)	464259979.22 (417290788.31-516020477.89)	17596.41 (15842.14-19525.24)	0.66 (0.62-0.69)	1347118.90 (956498.31-1772557.91)	52.41 (38.16-68.22)	-0.98 (-1.52 to -0.44)
Middle-high SDI	25074.30 (18164.41-33237.26)	1.27 (0.93-1.67)	-0.75 (-4.08-2.69)	278080918.57 (251203100.31-308471139.19)	15336.20 (13798.84-17095.00)	0.79 (0.75-0.83)	676567.46 (480709.11-915257.33)	34.81 (25.11-46.87)	-0.52 (-1.16-0.14)
High SDI	16984.00 (12138.02-22893.89)	0.95 (0.67-1.30)	-0.79 (-4.75-3.33)	138619942.87 (125499384.96-153306375.41)	10528.91 (9426.64-11724.43)	1.22 (1.17-1.26)	400520.00 (273854.75-550359.22)	25.46 (17.47-35.02)	-0.84 (-1.61 to -0.05)
Region									
Andean Latin America	2935.06 (2012.67-4051.53)	5.31 (3.62-7.30)	-0.23 (-1.96-1.54)	8442278.35 (7603147.20-9329779.19)	13689.71 (12376.75-15086.12)	0.50 (0.47-0.54)	70058.17 (47362.01-98970.21)	121.70 (82.37-172.61)	-0.69 (-1.05 to -0.34)
Australasia	413.12 (297.05-538.48)	0.87 (0.63-1.13)	-0.22 (-4.56-4.32)	3459804.59 (3121214.49-3821041.92)	9444.14 (8455.34-10478.02)	0.91 (0.86-0.95)	9514.38 (6661.05-12694.41)	22.52 (15.80-30.52)	-0.22 (-1.09-0.65)
Caribbean	1607.95 (1113.98-2236.69)	3.11(2.15-4.30)	-0.71 (-3.01-1.65)	8176172.43 (7382389.69-9017056.63)	16169.48 (14591.11-17866.53)	0.40 (0.37-0.43)	41576.95 (28066.48-60045.06)	80.64 (54.52-115.96)	-0.75 (-1.21 to -0.29)
Central Asia	2633.58 (1802.74-3693.53)	3.56 (2.51-4.95)	2.00 (-0.39-4.45)	12814865.72 (11452914.10-14330325.35)	14150.39 (12737.16-15748.23)	0.50 (0.46-0.54)	84039.15 (57215.03-120570.79)	97.76 (67.81-137.00)	1.97 (1.51-2.43)
Central Europe	2061.07 (1378.12-2972.76)	1.05 (0.71-1.49)	-0.36 (-4.12-	18627848.59 (16912570.59-	11894.85 (10754.82-	0.34 (0.30-	54731.91 (36055.44-	30.45 (19.95-43.92)	-0.35 (-1.06-

			3.56)	20480313.45)	13109.51)	0.38)	79057.76)		0.37)
Central Latin America	13972.52 (10310.77-18101.39)	5.90 (4.32-7.66)	-0.61 (-2.24-1.06)	42166216.88 (37817332.61-46840542.82)	16617.99 (14959.55-18388.64)	0.43 (0.40-0.46)	379421.91 (275350.09-504051.39)	154.08 (112.26-204.63)	-0.84 (-1.16 to -0.52)
Central Sub-Saharan Africa	1464.95 (913.12-2215.29)	2.71 (1.74-4.05)	-0.79 (-3.19-1.67)	11343938.83 (9886866.10-12995919.12)	13331.21 (11795.46-15097.36)	0.16 (0.13-0.20)	47553.59 (29400.33-74006.26)	70.04 (43.82-106.28)	-0.78 (-1.26 to -0.30)
East Asia	15620.62 (11027.12-21039.77)	0.80 (0.58-1.07)	-2.35 (-6.22-1.68)	303112259.13 (272363603.29-339481359.63)	15680.86 (14022.56-17552.19)	0.85 (0.81-0.89)	391872.94 (268774.96-535743.63)	18.91 (13.28-25.56)	-2.71 (-3.50 to -1.91)
Eastern Europe	7354.24 (5071.40-10094.04)	2.41 (1.67-3.30)	3.04 (0.05-6.12)	34110583.23 (30980982.64-37557624.89)	12295.07 (11098.49-13601.74)	0.39 (0.35-0.42)	245483.77 (166670.25-344145.21)	85.82 (58.40-120.98)	3.58 (3.06-4.11)
Eastern Sub-Saharan Africa	6094.93 (4211.94-8428.57)	3.98 (2.74-5.49)	-0.66 (-2.62-1.34)	36093305.19 (31609582.82-41132257.66)	13709.06 (12222.30-15370.26)	0.29 (0.26-0.33)	175459.64 (119576.20-250541.44)	91.96 (62.83-129.95)	-0.82 (-1.22-0.41)
High-income Asia Pacific	1462.94 (990.41-2089.17)	0.31 (0.21-0.42)	-3.18 (-8.97-2.97)	20933934.70 (18773194.85-23278277.91)	7671.66 (6829.94-8629.15)	0.56 (0.51-0.61)	26564.50 (18456.53-35825.79)	7.20 (4.90-9.83)	-3.36 (-4.57 to -2.14)
High-income North America	7014.45 (4867.08-9682.42)	1.20 (0.83-1.63)	0.34 (-3.56-4.39)	44312078.16 (39693504.66-49544059.79)	9395.90 (8403.86-10542.43)	0.98 (0.94-1.03)	178065.34 (120160.62-251423.01)	33.23 (22.97-46.98)	0.36 (-0.40-1.12)
North Africa and Middle East	11756.00 (7699.85-17161.92)	3.18 (2.07-4.60)	-0.35 (-2.59-1.94)	161456578.66 (146407605.69-177659074.34)	27748.49 (25409.87-30283.48)	0.47 (0.45-0.50)	265596.53 (171336.92-375177.75)	61.14 (39.58-87.12)	-0.31 (-0.83-0.21)
Oceania	87.79 (55.51-129.24)	1.13 (0.77-1.60)	-0.17 (-3.94-3.75)	1744101.91 (1541892.36-1965419.91)	16869.18 (15133.44-18726.79)	0.18 (0.15-0.21)	3194.63 (1975.61-4878.17)	33.00 (20.97-48.54)	-0.21 (-0.91-0.50)
South Asia	17739.51 (12596.43-24044.26)	1.28 (0.90-1.73)	-1.55 (-4.85-1.87)	241838470.86 (214848138.41-273682478.00)	14513.87 (12969.20-16400.60)	0.55 (0.51-0.58)	546376.19 (385933.87-757708.62)	34.54 (24.64-47.48)	-1.76 (-2.41 to -1.11)
Southeast Asia	19624.56 (13971.70-26356.88)	3.43 (2.46-4.62)	-0.23 (-2.40-1.99)	128065513.81 (114729541.70-142457456.46)	18299.21 (16508.63-20309.58)	0.46 (0.42-0.49)	539043.89 (379432.49-734864.03)	82.56 (58.82-110.61)	-0.58 (-1.01 to -0.14)
Southern Latin America	1429.48 (982.16-1989.97)	1.73 (1.18-2.41)	-0.18 (-3.30-3.02)	6483740.60 (5831421.14-7191963.86)	8602.52 (7719.35-9564.23)	0.90 (0.85-0.95)	34294.28 (22901.01-48792.78)	42.86 (28.61-61.04)	-0.52 (-1.14-0.11)
Southern Sub-Saharan Africa	888.05 (632.75-1213.53)	1.61 (1.14-2.18)	-0.53 (-3.41-2.44)	13161620.39 (11810250.76-14681736.21)	18075.91 (16347.92-19997.66)	0.41 (0.37-0.44)	26198.48 (18261.46-36311.83)	40.87 (28.84-55.60)	-0.69 (-1.26 to -0.12)
Tropical Latin America	4447.37 (3232.26-5827.27)	1.84 (1.35-2.40)	-0.44 (-3.42-2.63)	37986300.10 (34231442.55-41945927.58)	15241.19 (13723.17-16818.90)	0.49 (0.45-0.52)	120114.10 (85232.80-161026.13)	48.00 (34.37-63.83)	-0.60 (-1.18 to -0.02)
Western Europe	9604.81 (6833.82-12825.54)	1.09 (0.78-1.46)	-1.81 (-5.22-1.72)	59002155.13 (53307928.02-65208561.44)	9932.86 (8931.95-11044.15)	0.80 (0.76-0.85)	201120.00 (140612.13-273526.30)	26.97 (18.77-37.08)	-2.03 (-2.72 to -1.35)
Western Sub-Saharan Africa	6027.57 (4018.12-8572.80)	3.31 (2.21-4.67)	-0.67 (-2.78-1.48)	42321112.21 (37193511.05-48136808.11)	14283.82 (12756.86-15952.84)	0.22 (0.18-0.25)	181191.57 (116196.70-270466.76)	79.06 (51.99-113.80)	-0.70 (-1.13 to -0.26)

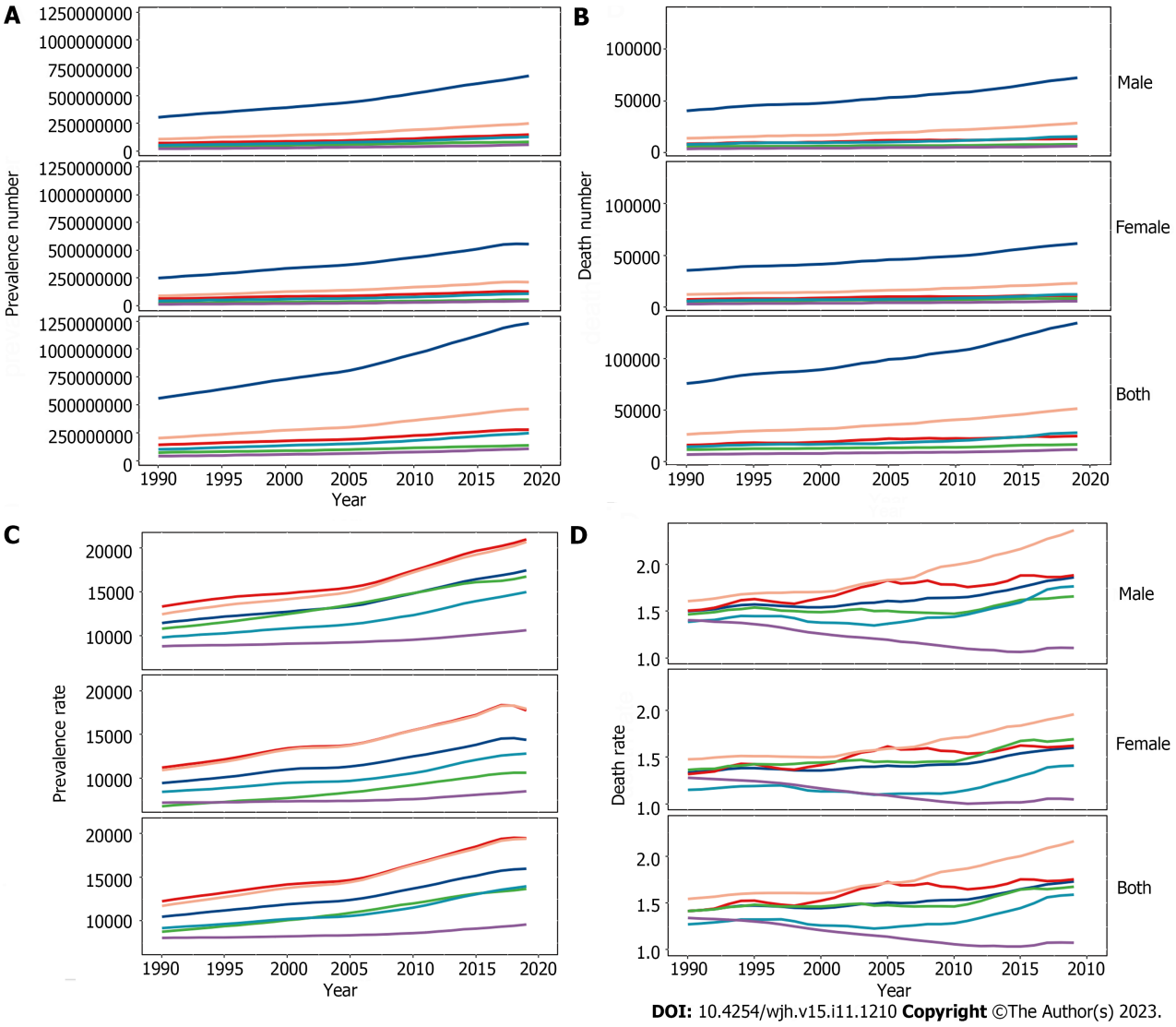
ASDAR: Age-standardized disability-adjusted life-years rate; ASDR: Age-standardized death rate; ASPR: Age-standardized prevalence rate; CI: Confidence interval; COCLD: Cirrhosis and other chronic liver diseases; DALYs: Disability-adjusted life-years; EAPC: Estimated annual percentage change; NAFLD: Nonalcoholic fatty liver disease; SDI: Sociodemographic index; UI: Uncertainty interval.



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Figure 1 Global disease burden of cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease by countries and territories for both sexes combined. A: The age-standardized prevalence rate (ASPR) of cirrhosis and other chronic liver diseases (COCLDs) due to

nonalcoholic fatty liver disease (NAFLD) in 2019; B: The percentage change in prevalence cases of COCLDs due to NAFLD between 1990 and 2019; C: The estimated annual percentage change of COCLDs due to NAFLD ASPR from 1990 to 2019. EAPC: Estimated annual percentage change.



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Figure 2 Cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease at the global and regional levels from 1990 to 2019. A: Prevalence number; B: Death number; C: All-age prevalence rate; D: All-age death rate.

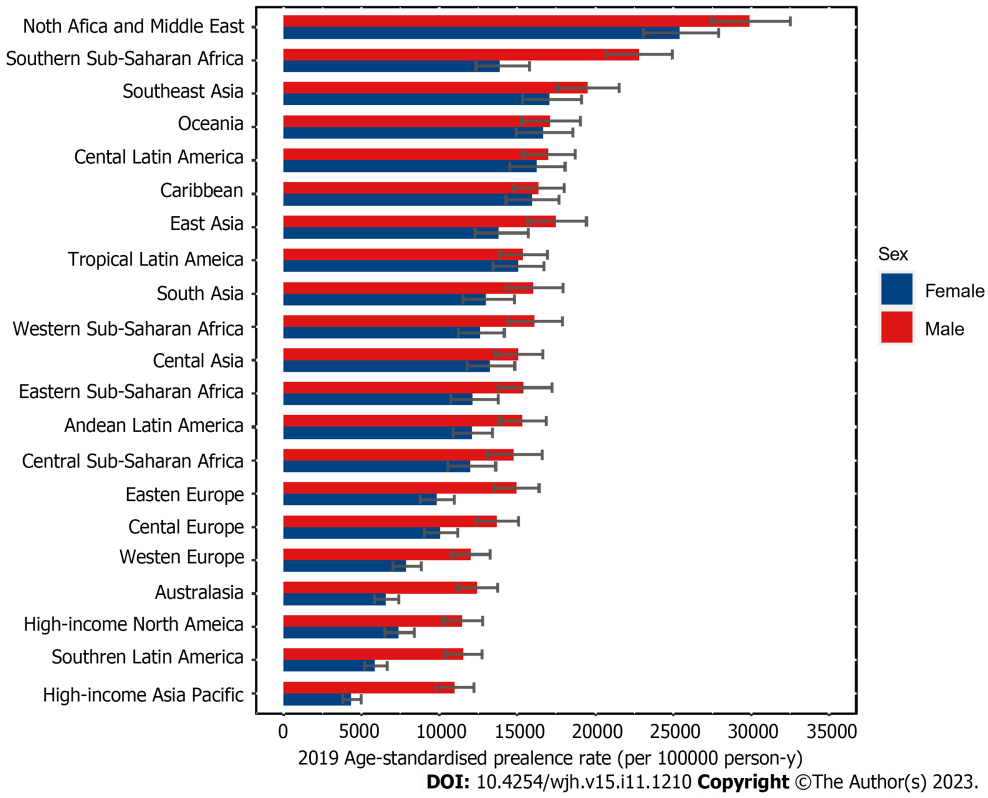
tary Figure 4).

Among the 21 GBD regions, both the highest ASDR and ASDAR were observed in Central Latin America in 2019. Both the lowest ASDR and ASDAR were observed in high-income Asia Pacific in 2019 (Supplementary Tables 1 and 2, Supplementary Figures 5 and 6). The highest increases in the ASDR (EAPC = 3.04; 0.05-6.12) and ASDAR (EAPC = 3.58; 3.06-4.11) were found in Eastern Europe, followed by Central Asia and high-income North America. In contrast, high-income Asia Pacific exhibited the most pronounced decreases in the ASDR (EAPC= -3.18; -8.97-2.97) and ASDAR (EAPC= -3.36; -4.57 to -2.14) (Table 1).

The highest ASPR of COCLDs due to NAFLD was found in North Africa and the Middle East regions, followed by Southeast Asia and Southern Sub-Saharan Africa (Figure 3, Supplementary Table 3). The highest increase in the ASPR was observed in high-income North America, followed by Australasia and Southern Latin America. Central Sub-Saharan Africa showed the lowest increases from 1990 to 2019 (Table 1).

National levels

At the national level, the ASPR of COCLDs due to NAFLD ranged from 6680.34 to 34515.88 per 100000 population in 2019. In that year, Egypt [34515.89 (31796.95-37253.06) per 100000] had the highest ASPR in 2019, followed by Qatar and Kuwait. Conversely, Finland, Canada, and Greenland had the lowest ASPR in 2019. The most pronounced changes in prevalent cases from 1990 to 2019 were seen in Qatar and Georgia. The largest increase in ASPRs was observed in the Republic of Korea [EAPC = 1.08; 95% confidence interval (CI): 1.03-1.13] and Equatorial Guinea between 1990 and 2019.



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Figure 3 Age-standardized prevalence rate for cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease by region and sex, 2019.

Only Zimbabwe (EAPC = -0.06; 95%CI: -0.10 to -0.03) showed a decreasing trend during this period (Supplementary Table 4, Figure 1C).

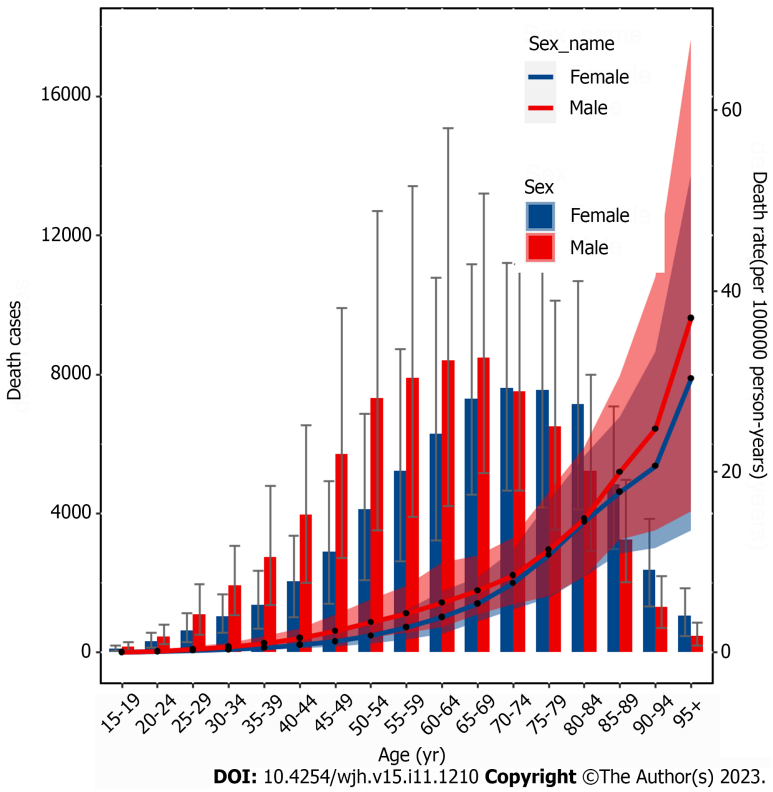
The highest ASDR of COCLDs due to NAFLD was observed in Egypt [14.02 (8.42-22.43)] in 2019, followed by Honduras and Guatemala. In contrast, Montenegro, Japan, and Singapore had the lowest ASDRs. Between 1990 and 2019, the United Arab Emirates [828.78% (368.37%-1461.06%)] showed the most significant increase in the number of deaths caused by COCLDs due to NAFLD, whereas Hungary showed a decrease of 39.94%. The largest increases in the ASDRs of COCLDs due to NAFLD were observed in Armenia (EAPC = 4.21; 95%CI: 0.98-7.54) and Kazakhstan (EAPC = 4.11; 95%CI: 1.33-6.97) from 1990 to 2019. In contrast, 143 countries or territories experienced decreasing trends, with the Republic of Korea presenting the largest decrease in ASDR during this period (EAPC = -4.19; 95%CI: -8.84 to -0.70) (Supplementary Table 5, Supplementary Figure 2).

The highest ASDAR of COCLDs due to NAFLD was observed in Guatemala [258.61 (170.42-329.98)] in 2019, followed by Honduras and Egypt. In contrast, Montenegro, Japan, and Singapore had the lowest ASDAR. Between 1990 and 2019, the United Arab Emirates [905.78% (402.13%-1627.41%)] showed the most significant increase in the number of DALYs from COCLDs due to NAFLD, whereas Hungary exhibited a decrease of 49.82% (-61.59% to -35.81%). The countries with the largest increases in ASDR during this period were Kazakhstan (EAPC = 4.02; 95%UI: 3.50-4.55) and Belarus. In contrast, the Republic of Korea experienced the largest decrease in ASDR, with the greatest reduction in ASDAR over the same period (Supplementary Table 6, Supplementary Figure 3).

Age and sex patterns

Globally, the prevalent number of COCLDs due to NAFLD exhibited an age-dependent pattern, reaching its peak at 45-49 years for males and 50-54 years for females. There was a declining trend in prevalence as age increased (Supplementary Figure 7). Similarly, the disease prevalence rate showed increasing and then decreasing trends with age in both sexes, with the highest prevalence rate observed in people aged 70-74 years, decreasing after this age group.

Globally, mortality rates increased with age and peaked at 65-69 years for males and 70-74 years for females before declining. The mortality rate steadily increased with age up to the 95+ age group for both sexes in 2019 (Figure 4). In the same year, the 50-54 age group for males and the 60-64 age group for females had the highest number of DALY cases, which decreased as age increased. The rate of DALYs peaked in the 80-84 age group, decreased in the 85-94 age group, and subsequently increased in the 95+ age group (Supplementary Figure 8). Among individuals under 70 years old, the numbers of prevalent cases, deaths, and DALYs lost was higher among males than females. However, among those aged 70 years and older, all three numbers were lower among males than among females (Figure 4, Supplementary Figures 7 and 8).



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Figure 4 All-age numbers and rates of deaths for cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease in 2019 are illustrated by sex for females and males.

Burden of COCLDs due to NAFLD by SDI

From 1990 to 2019, there was generally a negative correlation between SDI and global and regional ASDARs of COCLDs due to NAFLD. Globally, the observed burden of COCLDs due to NAFLD was lower than expected. In most regions, higher SDI values were associated with decreased ASDAR, except in Central Asia, high-income North America, and Eastern Europe, which showed an increasing trend during the study period. At the regional level, the observed burden of ASDAR of COCLDs due to NAFLD in Central Latin America, Andean Latin America, Eastern Sub-Saharan Africa, Western Sub-Saharan Africa, Southeast Asia, and Western Europe was found to be higher than the expected level based on the SDI from 1990 to 2019 (Figure 5). The link between the SDI and ASDR of COCLDs due to NAFLD had a similar pattern from 1990 to 2019 (Supplementary Figure 9). The predicted relationship between SDI and ASPR of COCLDs due to NAFLD exhibited an initial increasing trend, followed by a decreasing trend at an SDI value of 0.58 (Supplementary Figure 10).

At the national level, the ASDAR of COCLDs due to NAFLD in 2019 generally displayed a negative correlation with SDI. In numerous countries/territories, including Egypt, Guatemala, Honduras, and Mexico, the ASDAR was higher than the expected level based on SDI in 2019; conversely, in countries such as the Maldives, Bangladesh, Papua New Guinea, and Mozambique, the burden was lower than expected (Figure 6). Negative correlations between the national-level ASDR and ASPR of COCLDs due to NAFLD and SDI in 2019 were also found (Supplementary Figures 11 and 12).

DISCUSSION

This study comprehensively described the trends and patterns in prevalence, DALYs, and deaths caused by COCLDs due to NAFLD at the global, regional, and national levels over the past three decades. Globally, there were an estimated 123.56 million prevalent cases, 0.13 million deaths, and 3.62 million DALYs lost in 2019. Our findings indicated a substantial increase in the number of all-age deaths, prevalent cases, and DALYs. The global prevalence and ASPR of COCLDs due to NAFLD both showed increasing trends from 1990 to 2019. Although the ASDR and ASDAR of COCLDs due to NAFLD decreased between 1990 and 2019, the total number of deaths and DALYs experienced an increasing trend, which can be partly explained by population growth, longer life expectancy, and higher prevalence in older age groups. COCLDs due to NAFLD are an increasing threat to our population and place a strain on valuable health resources. As there is currently no effective treatment for NAFLD, this trend is likely to continue, driven by its increasing prevalence.

To highlight the vital role of metabolic dysfunction in the pathogenesis of fatty liver disease, the Asian Pacific Association for the study of the Liver proposed that NAFLD be renamed metabolic- or metabolic dysfunction-associated fatty liver disease (MAFLD) in 2020[22,23]. However, there exist slight discrepancies in the definitions of MAFLD and

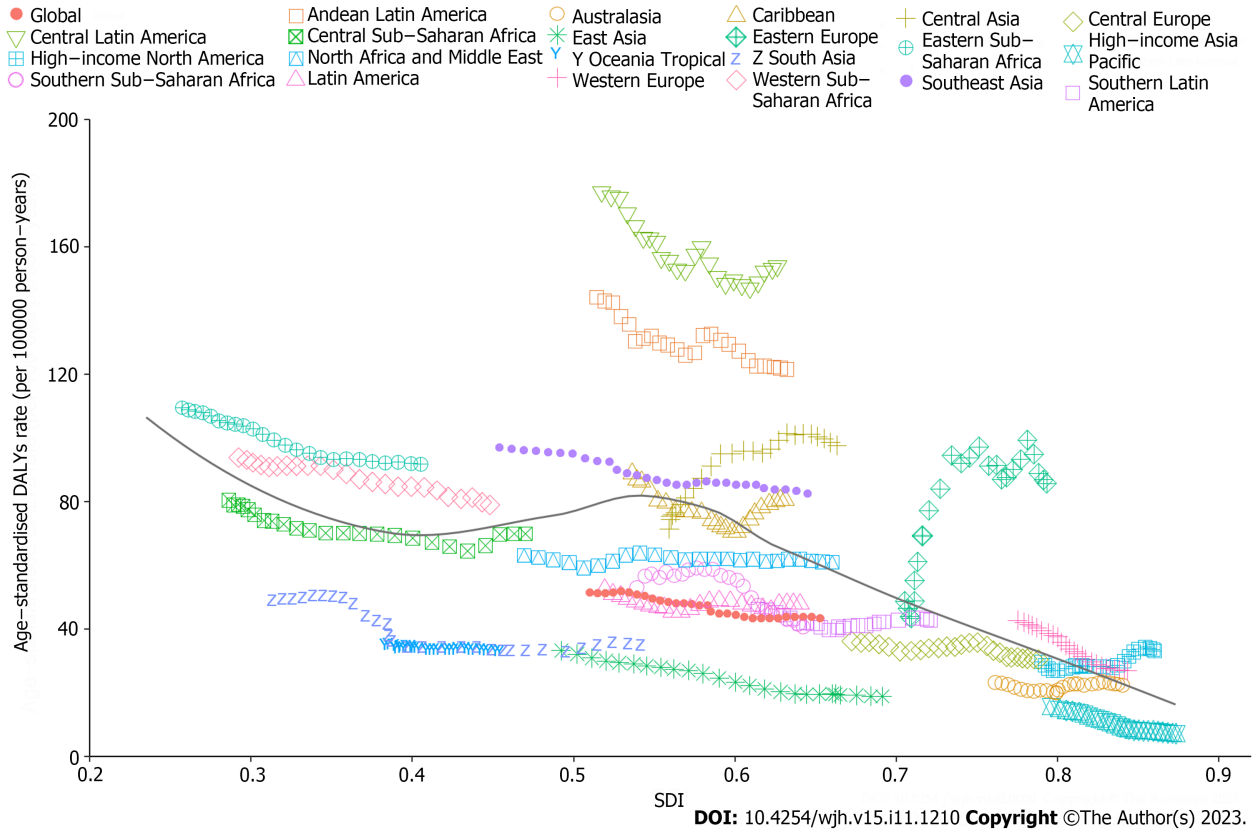


Figure 5 Age-standardized disability-adjusted life years rate of cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease by sociodemographic index globally and in 21 regions, 1990-2019. SDI: Sociodemographic index; DALYs: Disability-adjusted life years.

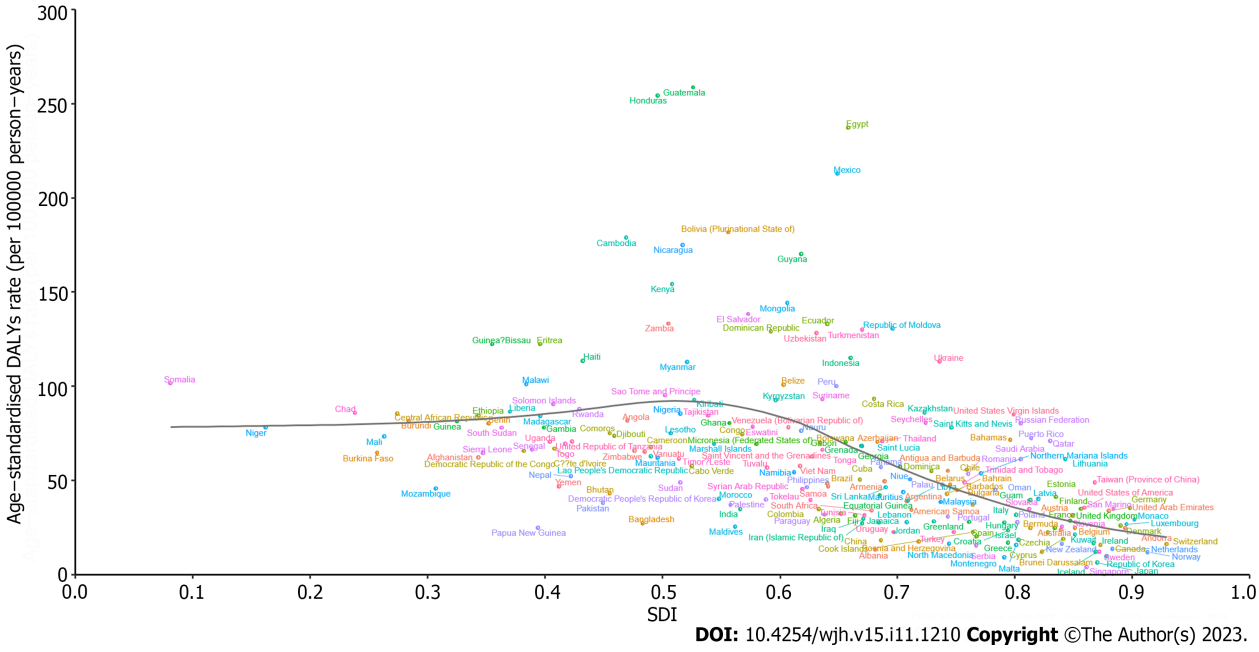


Figure 6 Age-standardized disability-adjusted life years rate of cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease by sociodemographic index in 204 countries and territories, 2019. DALYs: Disability-adjusted life years; SDI: Sociodemographic index.

NAFLD among different populations. The key distinctions between NAFLD and MAFLD lie in the requirement for NAFLD to exclude alcohol consumption and other risk factors, such as chronic viral hepatitis, whereas the MAFLD diagnosis focuses on detecting fatty liver in conjunction with metabolic risk factors, without necessitating the exclusion of other liver disorders[24]. In addition, GBD 2019 only estimated the burden of COCLDs due to NAFLD, and the MAFLD burden was not estimated. Therefore, in this study, we focused on the burden of COCLDs due to NAFLD.

NAFLD is closely associated with overweight and obesity, affecting an estimated 25% of the general adult population. Obesity significantly elevates the risk of NAFLD development. In a previous study, obesity was found in approximately 81% of patients with NASH and 50% of patients with NAFLD[25]. Lifestyle modifications and bariatric surgery have significantly improved NAFLD activity scores[26,27]. Earlier epidemiological investigations projected that the worldwide count of obese or overweight individuals surpassed 2.1 billion, constituting a pervasive global health concern[28]. For 2019, we identified the prevalent count of COCLDs attributed to NAFLD as 1.2 billion individuals, which was slightly less than the number of obese individuals.

Interestingly, reports indicate that around 20% of Asian individuals diagnosed with NAFLD have body mass index and waist circumference measurements falling within the range designated as lean, despite the prevailing association of NAFLD with overweight or obesity[29]. Notably, regions with higher incomes, such as the United States, France, and Japan, tend to exhibit an elevated prevalence of lean MAFLD[30]. However, certain developing countries, such as India and Sri Lanka, have an even higher prevalence rate of lean MAFLD, which may be attributed to racial and dietary factors. United States-based studies also indicate that people with ancestry from Latin America have a higher prevalence rate of lean MAFLD, whereas African Americans have a lower prevalence rate. The suggested racial and ethnic variations are important risk factors for the prevalence of lean MAFLD[30,31]. In addition, lean MAFLD patients have worse long-term outcomes than healthy people and have a similar prognosis to overweight or obese MAFLD patients. Therefore, more attention should be focused on normal-weight MAFLD patients, and a 3%-5% weight reduction and improvement in diet quality are strongly recommended to improve lean MAFLD[30]. Beyond body weight, additional factors such as diabetes, ethnic disparities, and genetic variations could also contribute to the prevalence and outcomes of lean NAFLD[32]. Given the ongoing global epidemic of obesity and diabetes, the burden of COCLDs attributed to NAFLD is expected to rise in the coming years.

According to previous research, the number of deaths due to cirrhosis caused by NAFLD/NASH was 102615 in 2012 and 118030 in 2017. The annual percent change in the ASDR of cirrhosis due to NAFLD/NASH from 2012 to 2019 was 0.29[33]. In contrast, our study found that the EAPC of the ASDR of COCLDs due to NAFLD was -0.65 from 1990 to 2019, indicating a decreasing trend. This variation in findings can be ascribed to disparities in the time frames of participant enrolment: Our study encompassed data from 1990 to 2019, while the study by Paik *et al*[33] included data from 2012 to 2017. Notably, we also calculated the EAPC of the ASDR of COCLDs due to NAFLD from 2012 to 2019 and found a decreasing trend with a value of -0.05. This indicates that there may have been a decreasing trend from 2017 to 2019.

The global upsurge in type 2 diabetes and obesity has raised the prevalence of NAFLD in both developed and developing nations[4]. Our study found that the highest ASPRs of COCLDs due to NAFLD were in some developing regions, such as North Africa, the Middle East regions, and Southeast Asia, and in specific countries such as Egypt, Qatar, and Kuwait. Previous research reported an NAFLD prevalence of 42.04% in South Asia and 31.79% in the Middle East, which is consistent with our finding that these regions had the highest prevalence rates[25,34].

According to previous research, approximately half of the global burden of liver complications associated with NAFLD is concentrated in the Middle East, North Africa, and Asia[35]. The escalated prevalence in these geographical areas can be attributed to intricate interplays of lifestyle choices, economic conditions, and ethnic factors. The tandem epidemics of obesity and NAFLD are predominantly propelled by unhealthy dietary practices and sedentary habits characterized by consumption of calorie-dense foods and insufficient physical activity[34]. Studies have indicated that Middle East and North African countries have a high prevalence of overweight and obesity, with more than 30% of females and more than 20% of males being obese in most countries in the region. This trend is attributed to unhealthy diets and the lowest levels of physical activity worldwide[36]. Concurrently, the Middle East and North Africa register the highest prevalence rates of cirrhosis of the liver attributable to NAFLD. This underscores the critical need for effective interventions aimed at mitigating the escalating prevalence of obesity and diabetes to ameliorate the burden of COCLDs attributed to NAFLD [37].

According to our study, the prevalence, deaths, and DALYs lost due to COCLDs stemming from NAFLD were higher in males than in females across all age groups before the age of 65-69 years. Similarly, the rates of these occurrences were comparable when compared with their female counterparts across all age groups. This sex disparity is consistent with previous research demonstrating that from 1990 to 2017, the burden of cirrhosis in males was universally higher in males than in females[38]. Hormonal factors could underlie this pattern, where estrogen, acting as an antioxidant, mitigates the activity of stellate cells and the advancement of liver fibrosis[39]. After menopause, women lose the protective effect of estrogen[40], and physiological changes associated with hypoestrogenism, such as insulin resistance, dysglycemia, dyslipidemia, and visceral fat accumulation, may be associated with the higher prevalence of COCLDs due to NAFLD in postmenopausal women[41,42]. This may partly elucidate the higher prevalence in males older than 70 years in our study.

We found that the number of prevalent cases, deaths, and DALYs lost due to COCLDs due to NAFLD was the highest among middle-aged groups (approximately 45-69 years). Within this range, the 75-79 age group presented the highest prevalence rate, while the 95+ age group exhibited the highest mortality rate. The underlying reasons for these findings are multifactorial. One potential explanation involves the metabolic alterations that transpire in older age groups[43]. Another possible reason is the natural history of liver cirrhosis, which is characterized by a compensated phase that lasts significantly longer than the rapidly progressive decompensated phase, with a median survival time of more than 12 years. A large proportion of patients with cirrhosis die after transitioning from the compensated phase to the decompensated phase[5]. Given this context, the imperative need for accurate, noninvasive methodologies to enable early identification of COCLDs stemming from NAFLD becomes evident.

We found a negative correlation between the SDI and the ASDAR of COCLDs due to NAFLD in the 21 GBD regions from 1990 to 2019 and in 204 countries in 2019. Generally, regions with higher SDI had a lower burden of cirrhosis due to NAFLD, which may be attributed to accessible high-quality health care and enough safe spaces to exercise[44,45].

Conversely, low-SDI and low-middle-SDI regions tended to bear a higher burden. The Sub-Saharan African region showed a high ASDAR of COCLDs due to NAFLD in 2019. Some regions such as Western Sub-Saharan Africa, Southeast Asia, Central Latin America, Andean Latin America, and Eastern Sub-Saharan Africa, as well as some countries and territories, such as Egypt, Honduras, and Guatemala, had burdens higher than expected based on their SDIs, indicating that these regions and countries should receive more investment and public health programs.

The escalating prominence of COCLDs emanating from NAFLD has positioned it as a preeminent public health challenge. Notably, an absence of effective pharmaceutical interventions to fully eradicate NAFLD persists. Consequently, interventions targeting weight loss could be efficacious and cost-effective strategies to avert the progression of NAFLD to COCLDs. In addition, exercise interventions without significant weight loss have also had a beneficial effect on alleviating NAFLD[46]. Thus, it is imperative to emphasize the critical role of weight management and exercise within public health programs. Furthermore, an enhancement of noninvasive diagnostic methods and the development of effective treatment strategies will be pivotal in alleviating the burden of COCLDs stemming from NAFLD. Importantly, public awareness regarding NAFLD and its associated complications remains inadequate. A concerted effort to raise population awareness about the implications of NAFLD is paramount.

In this study, we pioneered a comprehensive analysis of the relative burden of COCLDs attributed to NAFLD on global, regional, and national scales spanning the period from 1990 to 2019. Nevertheless, several limitations warrant consideration. First, the data from GBD 2019 have the general limitations of the GBD approach that have been described. The GBD estimates depended on robust statistical methods and trends from neighboring countries to overcome data scarcity and low data quality in some countries. Second, liver biopsy remains the gold-standard diagnostic test for patients with COCLDs due to NAFLD, but its poor acceptability during compensation and sampling variability may lead to misdiagnosis and underdiagnosis[47]. A dearth of diagnostic techniques may cause an underestimation of COCLDs due to NAFLD, which may be more severe in regions with low SDIs. Third, patients admitted to hospitals mostly had decompensated cirrhosis. Therefore, the number of compensated cirrhosis cases may be underestimated. The underreporting of cirrhosis can bias the estimates. Fourth, GBD 2019 failed to adopt the new term MAFLD to replace NAFLD. NAFLD was defined only after the exclusion of other causes of hepatic steatosis, and there is an unclear differentiation between NAFLD and alcoholic liver disease owing to different adjustments for alcohol use. Finally, the exclusion of patients with cirrhosis with hepatocellular carcinoma from our study could result in an underestimation of the true mortality rate among individuals with liver cirrhosis due to NAFLD.

CONCLUSION

This study described the burden of COCLDs due to NAFLD in 204 countries and territories from 1990 to 2019 by age, sex, and SDI. COCLDs due to NAFLD are becoming a significant global public health concern. Over the past three decades, there has been a notable increase in the ASPR, while the ASDR and ASDAR have exhibited downward trends. Notably, substantial geographic disparities exist in the burden of COCLDs due to NAFLD, with the highest prevalence rates observed in North Africa and the Middle East. In 2019, males had a higher burden of prevalence, deaths, and DALYs lost than females before the 65-69 age group. Furthermore, there is a negative correlation between SDI values and ASDAR. We hope this study raises public awareness of COCLDs due to NAFLD and broadcasts the need for more effective prevention strategies to minimize the future health care burden.

ARTICLE HIGHLIGHTS

Research background

The incidence and prevalence of nonalcoholic fatty liver disease (NAFLD) have been rapidly increasing worldwide over the past few decades, leading to cirrhosis and other chronic liver diseases (COCLDs). Cirrhosis is the leading cause of liver-related morbidity and contributes to more than 1 million deaths annually worldwide. NAFLD has become the leading cause of COCLDs.

Research motivation

A previous study reported the burden of liver cirrhosis caused by nonalcoholic steatohepatitis. However, no studies have focused on the epidemiology of COCLDs due to NAFLD across the globe.

Research objectives

We conducted a comprehensive and comparable updated analysis of the global, regional, and national levels of prevalence, death, and disability-adjusted life-years (DALYs) of COCLDs due to NAFLD in regards to age-standardized rates and numbers from 1990 to 2019, stratified by sex, age, and sociodemographic index.

Research methods

Data on COCLDs due to NAFLD were collected from the Global Burden of Diseases, Injuries, and Risk Factors Study 2019. Numbers and age-standardized prevalence, death, and DALYs were estimated through a systematic analysis of modeled data from the Global Burden of Diseases, Injuries, and Risk Factors Study 2019. Estimated annual percentage

change was used to determine the burden trend.

Research results

We found that the global age-standardized prevalence rate of COCLDs due to NAFLD was 15022.90 per 100000 population in 2019, with an estimated annual percentage change of 0.78. The age-standardized death rate and age-standardized DALYs rate per 100000 population were 1.66 and 43.69 in 2019, respectively. The highest prevalence rate was observed in North Africa and the Middle East. The numbers of prevalent cases, deaths, and DALYs cases of COCLDs due to NAFLD were higher in males than in females across all age groups before the age of 65-69 years. There was a negative correlation between sociodemographic index and age-standardized DALYs rate.

Research conclusions

COCLDs due to NAFLD have emerged as a large and growing public health burden worldwide. Globally, the ASPR has increased during the past three decades, whereas the ASDR and age-standardized DALY rate have decreased. There is geographical variation in the burden of COCLDs due to NAFLD. It is strongly recommended to improve the quality of COCLDs due to NAFLD health data across all countries and regions to facilitate better monitoring of the burden of COCLDs due to NAFLD.

Research perspectives

We believe that the findings of this study will provide insight into the global disease burden of COCLDs due to NAFLD and assist policymakers in formulating effective policies to mitigate modifiable risk factors.

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FOOTNOTES

Co-first authors: Zhi-Peng Liu, Guo-Qing Ouyang.

Co-corresponding authors: Song-Qing He, Guan-Dou Yuan.

Author contributions: Liu ZP, Ouyang GQ, He SQ, and Yuan GD conceived and designed the study; Liu ZP, Ouyang GQ, Huang GZ, Wei J, and Dai L were involved in collection and interpretation of the data; Liu ZP, Ouyang GQ, and Yuan GD edited the manuscript; Liu ZP, Ouyang GQ, and He SQ revised the manuscript; All authors were involved in reading and approving the final manuscript. We designated co-corresponding authors because our research was a collaborative team effort, and the designation of co-corresponding authors helped to assign responsibilities and tasks related to the paper. This helped improve the quality and reliability of the paper. Second, He SQ and Yuan GD made equally important contributions throughout the study. The selection of co-corresponding authors recognizes this equal contribution. In conclusion, we believe that the designation of He SQ and Yuan GD as co-corresponding authors is an appropriate choice for our manuscript because it accurately reflects the collaborative spirit and diversity of our team. Liu ZP and Ouyang GQ contributed equally as co-first authors to this work; He SQ and Yuan GD contributed equally as co-corresponding authors to this work.

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Clinical trial registration statement: This study was an analysis of the burden of cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease. The data were extracted from the Global Burden of Diseases, Injuries, and Risk Factors database, and clinical trial registration statements were not necessary.

Informed consent statement: This study was an analysis of the burden of cirrhosis and other chronic liver diseases due to nonalcoholic fatty liver disease. The data were extracted from the Global Burden of Diseases, Injuries, and Risk Factors database, and ethics approval and consent to participate were not necessary.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: Data are available from http://ghdx.healthdata.org/gbd-results-tool and can be acquired from the corresponding author at dr_hesongqing@163.com.

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ORIGINAL ARTICLE

Observational Study

Evaluation of a protocol for rifaximin discontinuation in critically ill patients with liver disease receiving broad-spectrum antibiotic therapy

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Abstract

BACKGROUND

Rifaximin is frequently administered to critically ill patients with liver disease and hepatic encephalopathy, but patients currently or recently treated with antibiotics were frequently excluded from studies of rifaximin efficacy. Due to overlapping spectrums of activity, combination therapy with broad-spectrum antibiotics and rifaximin may be unnecessary. A pharmacist-driven protocol was piloted to reduce potentially overlapping therapy in critically ill patients with liver disease. It was hypothesized that withholding rifaximin during broad-spectrum antibiotic therapy would be safe and reduce healthcare costs.

AIM

To determine the clinical, safety, and financial impact of discontinuing rifaximin during broad-spectrum antibiotic therapy in critically ill liver patients.

METHODS

This was a single-center, quasi-experimental, pre-post study based on a pilot pharmacist-driven protocol. Patients in the protocol group were prospectively identified *via* the medical intensive care unit (ICU) (MICU) protocol to have rifaximin withheld during broad-spectrum antibiotic treatment. These were compared to a historical cohort who received combination therapy with broad-spectrum antibiotics and rifaximin. All data were collected retrospectively. The primary outcome was days alive and free of delirium and coma (DAFD) to 14 d.

Safety outcomes included MICU length of stay, 48-h change in vasopressor dose, and ICU mortality. Secondary outcomes characterized rifaximin cost savings and protocol adherence. Multivariable analysis was utilized to evaluate the association between group assignment and the primary outcome while controlling for potential confounding factors.

RESULTS

Each group included 32 patients. The median number of delirium- and coma-free days was similar in the control and protocol groups [3 interquartile range (IQR 0, 8) *vs* 2 (IQR 0, 9.5), $P = 0.93$]. In multivariable analysis, group assignment was not associated with a reduced ratio of days alive and free of delirium or coma at 14 d. The protocol resulted in a reduced median duration of rifaximin use during broad-spectrum antibiotic therapy [6 d control (IQR 3, 9.5) *vs* 1 d protocol (IQR 0, 1); $P < 0.001$]. Rates of other secondary clinical and safety outcomes were similar including ICU mortality and 48-h change in vasopressor requirements. Overall adherence to the protocol was 91.4%. The median estimated total cost of rifaximin therapy per patient was reduced from $758.40 (IQR $379.20, $1200.80) to $126.40 (IQR $0, $126.40), $P < 0.01$.

CONCLUSION

The novel pharmacist-driven protocol for rifaximin discontinuation was associated with significant cost savings and no differences in safety outcomes including DAFD.

Key Words: Rifaximin; Hepatic encephalopathy; Critical illness; Antibiotics; Liver disease; Cirrhosis

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Core Tip: Critically ill patients with liver disease receiving broad-spectrum antibiotic therapy have been frequently excluded from clinical trials of rifaximin efficacy. Therefore, despite overlapping spectrums of antibacterial activity, it is not known if rifaximin provides additional clinical benefit in these patients. In this study, pharmacist-guided rifaximin discontinuation during broad-spectrum antibiotic therapy resulted in significant cost savings and was not associated with negative short-term cognitive effects or adverse events.

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INTRODUCTION

Hepatic encephalopathy (HE) encompasses a spectrum of neurocognitive alterations in patients with liver dysfunction and/or porto-systemic shunting, and is associated with symptoms that range in severity from minimal neuropsychiatric manifestations to cerebral edema and coma in the most severe cases[1,2]. The pathogenesis of HE has not been fully elucidated and is likely multifactorial. Ammonia has been implicated as a contributing factor to the development of HE due to its association with direct neurotoxicity mediated by astrocyte swelling and modification of glutamine and gamma-amino-n-butyric acid systems[3]. Rifaximin is an oral, non-absorbable rifamycin derivative with antibiotic activity against ammonia-producing gram-positive, gram-negative, and anaerobic species[4,5]. Long-term rifaximin use is associated with clinically important reductions in infections, hospital re-admissions, durations of hospital stays, and overt HE recurrence[6-8]. The exact mechanism by which rifaximin exerts benefit remains unclear[9]. Previous hypotheses focused on the control of ammonia-producing enteric bacteria *via* antibiotic activity[10,11]. However, a growing body of evidence depicts increasingly understood mechanisms of rifaximin activity including decreased circulating endotoxin burden, decreased microbiota-derived systemic inflammation, and improvement in cirrhosis-related dysbiosis which suggests the presence of multi-factorial benefits of rifaximin in the pathobiology of cirrhosis[12]. Rifaximin is recommended by the American Association for the Study of Liver Diseases (AASLD) as adjunctive therapy for the prevention of overt HE recurrence (grade I, A, 1)[13]. Similarly, the European Association for the Study of the Liver (EASL) recommends rifaximin as an adjunct to lactulose as secondary prophylaxis following ≥ 1 additional episodes of overt HE within 6 mo of the first episode (LoE 2, strong)[14]. Use of rifaximin for the treatment of HE is not recommended in these guidelines; however, efficacy has been demonstrated in randomized controlled trials and use is common in clinical practice[15-18].

Patients receiving or recently treated with antibiotics were frequently excluded from these trials[15-17]. These studies, which included patients with both acute and chronic HE, excluded cases with precipitants or recent medication exposures which could interfere with HE or therapeutic effect monitoring. Infection is a frequent precipitant of overt HE in critically ill patients, the treatment of which commonly relies on broad-spectrum antibiotics[19]. In many cases of infection, patients also receive rifaximin either as a continuation of home therapy or newly initiated treatment. Because these

patients are infrequently studied, it remains unclear if rifaximin provides additional therapeutic benefit when combined with broad-spectrum antibiotics.

In 2018, Cleveland Clinic established the medical intensive liver unit (MILU), a specialty unit of the MICU supported by a multidisciplinary team including intensivists, hepatologists, and critical care clinical pharmacy specialists caring for patients with a variety of hepatic pathologies. Many patients admitted to the MILU are initiated on broad-spectrum antibiotics for empiric or targeted therapy for infections in addition to HE treatment with rifaximin. A pharmacist-driven pilot protocol was implemented to reduce potentially overlapping therapy through the discontinuation of rifaximin during broad-spectrum antibiotic treatment. Pharmacists were also responsible for the coordination of rifaximin re-initiation following antibiotic therapy narrowing or discontinuation. This study aimed to evaluate the impact of this protocol on clinical, safety, and financial outcomes.

MATERIALS AND METHODS

Study design

This was an Institutional Review Board-approved, quasi-experimental, pre-post study conducted at a large quaternary academic medical and liver transplant center in the United States. The pharmacist-driven protocol for rifaximin discontinuation was implemented beginning August 1, 2020. Adult patients in the medical intensive care unit (ICU) (MICU) were prospectively screened by clinical pharmacy specialists and eligible for the protocol if they had orders for rifaximin and a qualifying antibiotic regimen (Table 1). Discontinuation of rifaximin was recommended and recorded by the pharmacist and research team. Duration of antibiotic therapy was tracked and reviewed daily by a small group of critical care clinical pharmacy specialists to ensure re-initiation of rifaximin upon antibiotic narrowing or discontinuation. Before implementation, education was provided to all pharmacists who would manage or verify orders for MICU patients to reduce time to rifaximin discontinuation for patients admitted to the MICU during evenings and weekends. Physician leadership and medical teams were also involved in education about the protocol and its implementation.

Patient selection

Patients[3] 18 years old were eligible for study inclusion if they received at least 3 d of broad-spectrum antibiotics and had an order for rifaximin during MICU admission. Additional inclusion criteria for the control group were: (1) Admission to the MICU between August 1 and October 31, 2019 and (2) rifaximin therapy for ≥ 3 d or 75% of the antibiotic treatment duration during MICU admission, whichever was longer. In the protocol group inclusion criteria were (1) admission to the MICU between August 1, 2020 and January 31, 2021; and (2) ≥ 3 d of broad-spectrum antibiotics without rifaximin and concomitant rifaximin for ≤ 25% of the antibiotic duration during MICU admission. Any patient with a positive test for severe acute respiratory syndrome coronavirus 2 during admission was excluded.

Outcomes

The primary outcome was days alive and free of delirium and coma (DAFD) to day 14. Secondary outcomes were days alive and free of delirium to day 14, days alive and free of coma to day 14, ICU length of stay, ICU mortality, time to first extubation, rate of reintubation, days of combination therapy during MICU admission, rate of protocol adherence, time to rifaximin discontinuation in the protocol group, and the per-patient cost of rifaximin therapy during the follow-up period. The cost of rifaximin therapy was calculated using the average wholesaler price as of January 2023 to reflect the increase in rifaximin cost since initial protocol implementation[20]. The minimum cost of rifaximin was calculated based on one tablet given (control) or saved (protocol) per day of therapy while the maximum cost assumed two tablets given or saved per day. Changes in vasopressor requirements and Glasgow Coma Score (GCS) during the first 48 h of MICU combination therapy or withholding rifaximin were evaluated as additional safety measures.

Study definitions

Day one for the 14-d study period was defined as the first day during MICU admission on which patients received broad-spectrum antibiotics and rifaximin (control group) or broad-spectrum antibiotics without rifaximin (protocol group). Broad-spectrum antibiotic regimens were defined as providing gram-positive, gram-negative, and anaerobic coverage (Table 1). A day of therapy was defined as a 24-h period from midnight to 11:59 pm during which at least one-half of the scheduled doses of rifaximin and/or broad-spectrum antibiotics were received. Days were considered delirium-free if patients were alive and without a positive confusion assessment method for the ICU (CAM-ICU) assessment during the 24-h period and coma-free if patients were alive and with zero hours spent with a Richmond Agitation Sedation Scale (RASS) score of -4 or -5 or with GCS of 3 during the 24-h period. Days of mechanical ventilation were defined as the use of positive pressure ventilation during any one hour of the 24-h period from midnight to 11:59 pm for use in the multivariable model. All admission days in non-ICUs during which the patient was alive were considered to be free of delirium as brief CAM (bCAM) and West-Haven grades (WHG) were not routinely recorded. Protocol adherence was defined as the discontinuation of rifaximin occurring within 72-h of protocol-defined broad-spectrum antibiotic therapy initiation. All vasopressor doses were converted to norepinephrine equivalents according to the following equation: [norepinephrine (mcg/min) + (epinephrine (mcg/min)] + [(dopamine (mcg/kg/min) ÷ 2] + [(phenylephrine (mcg/min) ÷ 10] + [vasopressin (units/hour) × 8.33][21,22]. Sedative agents included propofol, dexmedetomidine, ketamine, lorazepam, or midazolam when administered as a continuous infusion. Ileus was defined as > 48 h with zero bowel movements or fecal management system output recorded. Orders for octreotide continuous infusions were used as a

Table 1 Protocol-defined broad-spectrum antibiotic regimens

Monotherapy	Gram-positive/negative	Gram-positive	Gram-negative	Anaerobic
Ampicillin-sulbactam	Cefazolin	Vancomycin	Aminoglycosides	Metronidazole
Amoxicillin-clavulanate	Cephalexin	Linezolid	Polymyxin B and colistin	Clindamycin
Piperacillin-tazobactam	Cefuroxime	Daptomycin	Aztreonam	
Cefoxitin	Cefdinir	Quinupristin-dalfopristin	Cefiderocol	
Meropenem +/- vaborbactam	Cefixime	Rifampin		
Imipinem-cilastatin +/- relebactam	Ceftazidime +/- avibactam	Rifabutin		
Ertapenem	Ceftriaxone	Penicillins		
Tigecycline	Cefepime			
Eravacycline	Ceftaroline			
	Ceftolozane-tazobactam			
	Ciprofloxacin			
	Levofloxacin			
	Doxycycline			
	Sulfamethoxazole-trimethoprim			

Broad-spectrum antibiotic therapy is defined as: (1) Any agent from "Monotherapy"; (2) one agent from each of "Gram-positive/negative" and "Anaerobic"; (3) one agent from each of "Gram-positive," "Gram-negative," and "Anaerobic."

surrogate to identify episodes of gastrointestinal bleeding according to routine institutional practice. Occurrences of *Clostridioides difficile* infection were recorded if the patient had either a positive polymerase chain reaction or enzyme-linked immunosorbent assay test.

Statistical analyses

Continuous data were assessed for normality by the Shapiro-Wilk test. Parametric continuous data are reported as mean ± SD and were analyzed using a two-sample *t*-test. Non-parametric continuous data are reported as median (IQR) and were analyzed using the Wilcoxon rank-sum test. Categorical data are presented as number (%) and were analyzed by chi-squared or Fisher's exact tests, based on sample size. The primary outcome of DAFD was compared using a one-sided Wilcoxon rank-sum test, assuming greater median DAFD in the control group. It was calculated that the inclusion of 32 patients in each group would provide 80% power to detect a 0.65-d difference in DAFD, with a one-sided significance level of 0.025. Multivariable analysis of the primary outcome was planned to include covariates of biologic plausibility (duration of mechanical ventilation, use of deep sedation, MELD-Na score, gastrointestinal bleeding) and those with a *P*-value of < 0.05 in univariable analysis. A negative binomial model was selected for the multivariable analysis to account for the observed over-dispersion of the primary outcome which violated foundational assumptions of a Poisson distribution that was attempted after similar violations of linear regression despite log-transformation of the variables. All variables included in the model were selected based on prior literature and biological plausibility to contribute to or interfere with the assessment of delirium and coma or to indicate a clinically significant baseline difference in illness severity between the groups. All analyses were performed based on an overall significance level of 0.05 using either SAS software (version 9.4, Cary, NC) or Stata/IC software, v.14 (StataCorp LP, College Station, TX). Data extracted from the electronic medical record were collected and managed using REDCap electronic data capture tools hosted by Cleveland Clinic[23,24].

RESULTS

Overall characteristics of patients

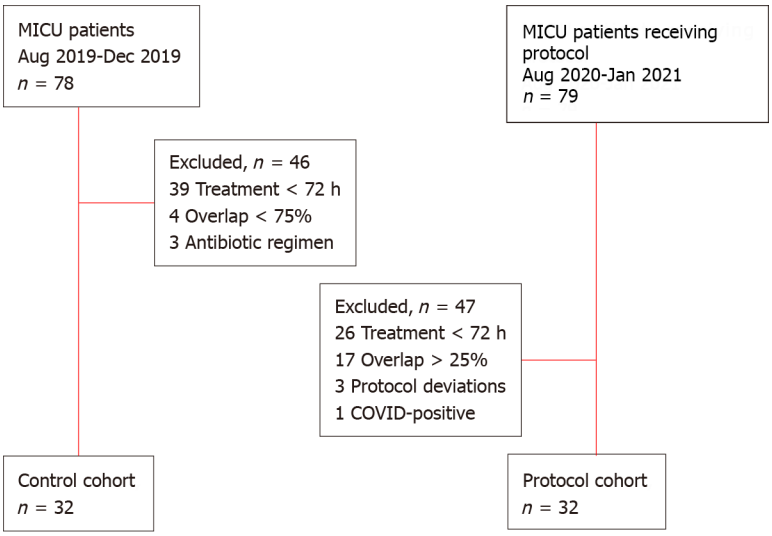
A total of 159 patients were screened for inclusion and 32 were included in both groups (Figure 1). The most common reason for exclusion in both groups was insufficient treatment duration. The two groups were well-balanced at baseline with the exception of norepinephrine requirements on the day of MICU admission, which were higher in the protocol group, and race (Table 2). There was no difference in high-grade HE at baseline; however, due to intubation and sedation on study day 1, many patients were unable to be assigned baseline WHG. Though none of these differences were statistically significant, deep sedation, paralysis, and gastrointestinal bleeding were more common among protocol patients while control patients more often received scheduled benzodiazepines and were admitted directly to the ICU.

Table 2 Baseline characteristics

Variable	Control (*n* = 32)	Protocol (*n* = 32)	*P* value
Male	19 (59.4)	18 (56.2)	0.21
Age (yr)	57.9 (± 12.9)	53.0 (± 12.7)	0.13
Race			0.01
White	26 (81.3)	24 (75)	
Unavailable	4 (12.5)	2 (6.3)	
Black	1 (3.1)	1 (3.1)	
American Indian/Alaskan native	1 (3.1)	0 (0)	
Asian	0 (0)	0 (0)	
Multiracial-Multicultural	0 (0)	0 (0)	
Weight (kg)	85.4 (± 23.1)	91.3 (± 29.2)	0.37
Direct Intensive Care Unit Admission	15 (46.9)	12 (37.5)	0.45
SOFA score	10.2 (± 3.0)	11 (± 3.2)	0.34
NE requirements, mcg/kg/min	0 (0, 0.136)	0.023 (0, 0.309)	0.02
MELD-Na	28.5 (± 8.5)	30.0 (± 8.2)	0.48
West-Haven grade			0.31
Unavailable	10 (31.3)	6 (18.8)	
0	6 (18.8)	8 (25)	
1	6 (18.8)	6 (18.8)	
2	4 (12.5)	6 (18.8)	
3	5 (15.6)	5 (15.6)	
4	1 (3.1)	1 (3.1)	
Glasgow Coma Score	9 (6, 14)	11 (7, 15)	0.69
Cirrhosis Etiology			0.21
Ethanol	17 (53.1)	21 (65.6)	
Non-alcoholic steatohepatitis	9 (28.1)	5 (15.6)	
Primary biliary cholangitis	2 (6.3)	1 (3.1)	
Autoimmune hepatitis	2 (6.3)	1 (3.1)	
Primary sclerosing cholangitis	0 (0)	1 (3.1)	
Unknown	2 (6.3)	0 (0)	
Other	0 (0)	3 (9.4)	
Pre-ICU rifaximin treatment	12 (37.5)	17 (48.6)	0.21
Antibiotic Indication			0.80
Empiric; source unknown	20 (62.5)	23 (71.9)	
Pneumonia	6 (18.8)	3 (9.4)	
Intraabdominal	4 (12.5)	4 (12.5)	
Bloodstream infection	1 (3.1)	1 (3.1)	
Skin and soft tissue	1 (3.1)	0 (0)	
Bone and joint infection	0 (0)	1 (3.1)	
Rifaximin regimen			0.37
550 mg BID	31 (96.9)	31 (96.9)	
400 mg BID	1 (3.1)	0 (0)	

200 mg BID	0 (0)	1 (3.1)	
Deep sedation use	5 (15.6)	9 (28.1)	0.38
Benzodiazepine use	3 (9.4)	0 (0)	0.29
Continuous neuromuscular blockade use	2 (6.3)	3 (9.4)	1.00
Polyethylene glycol use	9 (28.1)	13 (40.6)	0.29
Lactulose use	29 (90.6)	31 (96.9)	0.30
Gastrointestinal bleeding treatment	8 (25)	14 (43.8)	0.11
Alcohol withdrawal diagnosis	1 (3.1)	0 (0)	1.00
Ileus	6 (18.8)	9 (28.1)	0.56
C. difficile infection	1 (3.1)	2 (6.3)	1.00

All values are presented as mean ± SD, median (interquartile range), or N (%). BID: bis in die; ICU: Intensive care units; MELD-Na: Model for End-Stage Liver Disease Sodium score; NE: Norepinephrine; SOFA: Sequential Organ Failure Assessment score.



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Figure 1 Consort diagram.

Primary outcome

No significant differences were observed in the primary outcome (DAFD to day 14), between the control and protocol groups [3 (interquartile range (IQR 0, 8) *vs* 2 (IQR 0, 9.5); $P = 0.93$] (Table 3). After adjustment for deep-sedation, gastrointestinal bleeding treatment, MELD-Na score, and duration of mechanical ventilation in a negative binomial regression there remained no significant difference in the primary outcome between the control and protocol groups (ratio 0.78, 95% confidence interval 0.39-1.56, $P = 0.48$) (Table 4).

Secondary outcomes including safety analyses

The observed ICU mortality rate was high in both groups [control 13 (40.6%) *vs* protocol 15 (46.9%); $P = 0.61$]. Days alive and free of either delirium or coma, ICU length of stay, and time to extubation were similar between the groups. Similarly, no significant differences in vasopressor requirements and GCSs in the 48 h following rifaximin discontinuation were observed between groups (Table 3). For patients included in the protocol group, the median time to rifaximin discontinuation was approximately 24 h from MICU admission. Protocol adherence was 91.4% with the most common reason for non-adherence being rifaximin discontinuation during antibiotic therapy not meeting the protocol definition of broad-spectrum. Days of rifaximin therapy during broad-spectrum antibiotics were significantly reduced in the protocol group [6 (IQR 3-9.5) *vs* 1 (IQR 0-1); $P < 0.001$]. The cost of rifaximin therapy was also significantly reduced in the protocol group with an estimated cost savings of $316.00 [United States dollar (USD)] to $632.00 (USD) per patient.

Table 3 Clinical, safety, and cost outcomes

Outcome	Control (*n* = 32)	Protocol (*n* = 32)	*P* value
Primary outcome			
Days alive and free of delirium and coma to day 14	3 (0, 8)	2 (0, 9.5)	0.93
Secondary outcomes			
Days alive and free of delirium	3 (0, 8.5)	2 (0, 9)	0.85
Days alive and free of coma (RASS)	6 (3.5, 12)	8 (4, 11)	0.81
Days alive and free of coma (GCS)	8 (5.5, 13)	7.5 (5.5, 9)	0.21
Glasgow Coma Score 48-h change	0 (-3, 1.5)	0 (-1, 0.5)	0.43
ICU mortality (%)	13 (40.6)	15 (46.9)	0.61
ICU length of stay	10 (4.5, 20.5)	11 (7, 17)	0.73
Time to first extubation from day 1 of intubation	6 (4, 14) (*n* = 23)	5 (4, 9) (*n* = 26)	0.49
Rate of reintubation (%)	4 (17.4)	3 (11.5)	0.56
IVVP requirement 48-h change, NE mcg/kg/min equivalents	0 (0, 0.12)	0 (0, 0.01)	0.45
Days of MICU combination therapy	6 (3, 9.5)	1 (0, 1)	< 0.01
Protocol adherence	-	91.4%	-
Time to rifaximin discontinuation, days	-	1 (0, 1)	-
Cost of rifaximin therapy per patient to day 14, USD			
Minimum	379.20 (189.60, 600.40)	63.20 (0, 63.20)	< 0.01
Maximum	758.40 (379.20, 1200.80)	126.40 (0, 126.40)	< 0.01

GCS: Glasgow Coma Score; ICU: Intensive care unit; IVVP: Intravenous vasopressor; MICU: Medical intensive care unit; NE: Norepinephrine; RASS: Richmond Agitation-Sedation Scale; USD: United States dollar.

Table 4 Negative binomial multivariable model

(*n* = 60)	DAFD ratio (95%CI)
Group assignment (protocol)	0.78 (0.39, 1.56)
Deep sedation (yes)	0.89 (0.39, 2.02)
Gastrointestinal bleeding (yes)	0.65 (0.32, 1.31)
MELD-Na (per unit increase)	0.97 (0.94, 1.01)
Mechanical ventilation duration (per day)	0.79 (0.72, 0.87)

Reference value listed in parentheses. DAFD: Days alive and free of delirium and coma to day 14; MELD-Na: Model for End-Stage Liver Disease Sodium score; CI: Confidence interval.

DISCUSSION

The addition of rifaximin to broad-spectrum antibiotic therapy may provide overlapping antibacterial activity without additional therapeutic benefit in critically ill patients with HE. Notably, patients on broad-spectrum antibiotics have been generally excluded from studies of rifaximin efficacy in HE. This gap in the literature represents a need to better understand the role of rifaximin in this unique patient population, as ICU hospitalizations for patients with HE are typically characterized by severe disease and increased morbidity and mortality. This study is the first to evaluate the feasibility and safety of rifaximin discontinuation during broad-spectrum antibiotic therapy.

In our pilot investigation, rifaximin discontinuation during broad-spectrum antibiotic therapy in critically ill patients with liver disease was not associated with higher rates of delirium or coma. This result was robust to adjustment in multivariable analysis. As demonstrated in Table 3, no negative associations were observed between rifaximin discontinuation and short-term cognitive outcomes. Neither was rifaximin discontinuation associated with increased adverse effects, which adds support to the hypothesis that withholding rifaximin during broad-spectrum antibiotic therapy is

safe. The lack of observed differences in cognitive outcomes is an important contribution to the existing understanding of the interaction between treatment with rifaximin and other broad-spectrum antibiotics.

Much of the promising data for the benefit of rifaximin therapy in patients with cirrhosis, including reduced infections and hospitalizations, have been produced in the outpatient setting and reflect chronic use (> 30 d in most cases)[6-8]. In the present study, rifaximin was held for a limited time in hospitalized, critically ill patients during concomitant broad-spectrum antibiotic treatment. Few trials of rifaximin efficacy have included or focused on a critically ill population, however, the very limited data available suggest potential for harm with rifaximin discontinuation[19,25,26]. Sharma *et al* [18] published the largest single cohort evaluating rifaximin efficacy including critically ill patients. The authors reported a decrease in mortality among patients treated with rifaximin and lactulose compared with those treated with lactulose and placebo. Importantly, this finding is limited by the high rate of sepsis-related mortality in the placebo group. In contrast to the study by Sharma *et al*[18], all patients included in the present study had known or suspected infection but observed mortality rates were similar among patients who continued rifaximin during broad-spectrum antibiotic therapy and those who did not. In an open-label single-center study including 15 patients, Kalambokis *et al*[24] demonstrated that rifaximin therapy was associated with increased systemic vascular resistance after four weeks. This finding raised a question about the potential impact sudden discontinuation of therapy might have in critically ill patients predisposed to clinically significant vasodilation exacerbated in the setting of active infection. To address this question, vasopressor requirements in the first 48-h after rifaximin discontinuation (protocol) or antibiotic initiation (control) were compared. In the current larger cohort, rifaximin discontinuation was not associated with changes in vasopressor requirements despite severe and progressive illness in many included patients. Finally, in a single-center retrospective cohort analysis of mechanically ventilated critically ill patients with decompensated cirrhosis, rifaximin administration within the first 24-h post-intubation was associated with shorter time to extubation [hazard ratios 1.74 (1.21-2.50)], although pre-intubation rifaximin and lactulose administration was associated with a delayed time to extubation[26]. Rifaximin discontinuation was not associated with delays in extubation or rates of reintubation in the present study, though the sample size was smaller. In summary, although several studies in critically ill patients had demonstrated potential associations with mortality, vasopressor requirements, and duration of mechanical ventilation, the current study evaluated several potential safety concerns in a highly vulnerable patient population and did not reveal any negative signals associated with rifaximin discontinuation.

Notably, the present study demonstrates the feasibility and benefit of a pharmacist-driven, manually applied protocol with multidisciplinary support aimed at antimicrobial stewardship in a critically ill population. Though likely to be applicable to many centers in the United States, opportunities to optimize protocol execution exist including streamlining patient identification and enrollment, minimizing delays in rifaximin discontinuation, and ensuring rifaximin re-initiation after broad-spectrum antibiotic therapy completion or narrowing.

Several limitations exist within this evaluation. First, this was a single-center study with retrospective data collection. Second acute (overt) HE was not a requirement for inclusion, nor was the chronicity of HE episodes able to be quantified. Patients were stratified according to WHG and/or GCS in order to describe clinical status and align with available guideline recommendations for HE assessment. Similarly, it was not possible to definitively identify the specific indication for rifaximin for every patient nor to confirm the prescription of rifaximin prior to hospital admission with the limited available insurance claim records. Though based on available records, pre-hospital rifaximin therapy was prescribed at a similar rate in both the control and protocol groups, 37.5% *vs* 48.6%, respectively. Third, the high frequency of missing WHG due to retrospective clinical assessment in the setting of critical illness requiring mechanical ventilation and sedation necessitated the use of multiple surrogate endpoints. The primary outcome of DAFD was selected based on previously published studies evaluating critically ill patients' level of awareness[27,28]. While CAM scoring is routine in ICUs at our institution, delirium assessments (*i.e.* bCAM) are not routine in non-ICU care areas. The decision to consider all patients in non-ICU care areas delirium-free was based on an understanding of the clinical improvements required to support ICU discharge and the lack of routinely available validated scores to collect for the endpoint. This may have led to an over-estimation of the days free of delirium, however, this is likely balanced by the high average proportion of time spent in the ICU compared to non-ICU care areas. The 48-h change in GCS was also collected as a sensitive marker for any negative cognitive effect rifaximin discontinuation may have exerted. GCS was selected as current AASLD guidelines recommend this score as an alternative measure to the WHG for the diagnosis of HE (grade II-2, B, 1)[13]. The EASL guidelines recommend the addition of GCS to West-Haven criteria in patients with impaired consciousness, including those treated in an ICU (LoE 5, strong)[14]. Several outcomes of interest were unable to be assessed due to the absence of baseline WHG including the achievement and time to resolution or improvement of HE. Despite a robust effort to characterize the patient population and describe the severity and extent of illness, there are illness-specific variables and outcomes that were unable to be feasibly assessed, including indication for MICU admission and infection resolution which may have contributed to cognitive and clinical outcomes. Additionally, this study was not designed to evaluate long-term outcomes or impact of withheld rifaximin therapy. Despite these limitations, no differences were found in the available and utilized markers of cognitive outcomes between patients who did or did not have rifaximin discontinued during broad-spectrum antimicrobial therapy. Finally, the few baseline differences observed in the two cohorts may have been smaller or eliminated in a larger sample size. However, the authors anticipate the strong left skew in the primary outcome with a predominance of zero or minimal days spent alive and free of delirium and coma to persist, even in a larger sample, given the tenuous nature of critically ill patients with liver disease. Similarly, rates of HE resolution in a comparable population would be expected to be very low. These data characteristics would likely render future non-inferiority trials difficult or impossible to complete.

CONCLUSION

In conclusion, no significant differences were noted between the control and protocol groups in key clinical or safety outcomes. The robustness of the primary outcome to multivariable analysis strengthens the conclusion that rifaximin discontinuation during broad-spectrum antibiotic therapy does not appear to negatively impact the cognitive status of critically ill liver patients. This study demonstrates the feasibility of a pharmacist-driven protocol to reduce combination therapy in critically ill patients with liver disease treated with rifaximin and broad-spectrum antibiotics. Given the significant cost savings achieved during ICU and hospital admission, a prospective, multi-center evaluation of a similar protocol in a larger sample is warranted, including investigation into longer-term outcomes. Investigation of the impact of this type of protocol in non-critically ill liver patients should likewise be considered.

ARTICLE HIGHLIGHTS

Research background

Rifaximin is frequently administered to critically ill patients with liver disease and hepatic encephalopathy (HE). However, data supporting the use of rifaximin in this population, particularly in combination with broad-spectrum antibiotics, are extremely limited. Due to the overlapping spectrums of antibiotic activity, it was hypothesized that withholding rifaximin during broad-spectrum antibiotic therapy would be safe and reduce healthcare costs. The present study is the first to evaluate the feasibility and safety of rifaximin discontinuation during broad-spectrum antibiotic therapy and represents a highly vulnerable patient population.

Research motivation

The gap in available evidence demonstrates the need to better understand the role of rifaximin in this unique population, as intensive care unit (ICU) hospitalizations for patients with HE are typically characterized by severe disease and increased morbidity and mortality. Therefore, after protocol development the need to assess clinical and safety outcomes was clear. Additionally, given the opportunity to reduce healthcare expenditures with decreased use of rifaximin during ICU admission, costs of therapy were quantified. This proof-of-concept evaluation also provides a foundation for future, larger-scale, well-controlled studies to confirm and expand on the findings.

Research objectives

The present study aimed to evaluate the safety, efficacy, and financial impact of discontinuing rifaximin during broad-spectrum antibiotic use. The efficacy of withholding rifaximin was evaluated using a surrogate marker for short-term cognitive impact, days alive and free of delirium and coma. Multiple, robust safety outcomes were considered including mortality, ICU length of stay, 48-h change in vasopressor requirements, duration of mechanical ventilation, and successful extubation. Cost avoidance was evaluated by comparing rifaximin drug costs during the observation period pre- and post-protocol. The outcomes utilized provided an initial, comprehensive assessment of the pilot protocol that could be replicated in further investigations.

Research methods

This was a single-center, quasi-experimental study evaluating outcomes pre- and post-implementation of a pharmacist-driven protocol for rifaximin discontinuation in critically ill liver patients being treated in a medical ICU. To address potential sources of bias, multivariable analysis of the primary outcome was performed with characteristics selected based on biological plausibility and univariate screening. Inferential statistics were performed in the usual fashion based on data type and distribution. The study achieved 80% power to detect a 0.65 d difference in the primary outcome.

Research results

In this pilot investigation, rifaximin discontinuation during broad-spectrum antibiotic therapy in critically ill patients with liver disease was not associated with more days of delirium or coma [3 (0, 8) *vs* 2 (0, 9.5); $P = 0.93$]. Protocol application was associated with a high rate of adherence (91.4%) and resulted in a significant reduction in days of combination therapy [6 (3-9.5), 1 (0-1); $P < 0.001$] and medication expenditures (estimated per patient cost avoidance $316.00 to $632.00 USD). No signals of harm were detected in any safety endpoint. The results of this study support the safety and feasibility of a protocolized discontinuation of rifaximin during broad-spectrum antibiotic therapy. Due to the limited sample size and retrospective nature of the present study, future evaluations should prioritize larger sample sizes and prospective designs to the greatest extent possible. Many questions remain regarding the optimal use of rifaximin among patients being treated with broad-spectrum antibiotics, including non-critically ill patients and those receiving long courses of therapy.

Research conclusions

This was a novel evaluation that provides new insight about the potential safety of discontinuing rifaximin during short-term, broad-spectrum antibiotic therapy in critically ill patients with liver disease which has not yet been investigated in the literature. The safety, efficacy, and cost-saving results of this study warrant confirmation in an investigation with a larger sample size and prospective, well-controlled methods which could lead to broader application of a similar protocol. Finally, this study provides further support that pharmacists may be leveraged to assist with antimicrobial

stewardship efforts in specific and dynamic patient populations.

Research perspectives

Future research related to this question should focus on: Confirmation of the reported findings; longer-term outcomes of withholding rifaximin therapy, particularly during prolonged courses of broad-spectrum antibiotics; the impact of a similar protocol among non-critically ill patients; and opportunities to optimize protocol application.

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ORIGINAL ARTICLE

Basic Study

Metabolomics in chronic hepatitis C: Decoding fibrosis grading and underlying pathways

Adriana Camargo Ferrasi, Samara Vitória Granja Lima, Aline Faria Galvani, Jeany Delafiori, Flavia Luísa Dias-Audibert, Rodrigo Ramos Catharino, Giovanni Faria Silva, Roberta Rodrigues Praxedes, Driele Bretones Santos, Dayane Trevisan de Macedo Almeida, Estela Oliveira Lima

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Abstract

BACKGROUND

Chronic Hepatitis C (CHC) affects 71 million people globally and leads to liver issues such as fibrosis, cirrhosis, cancer, and death. A better understanding and prognosis of liver involvement are vital to reduce morbidity and mortality. The accurate identification of the fibrosis stage is crucial for making treatment decisions and predicting outcomes. Tests used to grade fibrosis include histological analysis and imaging but have limitations. Blood markers such as molecular biomarkers can offer valuable insights into fibrosis.

AIM

To identify potential biomarkers that might stratify these lesions and add information about the molecular mechanisms involved in the disease.

METHODS

Plasma samples were collected from 46 patients with hepatitis C and classified into fibrosis grades F1 (n = 13), F2 (n = 12), F3 (n = 6), and F4 (n = 15). To ensure that the identified biomarkers were exclusive to liver lesions (CHC fibrosis), healthy volunteer participants (n = 50) were also included. An untargeted metabolomic technique was used to analyze the plasma metabolites using mass spectrometry and database verification. Statistical analyses were performed to identify differential biomarkers among groups.

RESULTS
Six differential metabolites were identified in each grade of fibrosis. This six-metabolite profile was able to establish a clustering tendency in patients with the same grade of fibrosis; thus, they showed greater efficiency in discriminating grades.

CONCLUSION
This study suggests that some of the observed biomarkers, once validated, have the potential to be applied as prognostic biomarkers. Furthermore, it suggests that liquid biopsy analyses of plasma metabolites are a good source of molecular biomarkers capable of stratifying patients with CHC according to fibrosis grade.

Key Words: Chronic Hepatitis C; Fibrosis; Metabolome; Biomarkers; Plasma; Liquid biopsy

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Core Tip: Chronic Hepatitis C affects 71 million people globally and leads to liver fibrosis, cirrhosis, cancer, and death. The accurate staging of fibrosis is crucial for treatment decisions and outcome prediction. Blood markers are a relevant source of information, and various molecular biomarkers have been investigated to characterize liver fibrosis. We analyzed plasma metabolites by mass spectrometry in 50 healthy participants, and in 46 patients with hepatitis C and classified them into fibrosis grades F1-F4. Six differential metabolites were identified in each grade of fibrosis; their biochemical pathways were analyzed and suggests molecular mechanisms involved in the disease.

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INTRODUCTION

Chronic hepatitis C (CHC) is an infectious disease caused by the hepatitis C virus (HCV) and is a serious public health problem, affecting an estimated 71 million people worldwide[1-3].

Approximately 50%-80% of HCV-infected individuals develop CHC, which can trigger a chronic inflammatory disease process leading to liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC), and death[4].

Natural progression of CHC occurs with sustained inflammation due to repetitive liver injury, followed by the activation of hepatic stellate cells, deposition of fibrillar collagen in the extracellular matrix (ECM), and progressive fibrosis[5,6]. These progressive processes may result in ECM degradation and, consequently, vascular and architectural alterations, leading to cirrhosis (10%-20% of patients)[7] or HCC (1%-5%)[8].

Early diagnosis and treatment can prevent liver cirrhosis and HCC, especially with screening and recent advances in CHC treatment based on direct-acting antiviral therapy. However, effective reduction of disease morbidity and mortality requires better characterization of liver involvement, more accurate prognosis, and follow-up[9]. Under this scenario, accurate identification of the liver fibrosis stage is critical for the clinical management of HCC, guiding therapeutic options and helping to predict prognosis[10]. However, this approach is challenging. Tests used to stage fibrosis include histological analysis of liver biopsies and imaging tests. Liver biopsy is considered the "gold standard" for the diagnosis and staging of liver fibrosis. However, it is an invasive and uncomfortable procedure with a risk of minor (10%-20%) or serious (0.5%-1%) complications[7,11]. In addition, the interpretation of histological results is subject to sampling errors and inter-observer subjectivity in the interpretation of histological results[7,12,13]. For staging the grades of fibrosis in biopsied liver tissue, the 0-4 scale of the Metavir classification system[14] is commonly used; however, the main limitations are related to the representativeness of liver samples and histopathological interpretation. Conventional imaging tests include ultrasonography, computed tomography, and magnetic resonance imaging. Although they represent important tools for detecting cirrhosis, nodules on the liver surface, and splenomegaly, they present low sensitivity for moderate or even advanced fibrosis. Newer acoustic technologies, such as hepatic elastography, can increase the accuracy of imaging techniques. For these tests, acoustic vibrations are applied to the abdomen and, according to how quickly these vibrations are transmitted along the liver tissue, the stiffness (fibrosis) of the liver is indicated. However, conditions other than fibrosis also increase liver stiffness[7], which requires further study and standardization. Another important limitation is the cost of the equipment[15], which is unaffordable in places with limited financial resources. In clinical practice, blood markers should be considered a relevant source of information. Current approaches are limited to combining commonly available tests (*e.g.*, aspartate transaminase, alanine aminotransferase, albumin, serum bilirubin, and international normalized ratio) with clinical information (*e.g.*, age, body mass index, and diabetes) and, in some cases, direct markers of liver function. However, this approach is most useful in distinguishing between two levels of fibrosis: Absent to minimal *vs* moderate to severe and fails to stratify the grades.

Undeniably, the search for blood biomarkers is a less invasive method for diagnosis and prognosis, and as blood circulates through most tissues, it can be a relevant source of information about diseases. Therefore, different molecular biomarkers, particularly those with easier and more accessible analytical methodologies, have been investigated for the characterization of liver fibrosis[16-20].

The present study focused on analyzing the plasma metabolome of patients with CHC with different grades of fibrosis aiming to identify potential biomarkers for stratifying these lesions. The metabolome is the set of endogenously synthesized metabolites in a specific physiological condition and may represent the final product of gene expression. Thus, as a secondary aim of this study, we analyzed the pathways linked to the main metabolites detected, contributing information about the molecular mechanisms involved in the disease.

MATERIALS AND METHODS

Study participants

This study was approved by the Ethics Committee on Research of São Paulo State University in accordance with the provisions of the Declaration of Helsinki. Plasma samples from 46 volunteer participants diagnosed with hepatitis C were obtained from peripheral blood. The inclusion criteria were as follows: Patients > 18 years, unrelated, diagnosed by detection of HCV RNA, with identification of HCV genotype, naïve patients (with no previous hepatitis C treatment), and patients with a known fibrosis stage or clinical diagnosis of cirrhosis by imaging. The exclusion criteria were as follows: Volunteers with a history of liver transplantation, hepatic steatosis unrelated to chronic hepatitis C and other liver diseases. To ensure that the biomarkers identified were exclusive to liver lesions (hepatitis C fibrosis), 50 healthy volunteer blood bank donors [healthy control group (CG)] were included in this study. Participants were recruited from the Viral Hepatitis Outpatient Clinic of Botucatu Medical School, UNESP, Brazil. The demographic and clinical characteristics of the study participants are summarized in Table 1.

Fibrosis was classified based on the Metavir score[14]. Liver samples were collected by percutaneous biopsy before treatment and analyzed histologically. Peripheral blood was collected at the same time as the liver biopsy.

Sample preparation

Samples were collected in tubes with ethylenediamine tetraacetic acid anticoagulant, followed by centrifugation to separate the plasma, which was stored at -80 ℃ until metabolite extraction. At the time of extraction, 20 μL of blood plasma was solubilized in 200 μL of tetrahydrofuran, vortexed, and centrifuged at 3200 rpm for 5 min. Then, the collected supernatant was solubilized in 780 μL of methanol and again centrifuged as above. Afterward, 50 μL of this supernatant was solubilized in 500 μL methanol q.s., homogenized, and subjected to chemical ionization with 0.1% formic acid.

Mass spectrometry

For mass spectrometry analysis, the ionized solution was directly injected into an LTQ Mass Spectrometer (ESI-LTQ-XL Discovery, Thermo Fisher Scientific, Waltham, MA, United States) using electrospray ionization. Ten replicates were used for each biological replicate. The parameters for analysis were set as the following configuration: Sample flow rate of 10 μL/min, capillary temperature of 180 ℃, 7 kV spray voltage, and carrier gas of 2 arbitrary units. After direct injection, the samples were analyzed in the positive ion mode in the mass range of 100-1400 (mass-to-charge ratio), and the signal intensity was detected, which resulted in a set of ions *m/z* for each sample. XCalibur software (v. 2.4, Thermo Scientific) was used to acquire and process the spectrometer data, which were submitted for statistical analysis.

Statistical analyses

Statistical analysis was performed using MetaboAnalyst 4.0 platform[21], in which raw data were evaluated using partial least squares discriminant analysis (PLS-DA). As a result, a list of markers was generated according to the intensity of the most differential and important markers for each group evaluated; that is, the variable importance score (VIP score) was obtained. From this, six ions with the highest VIP score for each grade of fibrosis, with scores > 2.0, were selected. The accuracy of the identified biomarkers was assessed by receiver operating characteristic (ROC) curve analysis.

Identification of biomarkers

From the selected biomarkers, a search was performed using the METLIN online metabolomics database (http://metlin.scripps.edu) to identify molecules compatible with the mass/charge values selected for each grade of fibrosis. The molecules of interest were added to the candidate list and fragmented in silico using the MassFrontier tool (v. 6.0, Thermo Fisher Scientific). After the fragmentation in silico, the molecules whose fragments were compatible with those generated experimentally were selected.

RESULTS

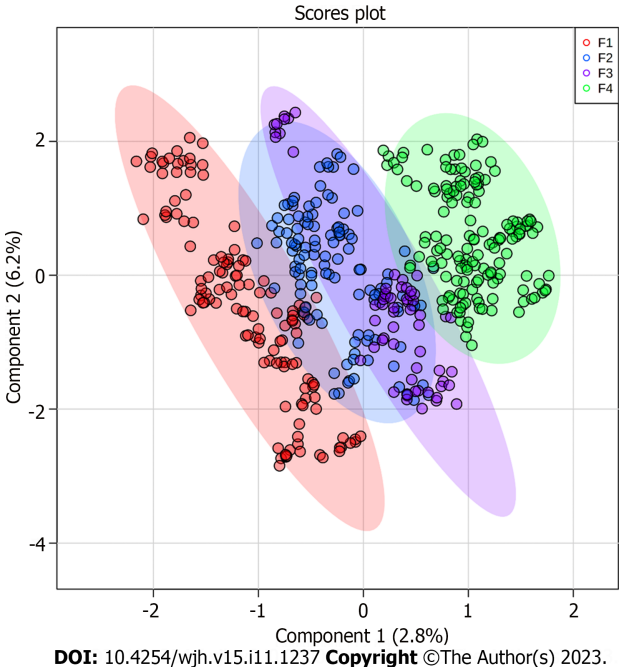
Selection of biomarkers

Based on the PLS-DA, the ions were grouped according to the signal intensity profile within each staging grade, making it possible to analyze the separation between fibrosis grades, as represented in the PLS-DA score plot (Figure 1).

Table 1 Demographic and clinical characteristics of all study participants, distributed by fibrosis grade (Metavir score)

Variables	Healthy control	Fibrosis grade (Metavir)[1]			
		F1	F2	F3	F4
Age (yr)	44 ± 12.2	50 ± 10.78	49 ± 8.6	55 ± 8.8	54 ± 11.22
Sex					
Male	24 (65.0)	8 (61.5)	7 (58.3)	5 (100)	9 (60.0)
Female	26 (52.0)	5 (38.5)	5 (41.7)	0	6 (40.0)
BMI (kg/m^2)	22.9 ± 2.98	28.3 ± 8.89	26.5 ± 6.26	26.9 ± 3.60	27.4 ± 7.14
HCV genotype					
1[2]	-	10 (77.0)	8 (66.7)	3 (50.0)	12 (80.0)
Not 1[3]	-	3 (23.0)	4 (33.3)	3 (50.0)	3 (20.0)

[1]Metavir score to assess the fibrosis grade by histopathological examination of a liver biopsy.
[2]HCV 1: Hepatitis C Virus genotype 1.
[3]HCV not 1: Hepatitis C Virus other genotypes.
BMI: Body mass index; HCV: Hepatitis C virus.



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Figure 1 Score plot generated from partial least squares discriminant analysis comparing fibrosis grades (Metavir). Each plasma metabolite profile is highlighted by different colors: F1 (red dots), F2 (blue dots), F3 (purple dots), and F4 (green dots). The shaded regions around the points represent the 95% confidence interval for each group.

To identify the biomarkers responsible for the separation between the groups (Figure 1), a VIP score was used in the projection. This score allows visualization of the relevance of each marker within each grade analyzed according to the mass/charge ratios of the metabolites[22]. Considering a VIP score of > 2.0 (Figure 2), the six most important ions were selected for each group (Table 2).

To ensure that the identified biomarkers were exclusive to liver lesions (fibrosis, CHC), CG were included. The plasma samples from the two groups (CHC *vs* CG) were compared and this analysis showed that the fibrosis biomarkers (Table 2) were not detected in CG. The PLS-DA and VIP score graphs comparing the two groups are shown in Figures 3 and 4, respectively.

Identification of biomarkers

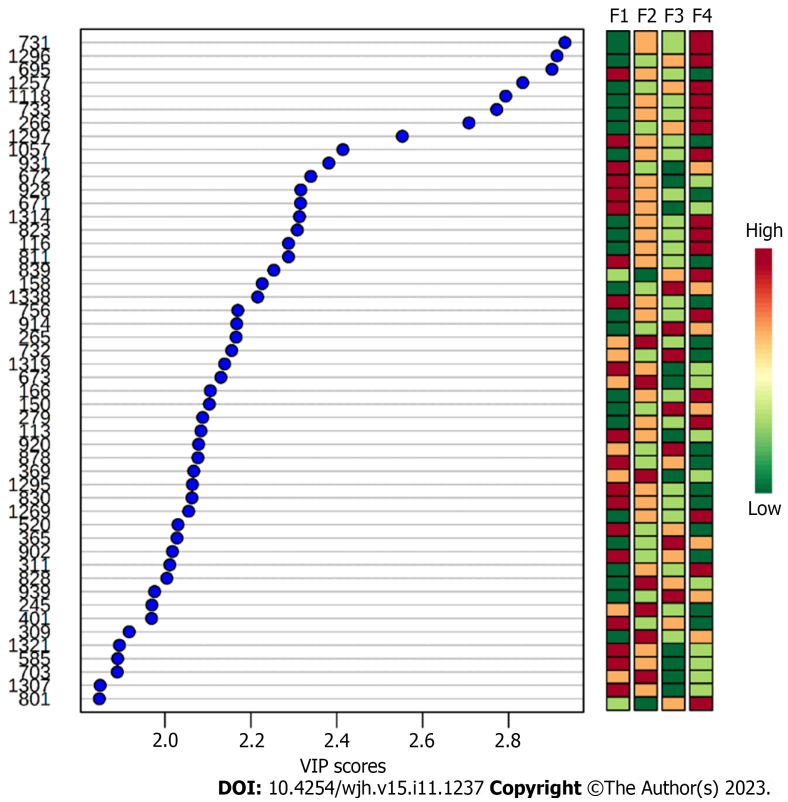
The most relevant biomarkers, represented by *m/z* values, were identified according to fibrosis grade, as shown in Table 2.

Table 2 Metabolites identified as important in discriminating grades of fibrosis (Metavir) in chronic hepatitis C

Class	Molecule	Ion	VIP score	Formula	Adducts	MSMS	Metlin ID
F1							
Sterols	18:0 Cholesteryl ester	671	2.3	$C_{45}H_{80}O_2$	$[M+NH_4]^+$	303-369-583-437-147-161-135-109-95	83955
	20:5 Cholesteryl ester	672	2.35	$C_{47}H_{74}O_2$	$[M+H]^+$		41710
Glycerolipids	DG(44:12)[1]	695	2.9	$C_{47}H_{68}O_5$	$[M+H-H_2O]^+$	311-119-95-81-69-57-97-113-339	4681
	DG(42:7)[1]			$C_{45}H_{74}O_5$	$[M+H]^+$	311-119-95-81-69-57-97-113-339-437	4605/59181
Coenzyme A	cis,cis-3,6-Dodecadienoyl-CoA	928	2.35	$C_{33}H_{54}N_7O_{17}P_3S$	$[M+H-H_2O]^+$	95-112-119-720-184	58193
Polypeptide	Angiotensin III	931	2.4	$C_{46}H_{66}N_{12}O_9$	$[M+H]^+$	400-311-112-113-96-97-437-659-720-146-147	58017
Unknown	-	1297	2.55	-	-	-	-
F2							
Methyladenosine	N6-Methyladenosine	265	2.18	$C_{11}H_{15}N_5O_4$	$[M+H-H_2O]^+$	81-85-63-99-117-135-149-163-177	58196
	3'-O-Methyladenosine					81-85-63-117-135-163	58340
	1-Methyladenosine					81-84-63-99-117-135-163	6888
	2'-O-Methyladenosine					81-85-63-99-117	58235
	O6-Methyl-2'-deoxyguanosine					81-85-63-99-149-163-177	66286
Eicosanoids	8,15-diHPETE	369	2.08	$C_{20}H_{32}O_6$	$[M+H]^+$	95-81-109-147-161-135-69-93-107	75001
	5S,15S-diHPETE						75023
	5,15-diHPETE						75000
	15S-hydroperoxy-PGD2						74985
	20-hydroxy-PGD2						74981
Sphingolipids	Cer (42:1)[1]	673	2.15	$C_{42}H_{83}NO_3$	$[M+Na]^+$	303-370-95-81-109-60-69-93-107	41569
Unknown	-	828	2.05	-	-	-	0
F3							
Aminoacid derivative	(S)-2,3,4,5-Tetrahydropiperidine-2-carboxylate	150	2.1	$C_6H_9NO_2$	$[M+Na]^+$	106-134-84-61-56-52-105-120	62803
Prenol lipid	Farnesylcysteine	365	2.05	$C_{18}H_{31}NO_2S$	$[M+K]^+$	203-185-112-81-71-307	62388
Glycerophospholipid	PE(34:5)[1]	732	2.17	$C_{39}H_{68}NO_8P$	$[M+Na]^+$	184-437-438-660-83-113-133-97	60844
Coenzyme A	S-2-Octenoyl CoA	914	2.18	$C_{29}H_{48}N_7O_{17}P_3S$	$[M+Na]^+$	86-80-119-112-95-67-104-184-720	58140
Unknown	-	158	2.2	-	-	-	-
		920	2.1				
F4							
Glycerophospholipids	PE(36:1)[1]	731	3	$C_{41}H_{80}NO_7P$	$[M+H]^+$	184-659-437-393-113	62180
	PE(34:6)[1]			$C_{39}H_{66}NO_8P$	$[M+Na]^+$	184-659-437-393-316-113	77367
	PE(O-36:1)[1]	733	2.6	$C_{41}H_{82}NO_7P$	$[M+H]^+$	184-97-304-437-369	77526

	PE(P-36:0)[1]						77623
	PE(34:5)[1]			$C_{39}H_{68}NO_8P$	$[M+Na]^+$	184-97-304-437-675-369	60812
Coenzyme A	CoA(22:2)[1]	1118	2.8	$C_{43}H_{74}N_7O_{17}P_3S$	$[M+CH_3OH+H]^+$	453-703-437-338-113-780-675-799	75415
Acyl-carnitines	Malonylcarnitine	266	2.7	$C_{10}H_{17}NO_6$	$[M+NH_4]^+$	172-94-95-116-57-90-204	6484
Unknown	-	1257	2.85	-	-	-	-
		1296	2.95				

[1]Carbon number: Double bond number. MSMS: In tandem mass spectrometry; DG: Diacylglycerol; CoA: Coenzyme A; HPETE: Hydroxy/hydroperoxyeicosatetraenoic acids; Cer: Ceramide; PE: Phosphoethanolamine; PGD: Prostaglandin; VIP score: Variable importance score.



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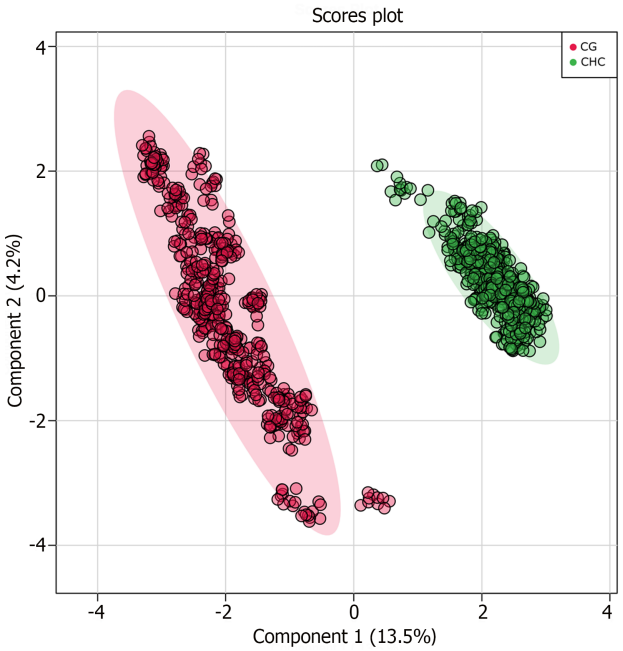
Figure 2 Variable importance score of the biomarkers identified for grades F1, F2, F3, and F4. The Y axis represents the metabolites *m/z* ratio. The X axis indicates the importance of projection for each biomarker. Laterally to the right, the relevance of each specific marker within the group analyzed is represented according to the color gradations of each biomarker for each grade of fibrosis. The red color represents the most relevant biomarkers; as the red intensity decreases and approaches the green color, the relevance of the biomarkers reduces. Red = up-regulation; Green = down-regulation. VIP Score: Variable importance score.

ROC curve analysis

The accuracy of the biomarkers was assessed using the ROC curve analysis of the sets of metabolites identified for each fibrosis grade (Figure 5). ROC curves were used to analyze the sensitivity, specificity, and area under the curve (AUC) of each group of metabolites identified at each grade of fibrosis. The ROC curve of the selected metabolites for F1 (AUC = 0.806) was plotted with a sensitivity of 82% and a specificity of 68%, and the other selected metabolite groups for F2 (AUC = 0. 652), F3 (AUC = 0.807), and F4 (AUC = 0.864) showed sensitivities of 62%, 82%, and 83% and specificities of 57%, 74%, and 76%, respectively.

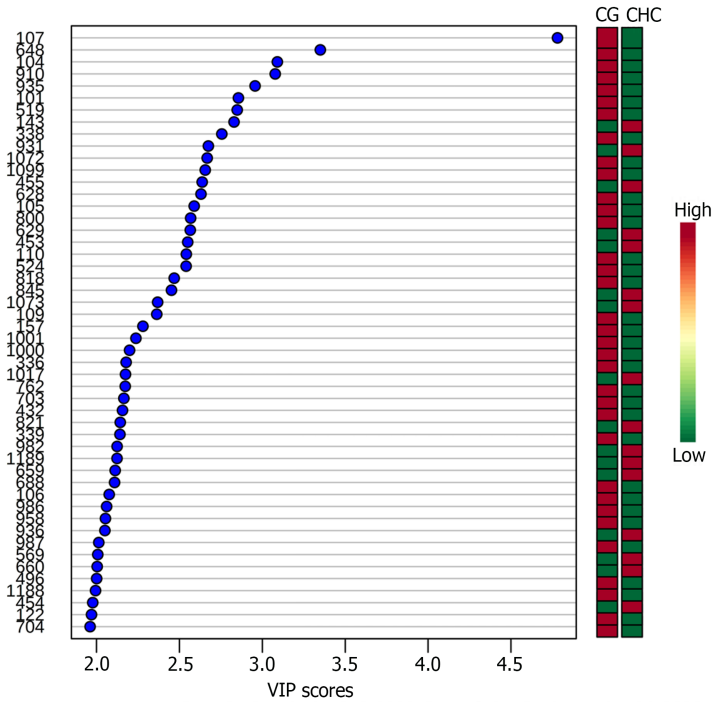
DISCUSSION

The metabolome was analyzed to identify new prognostic and diagnostic biomarkers. Thus, the present study investigated the differential metabolites in blood plasma as potential biomarkers of fibrosis stages. Our analysis identified potential biomarkers for each grade of liver fibrosis, which will increase our knowledge about the progression of CHC



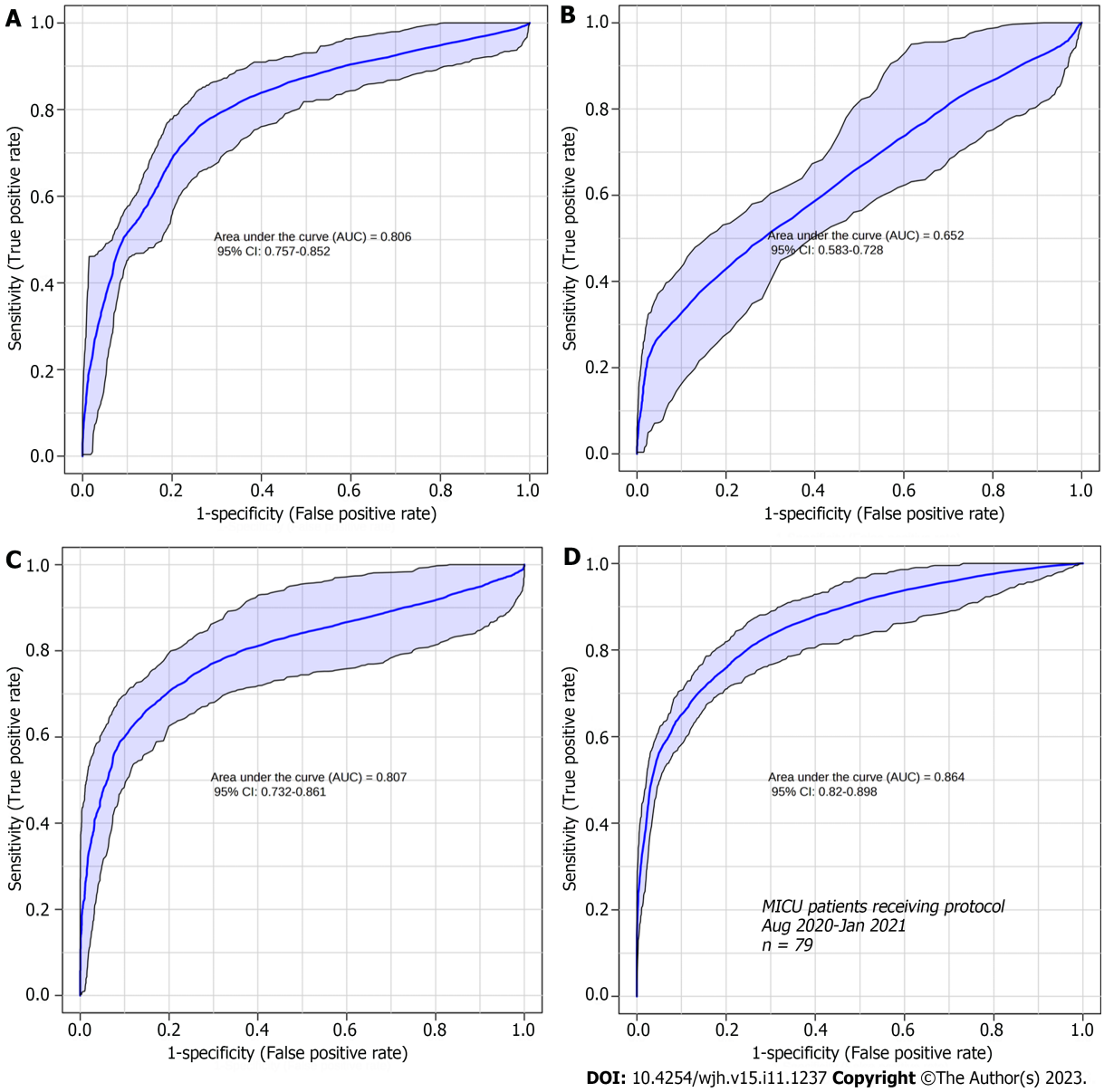
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Figure 3 Score plot generated from partial least squares discriminant analysis comparing the chronic hepatitis C *vs* healthy control groups. Each group is highlighted by different colors: Healthy control groups (red dots) and chronic hepatitis C (green dots), where each dot represents one analytical replicate. The shaded regions around the points represent the 95% confidence interval for each group.



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Figure 4 Variable importance score of the biomarkers identified when comparing the chronic hepatitis C *vs* healthy control groups. The Y axis represents the metabolites *m/z* ratio. The X axis indicates the importance in projection for each biomarker. Laterally to the right, the relevance of each specific marker within the group analyzed is represented according to the color gradations of each biomarker for each grade of fibrosis. The red color represents the most relevant biomarkers; as the red intensity decreases and approaches the green color, the relevance of the biomarkers reduces. Red = up-regulation; Green = down-regulation. VIP Score: Variable importance score.



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Figure 5 Receiver operating characteristic curve analysis of the sets of metabolites identified at each fibrosis grade. A: Receiver operating characteristic (ROC) curve analysis of the set of differential metabolites identified in F1 compared to the other grades (F2, F3, and F4); B: ROC curve analysis of the set of differential metabolites identified in F2 compared to the other grades (F1, F3, and F4); C: ROC curve analysis of the set of differential metabolites identified in F3 compared to the other grades (F1, F2, and F4); D: ROC curve analysis of the set of differential metabolites identified in F4 compared to the other grades (F1, F2, and F3).

and highlight targets for further investigation. The identified biomarkers were able to establish a clustering tendency in patients with the same grade of fibrosis despite some overlap. The score plot analysis showed greater efficiency in discriminating between the extreme grades (F1 and F4), with an overlap in grades F2 and F3. This result may be related to the analytical bias of histological classification, as the formation of groups was based on this criterion-Metavir[12-14], which is subject to some bias related to inadequate sample acquisition, incorrect sample representation or inter-observer variability[7,12,13]. To ensure that the identified biomarkers were exclusive to liver lesions caused by CHC, we compared them with the plasma samples of healthy donors (Figures 3 and 4). None of the biomarkers found in the patient within the CHC group were detected in the plasma of healthy controls, which reinforces their potential as biomarkers exclusive to the disease.

Analysis of the accuracy of the most relevant metabolites in each grade showed that the sets associated with grades F1, F3, and F4 were good biomarkers (AUC 0.806, 0.807, and 0.864, respectively; Figure 5) and had good sensitivity and specificity scores. However, the metabolites identified as grade F2 were less specific and showed poor sensitivity. Such findings could be useful for distinguishing grades F1, F3, and F4, where uncertainty exists when the analyses are based solely on histology. Some serum markers of fibrosis validated in patients with hepatitis C and correlated with liver biopsy as a reference standard showed a mean AUC suitable for clinical practice (> 0.80)[23]; however, an overlap was also observed between adjacent grades of liver fibrosis, particularly the lower grade[24].

Despite the histological bias, our analysis identified different metabolites from diverse chemical classes, including sterols, fatty acids, lipids, and coenzymes. However, for each grade of fibrosis, a metabolite profile has been identified, and as observed in Figure 2, the relevance of each molecule changes according to the fibrosis grade and may intensify or decrease during the disease.

Some studies have demonstrated the potential of metabolomics analyses for different scenarios in diverse diseases, particularly in cancer management[25]. One of the great achievements of metabolomics is the assessment of therapeutic responses and tumor progression, as shown by Rattner *et al*[26], in which serum blood metabolites indicated positive or negative responses to chemotherapy using gas chromatography-mass spectrometry. In addition, some methods for metabolomics analysis, such as nuclear magnetic resonance and multisegment injection-capillary electrophoresis-mass spectrometry, have also shown impressive results, and have also been used to evaluate the metabolome of serum samples from patients with CHC with fibrosis of different grades[27]. This study identified markers for the highest grades of fibrosis, which are compatible with our results, such as glycerophospholipid and acyl-carnitine markers. Therefore, the use of metabolomics approaches for liquid biopsies show promise as diagnostic, prognostic, and therapeutic monitoring tools.

In the context of viral infection, viruses are known to synthesize fatty acids by benefitting from their intermediate products. HCV alters the expression of lipid-related genes associated with cholesterol biosynthesis[28,29]. Interestingly, some metabolites found in different grades of fibrosis are associated with lipid alterations[30-32].

For grade F1, biomarkers that may be more related to HCV infection than to the development of fibrosis were observed when compared to patients with more advanced fibrosis. Thus, the first molecule identified in F1 belonged to the sterol class, with specific signatures for cholesterol ester (CE) (m/z = 671 and m/z = 672). Previous studies have pointed out that CE is a critical component of lipoviral particles whose synthesis has been linked to HCV infection in vitro when cholesterol and triglyceride accumulation is observed[29]. In agreement with our results, we suggest that HCV may modulate the environment, promoting a higher density and infectivity of viral particles and viral spread in the hepatic tissue, which intensifies infection[28,33,34].

Considering lipid metabolism and accumulation, it was possible to identify the sphingolipid class in intermediate-grade F2, represented by ceramide (m/z = 673). It is a central molecule in sphingolipid metabolism with anti-proliferative and pro-apoptotic effects[30]. In the context of HCV infection, lipid accumulation and, consequently, ceramide accumulation occur and may lead to steatosis[35], which may contribute to the development of liver fibrosis[5,35-37].

In addition, a glycerolipid was also identified in F1, specified as diacylglycerol (DG) (m/z = 695). Recent studies have shown that the conversion of DG to phosphatidic acid (mediated by diacylglycerokinases) results in lysophosphatidic acid production, which is involved in many chronic inflammatory diseases, including fibrosis and cancer[38,39]. Therefore, the present study highlights a potential relationship between high levels of DG and a less fibrotic state (low-grade fibrosis) compared to F4, where fibrosis is accentuated.

Another lipid class, glycerophospholipids, was identified in intermediate-grade F3 and advanced-grade F4, in which the biomarkers were identified as phosphoethanolamines (PE) (m/z = 731, m/z = 732, and m/z = 733). Some studies have suggested that PE gradually increases according to the grade of liver fibrosis and acts as a potential marker of carcinogenesis[40,41]. This finding suggests that patients diagnosed with F3 could be at the beginning of the carcinogenesis process; however, this hypothesis needs to be further investigated.

Other biomarkers related to changes in lipid signaling pathways have also been identified. One of these belongs to the eicosanoid class (m/z = 369) identified in F2. This molecule is a biologically active lipid that has several implications in biological processes and is a potent mediator of inflammation in infectious diseases and HCC[42,43]. In addition, it is associated with liver fibrosis staging and is a potential biomarker[44-46]. Another class of lipids, prenol lipids, was identified as F3, represented by farnesylcysteine (m/z = 365). This marker participates in the process of liver carcinogenesis by directly acting on the activity of oncogenic rat sarcoma virus protein[47,48]. Thus, these results encourage investigations into the use of this metabolite as a potential biomarker of the risk of tumor development.

Different intermediate metabolites of the coenzyme A (CoA) class have also been identified, and they are typically involved in the β-oxidation of medium- and long-chain fatty acids to acyl-CoA, a key intermediate in lipid metabolism. Some studies suggest the existence of a disruption in fatty acid lipid metabolic pathways during HCV infection[49,50]. This process results in the accumulation of acyl-CoA and fatty acid metabolic intermediates, such as the three molecules identified in the present study, described as follows. The cis,cis-3,6-dodecadienoyl-CoA (m/z = 928) was identified in the F1 cases in our study. For F3, the marker S-2-octenoyl CoA (m/z = 914) was found[51,52], and in advanced grade (F4), a CoA metabolite (m/z = 1118) was identified. Because different acyl-CoAs isoenzymes are expressed in the liver, some of which are overexpressed in activated hepatic stellate cells[51,53], the results of the present study indicate that there is a disruption in lipid metabolism throughout the infection; however, this is unclear and requires further investigation. Considering the presence of acyl-CoAs in three different fibrosis grades, these molecules are not good candidates for the classification of fibrosis stages but highlight their importance in CHC.

Another marker involved in β-oxidation was found in patients with F4, represented by malonyl carnitine (m/z = 266). Tumors require more energy for cell proliferation, which may lead to dysregulation of energy-supplying metabolic pathways, such as β-oxidation of fatty acids[54,55]. In the context of HCC, alterations in the metabolism of acylcarnitine are directly related to the worsening of the disease and to alterations of β-oxidation[56], which results in the accumulation of Acyl-CoA[57], as discussed previously. Thus, malonylcarnitine can be considered a potential HCC biomarker; however, further studies are needed to validate this hypothesis.

In addition to the lipid biomarkers, the polypeptide angiotensin III (Ang III) (m/z = 931) was identified in F1, which, according to some studies, exhibits physiologically relevant effects similar to those of angiotensin II. In the context of CHC and liver fibrosis, Ang III participates in the increase in collagen production through its interaction with the angiotensin type 2 receptor[58,59] Therefore, this pathway may be involved at the beginning of the fibrotic process once

Ang III is identified in F1.

The last two metabolites were identified as intermediate grades: methyladenosine (*m/z* = 265) in F2 and (S)-2,3,4,5-tetrahydropiperidine-2-carboxylate (*m/z* = 150) in F3. Adenosine methylation is a post-transcriptional modification of mRNAs that affects various biological functions[60-62]. In HCV infection, methyladenosine may represent an RNA modification that enhances the production of infectious particles by interacting with viral proteins[62-64]. These findings suggest that these modifications are involved in the progression of infections and liver fibrosis. Finally, (S)-2,3,4,5-tetrahydropiperidine-2-carboxylate identified in F3 may be related to the degradation of enzymatically inactive proteins and viral assembly[65]. Although this study related amino acid residues to the progression of infection and consequent worsening of fibrosis staging, further studies are necessary to clarify the actions of these protein residues in the viral cycle.

The main limitation of this research was the sample size. This study covered a regional sample and were limited to a single center, which may limit external generalization. However, the results encourage further research with a larger casuistry and the application of this methodology to other liver diseases.

The current study has innovative potential for the detection of markers in plasma, an easily accessible biological fluid. Besides, liquid biopsy could be used side by side with the other noninvasive tests (like elastography) for achieving more accuracy in predicting prognosis.

CONCLUSION

In conclusion, the results from this study suggest that some of the observed biomarkers, once validated, have the potential to be applied as prognostic biomarkers. In addition, they suggest that liquid biopsy analyses of plasma metabolites are a good source of molecular biomarkers capable of stratifying patients with CHC according to fibrosis grade.

ARTICLE HIGHLIGHTS

Research background

Chronic hepatitis C (CHC) is an infectious disease caused by the hepatitis C virus, leading to liver issues like fibrosis, cirrhosis, cancer, and death. The accurate fibrosis stage identification is crucial for treatment decisions and predicting outcomes. Thus, blood markers are a source of relevant information on the staging of fibrosis, in a less invasive and representative way, compared to percutaneous biopsies.

Research motivation

Currently, approaches to staging fibrosis are invasive, subject to sampling errors and subjectivity between observers. In clinical routine, blood markers should be considered a relevant source of information. However, current approaches are limited to routine biochemical tests associated with clinical information, which is not very informative. Analyses based on liquid biopsy are less invasive, and blood plasma, since it circulates throughout the body, can provide information on pathologies that have not yet manifested themselves clinically, positively impacting on prognosis.

Research objectives

Analyze the plasmatic metabolome of CHC patients, looking for potential biomarkers to stratify these lesions.

Research methods

Plasma metabolites from hepatitis C patients and 50 healthy volunteer participants were analyzed using the LTQ Mass Spectrometer. The sample and the control group were classified into Fibrosis grades was classified using the Metavir score. Liver samples were collected by percutaneous biopsy before any treatment and then analyzed histologically. The most relevant metabolites were categorized using the METLIN online metabolomics database. The molecules of interest were added to a list of candidates and subsequently fragmented in silico using the MassFrontier tool. Molecules compatible with those generated experimentally were then selected for functional analysis.

Research results

For each degree of fibrosis, six differential metabolites were identified that were able to establish an interesting grouping trend among patients with the same degree of fibrosis.

Research conclusions

The results of this study suggest that liquid biopsy analyzes of plasma metabolites are a good source of molecular biomarkers capable of stratifying patients with CHC according to their fibrosis grade.

Research perspectives

Some of the observed biomarkers, once validated, have the potential for application as prognostic biomarkers. This study has innovative potential regarding the detection of pre-clinical biomarkers in easily accessible plasma using minimally

invasive methods.

FOOTNOTES

Author contributions: Ferrasi AC, Lima EO, Delafiori J and Dias-Audibert FL performed mass spectrometry experiments; Ferrasi AC, Galvani AF, Santos DB, Silva GF, and Praxedes RR performed biofluid collection and selection and provided clinical support; Ferrasi AC, Almeida DTM, Lima EO, Praxedes RR, and Lima SVG analyzed the data and prepared the Figures; Ferrasi AC and Lima SVG wrote the manuscript; Lima EO, Silva GF, and Catharino RR revised the manuscript; Catharino RR provided the infrastructure and methodological support; Ferrasi AC and Lima EO designed, managed, and supervised the study; All authors approved the final version of the article.

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LETTER TO THE EDITOR

Letter to editor 'Non-invasive model for predicting high-risk esophageal varices based on liver and spleen stiffness'

Xin Gao, Xiao-Yan Guo, Long-Bao Yang, Zhong-Cao Wei, Pan Zhang, Ya-Tao Wang, Chen-Yu Liu, Dan-Yang Zhang, Yan Wang

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Abstract

This letter to the editor relates to the study entitled "Non-invasive model for predicting high-risk esophageal varices based on liver and spleen stiffness". Acute bleeding caused by esophageal varices is a life-threatening complication in patients with liver cirrhosis. Due to the discomfort, contraindications, and associated complications of upper gastrointestinal endoscopy screening, it is crucial to identify an imaging-based non-invasive model for predicting high-risk esophageal varices in patients with cirrhosis.

Key Words: Cirrhosis; High-risk esophageal varices; Non-invasive prediction model; Spleen stiffness measurement; Liver stiffness measurement; Upper gastrointestinal endoscopy

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Core Tip: Liver cirrhosis is the end of chronic liver disease. Rupture of esophageal varices (EVs) is a common and potentially fatal complication in patients with cirrhosis. In clinical practice, prophylactic treatment is primarily used to prevent events of esophageal venous bleeding, however, this strategy requires invasive and expensive upper gastrointestinal endoscopy testing, leading to poor patient adherence. In recent years, several studies have demonstrated an association between EVs and liver stiffness measurement (LSM) as well as spleen stiffness measurement (SSM). The main objectives of this paper are to elucidate the differences between EVs, SSM, and LSM and explore the feasibility of using LSM and SSM to develop a non-invasive model for predicting high-risk esophageal varices.

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TO THE EDITOR

We read with interest the retrospective study by Yang *et al*[1], which is titled "Non-invasive model for predicting high-risk esophageal varices based on liver and spleen stiffness". The results of this study highlight the potential use of liver stiffness measurement (LSM) and spleen stiffness measurement (SSM) for predicting high-risk esophageal varices (HEVs) in patients with cirrhosis.

Portal hypertension (PH) is a common and significant complication in patients with cirrhosis, leading to esophageal varices (EVs)[2]. Hepatic venous pressure gradient and upper gastrointestinal endoscopy (UGE) are considered the gold standard for assessing the severity of PH and the risk of EV bleeding. However, due to their invasiveness, discomfort, and high cost, it is crucial to identify non-invasive markers for screening HEVs in cirrhotic patients[3,4]. In recent years, LSM and SSM using transient elastography (TE), acoustic radiation force impulse elastography, two-dimensional shear wave elastography, and magnetic resonance elastography have been proven to be accurate diagnostic tools for evaluating chronic liver disease with liver fibrosis as well as predicting the presence or absence of HEVs[5].

We want to emphasize a few points about this study: In this study, the authors used Baveno VI as a comparator but did not include the more comprehensive Baveno VII as a comparator[6]. At the same time, the authors did not distinguish between the M and XL models of the FibroScan probe when measuring LSM and SSM, which may have an impact on the comprehensiveness and accuracy of the results[7]. Second, patients with current/past clinical cirrhosis were included in this study, but the proportion of patients with decompensated cirrhosis in this cohort is unclear, since the non-invasive measures used here were primarily used for endoscopic triage of patients with compensated cirrhosis. No guidelines recommend its use in patients with clinical decompensation, for whom screening by UGE is recommended. Additionally, while all subjects included in this study had viral hepatitis cirrhosis, they did not consider the possible effect of antiviral treatment on TE measurements. Furthermore, the effect of alcoholic and nonalcoholic steatohepatitis on cirrhosis has been underrepresented, which may limit the external validity of our findings across diverse populations and settings. To enhance the reliability of the conclusions of this study, we recommend a study with a larger sample size, especially in patients with nonalcoholic steatohepatitis and alcohol-induced cirrhosis, to verify the validity of the model in patients with different types of cirrhosis. Such a study would help improve the convenience and operability of clinical practice and more accurately assess the condition of patients.

The highlight of this study is that all enrolled patients completed UGE testing. Additionally, when SSM is unavailable or unsuccessful, the Baveno VI criterion can be used as a reasonable alternative according to Yang *et al*[1] Moreover, a screening strategy based on LSM and SSM could reduce the workload of endoscopy and optimize the use of health care resources while minimizing risk and patient discomfort. In summary, we acknowledge the efforts and contributions made by the authors. Furthermore, we recommend further prospective validation to facilitate future research on this topic.

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FOOTNOTES

Author contributions: Gao X contributed to this work; Guo XY, Wei ZC, Liu CY wrote this letter; Yang LB, Zhang P, Zhang DY edited this letter, and Wang YT and Wang Y revised this letter.

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EDITORIAL

From metabolic dysfunction-associated fatty liver disease to metabolic dysfunction-associated steatotic liver disease: Controversy and consensus

Li Chen

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Abstract

The newly released nomenclature of metabolic dysfunction-associated steatotic liver disease (MASLD) in the 2023 European Association for the Study of the Liver Congress has raised great clinical concerns. This marks the second instance of significant renaming of non-alcoholic fatty liver disease since the introduction of metabolic dysfunction-associated fatty liver disease (MAFLD) in 2020. The nomenclature and definitions of MASLD and MAFLD exhibit significant disparities as well as substantial consensus. The disparities regarding the framework of nomenclature, the definitions, the clinical management, and the impact on the clinical outcomes between MASLD and MAFLD were comprehensively compared in this editorial. Additionally, the consensus reached by the MASLD and MAFLD definitions also emphasizes positive diagnosis rather than negative diagnosis within the framework of establishing a diagnostic approach. Furthermore, they acknowledged the pivotal role of metabolic dysfunction in the pathogenesis of MAFLD or MASLD and the positive role of increasing the awareness of the disease in public. Fortunately, the non-invasive tests remains effective in the MASLD and MAFLD era. Elucidating these disparities would contribute to a more comprehensive comprehension of the nature of steatotic liver disease and enhance clinical practice. Thus, more efforts are required to reach more consensus about these important topics.

Key Words: Non-alcoholic fatty liver disease; Metabolic dysfunction-associated fatty liver disease; Metabolic dysfunction-associated steatotic liver disease; Nomenclature; Metabolic risk factors

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Core Tip: The nomenclature for non-alcoholic fatty liver disease has undergone name changes twice in a span of three years. However, there exist significant disparities and some consensus between the transition from metabolic dysfunction-associated fatty liver disease to metabolic dysfunction-associated steatotic liver disease. Clarifying these discrepancies would greatly benefit clinical practice and trials.

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INTRODUCTION

With the escalating prevalence of obesity and type 2 diabetes mellitus (T2DM) in the general population, fatty liver disease has become a predominant etiology of chronic liver disease worldwide[1,2]. Non-alcoholic fatty liver disease (NAFLD), first named by Jurgen Ludwig in 1986, was defined as hepatic steatosis affecting at least 5% of hepatocytes without any other causes of liver injury[3]. NAFLD encompasses a histological spectrum of disorders ranging from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) and can progress to liver cirrhosis or hepatocellular carcinoma[4]. Due to its exclusionary diagnostic criteria, the term NAFLD has encountered significant challenges in both diagnosis and treatment, particularly when patients with NAFLD present with concurrent alcohol consumption or viral hepatitis. In 2020, a novel nomenclature of "metabolic dysfunction-associated fatty liver disease (MAFLD)" was proposed by a panel of international experts from 22 countries to address these concerns.

The MAFLD framework emphasizes the importance of metabolic dysfunction, as demonstrated by the revised definitions of overweight/obesity, T2DM, or the presence of at least two metabolic risk abnormalities, regardless of the underlying etiologies and comorbidities such as alcohol consumption and viral hepatitis[5]. However, the revised definition of MAFLD, which encompasses alcoholism and acknowledges the coexistence of multiple etiologies, raises concerns regarding its impact on the natural history and therapeutic development of the condition, as well as the potential stigmatization associated with the term "fatty". Recently, three large pan-national liver associations, including the American Association for the Study of Liver Disease, European Association for the Study of the Liver, and Latin American Association for the Study of the Liver, proposed a novel nomenclature for steatotic liver disease (SLD) and metabolic dysfunction-associated SLD (MASLD) through a modified Delphi process. In this novel nomenclature, the umbrella term, SLD, is classified into two distinct subcategories based on the presence and absence of a cardiometabolic risk factor (CMRF). Furthermore, the subcategory of CMRF is further classified into MASLD and MetALD depending on the alcohol consumption. MASLD is defined as the presence of hepatic steatosis in conjunction with at least one CMRF and no other discernible cause[6]. Within a span of merely 3 years, two distinct nomenclatures were successively proposed. The magnitude of the controversy surrounding these nomenclatures remains substantial, with societies yet to reach a consensus. Therefore, elucidating the disparities and similarities between MASLD and MAFLD is imperative.

THE CONTROVERSY BETWEEN MASLD AND MAFLD

The disparity in the framework of nomenclature between MASLD and MAFLD

MASLD is a branched term under the overarching term of SLD, which encompasses a broad spectrum of causes contributing to hepatic steatosis. The term MASLD, which excludes alcoholic consumption and other concomitant etiologies, is parallel with MetALD, alcoholic liver disease (ALD), other specific etiology SLD, and cryptogenic SLD. From this perspective, MASLD can be regarded as a negative diagnosis. MASLD focuses on the intricate relationship between hepatic fat deposition and metabolic dysfunction while excluding the effects of alcohol. While MAFLD is a positive diagnosis regardless of alcohol consumption or other concomitant liver diseases. MAFLD is a single overarching term encompassing the primary and secondary fatty liver disease as long as it meets the criteria of metabolic dysfunction. Nevertheless, the MAFLD leads to more heterogeneity in the disease spectrum compared to MASLD. Given the global popularity of alcohol consumption, the coexistence of NAFLD and alcohol consumption poses challenges for diagnosing NAFLD; this issue can be readily resolved by adopting the term MAFLD. However, alcohol consumption interacts with components of the metabolic syndrome to exert synergistic or supra-additive effects on the development and progression of liver disease, further complicating the natural history of MAFLD[7]. The introduction of the term MetALD provides a framework to study the natural history of such a distinct subcategory that has both alcohol consumption and metabolic dysfunction.

The disparity in the definitions of MASLD and MAFLD

Regarding the identification of steatosis, the MASLD criteria recommend confirmation through either imaging or histological methods. While the MAFLD criteria confirm steatosis by histological methods, or non-invasive tests (NITs) based on imaging approach or blood biomarkers. Moreover, the MAFLD criteria enumerate the NITs including

ultrasound, vibration-controlled transient elastography, computed tomography or magnetic resonance imaging (MRI), MRI–derived proton density fat fraction, and fatty liver index. From this perspective, the MAFLD criteria provide more options for the identification of steatosis.

One of the most prominent distinctions between MASLD and MAFLD is the identification of metabolic risk factors, which intend to identify patients likely to have insulin resistance as the main cause of hepatic steatosis. According to the MAFLD criteria, two out of seven metabolic risk factors must be met for patients without T2DM or obesity as outlined below: waist circumference ≥ 102/88 cm in Caucasian men and women or ≥ 90/80 cm in Asian men and women; blood pressure ≥ 130/85 mmHg; plasma triglycerides ≥ 1.70 mmol/L; plasma high density lipoprotein-cholesterol < 1.0 mmol/L for men and < 50 mg/dL < 1.3 mmol/L for women; prediabetes (fasting glucose levels 5.6 to 6.9 mmol/L, or 2-h postload glucose levels 7.8–11.0 mmol or HbA1c 5.7% to 6.4%); homeostasis model assessment-insulin resistance (HOMA-IR) score ≥ 2.5; and plasma high-sensitivity C-reactive protein (hs-CRP) level > 2 mg/L. The MASLD criteria necessitate the fulfillment of at least one out of the five cardiometabolic risk factors, which are similar to those outlined in the MAFLD criteria, except HOMA-IR and hs-CRP levels. As HOMA-IR and hs-CRP levels are not universally measured in all clinical settings, the MASLD criteria are more accessible and intuitive than the MAFLD criteria. A recent study has demonstrated that the MASLD criteria outperforms the MAFLD criteria in identifying fatty liver disease among lean patients, despite the absence of hs-CRP data[6]. However, which criterion is superior in identifying metabolic dysfunction and insulin resistance remains unclear.

The difference in the management of steatohepatitis

The MAFLD definition abandons the dichotomous stratification into steatohepatitis and non-steatohepatitis, which was established during the NAFLD era. The dichotomous classification of steatohepatitis and non-steatohepatitis may fail to capture the complete spectrum of disease response to alterations in underlying metabolic dysfunctions or pharmacological interventions[5]. The MAFLD definition places greater emphasis on the degree of activity and stage of fibrosis, rather than being entangled in the presence or absence of steatohepatitis. These changes inevitably exerted a significant impact on clinical trials, particularly in terms of endpoint selection, as the current objective of drug development is to achieve the resolution of NASH without any deterioration in liver fibrosis[8]. However, the definition of MASLD emphasizes that the presence of steatohepatitis carries prognostic implications and should remain an important distinction. Consequently, retaining the term "steatohepatitis" is imperative in both clinical practice and trial endpoints. Moreover, the MASLD definition suggests replacing the term NASH with metabolic dysfunction-associated steatohepatitis (MASH), which may reduce the confusion in clinical practice and trials[6]. The MASLD definition also permitted the integration of MASH with additional assessment of fibrosis severity.

The difference in the impact on clinical outcomes

The divergent framework of nomenclature and definition between MASLD and MAFLD implies the likelihood of disparate impact on clinical outcomes. Due to the heterogeneous nature of MAFLD, MAFLD has a more detrimental impact on clinical outcomes compared to MASLD. Based on the Third National Health and Nutrition Examination Survey (NHANES III), studies have demonstrated that MAFLD is associated with an elevated risk of all-cause and cardiovascular mortality, whereas NAFLD alone does not increase the risk of all-cause mortality[9]. MAFLD criteria identified a significant group of individuals with more comorbidities and worse prognosis compared with those with NAFLD only [10]. However, another study demonstrated no observed differences in cumulative all-cause and cause-specific mortality between MAFLD and NAFLD after adjusting for alcohol-associated liver disease[11].

A recent NHANES III study demonstrated that both the MAFLD-only and MASLD/MAFLD overlap subgroups exhibited significantly higher all-cause mortality, as opposed to the MASLD-only subgroup. With regard to cause-specific mortality, MAFLD was significantly associated with an increased risk for cardiovascular disease -related and diabetes-related mortalities, whereas MASLD was independently related to a higher risk of diabetes-related mortality[12]. Another study based on NHANES III also failed to demonstrate the association between MASLD and all-cause mortality after adjustment for demographic and other factors such as body mass index and hepatitis B and C viral infections. On the contrary, MetALD and ALD were significantly associated with all-cause mortality[13].

The difference in stigmatization of nomenclature

The renaming is partly motivated by the stigmatization associated with terms such as "fatty" and "nonalcoholic." During the Delphi process, the terms "nonalcoholic" and "fatty" were deemed to be stigmatizing by 61% and 66% of panelists, respectively[6]. However, the vote percentage does not surpass the priori threshold of ≥ 67%, thus failing to achieve a consensus. Perceptions of stigma differ widely across different languages and cultures. In certain Indian languages, the term "fatty" is associated with robust health and would be considered a compliment. There is no justification for stigmatizing "non-alcoholic" fatty liver disease, as it is not "alcoholic"[14]. Depending on the regions and cultural contexts, the transition from "fatty" to "steatosis" may be either ambiguous or unattainable. Additionally, the term "steatosis" can be overly medicalized, which may confuse the patients[15]. Interestingly, a recent survey in Mexico revealed that 69.5% of participants expressed that incorporating the term "alcohol" in the disease nomenclature carries a stigmatizing connotation and advocated for its exclusion. In contrast, 85.6% of participants indicated that they do not perceive the inclusion of "fatty" as stigmatizing and preferred retaining it to enhance effective communication regarding the disease[16]. Some experts argued that the change from NAFLD to MASLD and destigmatization is driven by political correctness rather than scientific rationale[14]. To date, the Asian Pacific Association for the Study of the Liver still advocates MAFLD and does not endorse MASLD, highlighting the ongoing controversy among various associations.

THE CONSENSUS BETWEEN MASLD AND MAFLD

The consensus on positive diagnosis and metabolic dysfunction

Both MASLD and MAFLD definitions adapt positive criteria and abandon the exclusive criterion of "non-alcoholic." Once hepatic steatosis is identified, either by histopathology or by imaging method, the diagnosis of steatotic liver disease or fatty liver disease is established. Both MASLD and MAFLD acknowledge metabolic dysfunction as the primary driver of disease progression. Moreover, metabolic dysfunction independently contributes to cardiometabolic outcomes. The complex interplay of metabolic, inflammatory, and vascular mechanisms exacerbates systemic atherogenesis; thereby, promoting the development and progression of cardiovascular diseases[17].

The consensus on increasing the awareness of the disease

The previous term NAFLD, which has been in use for four decades, is an exclusive criterion that implies no association with alcoholic consumption and overlooks the role of metabolic dysfunction in its pathogenesis. The term MASLD or MAFLD conveys an intuitive message to patients regarding the etiology, facilitating their comprehension of pathophysiology and treatment. The wide spread of novel nomenclature will enhance public awareness about metabolic dysfunction as an etiology for liver disease.

The consensus on the effectiveness of NITs

The changes in nomenclature raise a concern regarding the continued effectiveness of the NITs established in previous decades, which were based on extensive epidemiological data. The worries about the potential negative impact of changes in diagnostic criteria for biomarkers is warranted. Fortunately, the extensively validated NITs still can be applied under novel criteria. A recent liver biopsy-based study demonstrated that the Index of NASH (ION) exhibited superior discriminatory ability for detecting the presence of MASLD, while the aspartate aminotransferase to platelet ratio index and fibrosis-4 score effectively differentiated severe fibrosis stages[18].

UNRESOLVED QUESTIONS

The novel MASLD nomenclature also raises new questions. Firstly, in some patients, the hepatic steatosis vanishes while progressing to cirrhosis. The absence of steatosis prompts the question of whether such patients should be diagnosed with cryptogenic SLD or MASLD-cirrhosis[19]. Secondly, should the patients who drink more than 350/420 g/wk and fulfill one out of five CMRFs be diagnosed with MetALD, or should they be diagnosed with ALD? Conversely, should the patients who intake alcohol below 350/420 g weekly and without CMRFs be diagnosed as ALD, or should they be diagnosed with MetALD? Thirdly, patients with coexisting fatty liver and hepatitis B should be diagnosed as MASLD or other specific etiology SLD?

CONCLUSION

The term NAFLD has been proposed for nearly four decades; however, with time, it no longer adequately encompasses the pathophysiology of fatty liver disease, necessitating a change in its nomenclature. Indeed, the modification of a well-established or entrenched term poses great challenges. Within more than just 3 years, two distinct nomenclatures, namely MAFLD and MASLD, have been proposed sequentially. The novel nomenclatures not only modify the framework and operational definitions but also provide fresh insights into the pathophysiology and treatment strategies. More importantly, the renaming has significantly enhanced public awareness. Nevertheless, the substantial disparities between MAFLD and MASLD imply controversies among various professional associations, thereby may confuse physicians and patients. More research and debates are needed to reach a consensus in the future.

FOOTNOTES

Author contributions: Chen L designed the overall concept and contributed to the writing, and editing of the manuscript, and review of the literature.

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REVIEW

Multifunctional roles of inflammation and its causative factors in primary liver cancer: A literature review

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Abstract

Primary liver cancer is a severe and complex disease, leading to 800000 global deaths annually. Emerging evidence suggests that inflammation is one of the critical factors in the development of hepatocellular carcinoma (HCC). Patients with viral hepatitis, alcoholic hepatitis, and steatohepatitis symptoms are at higher risk of developing HCC. However, not all inflammatory factors have a pathogenic function in HCC development. The current study describes the process and mechanism of hepatitis development and its progression to HCC, particularly focusing on viral hepatitis, alcoholic hepatitis, and steatohepatitis. Furthermore, the roles of some essential inflammatory cytokines in HCC progression are described in addition to a summary of future research directions.

Key Words: Inflammation; Primary liver cancer; Hepatocellular carcinoma; Nonalcoholic fatty liver disease; Hepatitis virus

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Core Tip: Primary liver cancer is the second most common tumor in the world, and the number of deaths due to this disease is increasing every year. A large number of studies have shown that inflammation has a certain regulatory effect in the occurrence and exacerbation of liver cancer. However, the function of inflammation in liver cancer remains to be studied. This review introduces the classification of hepatitis, the correlation between various inflammatory factors and hepatocellular carcinoma (HCC), and some of the anti-inflammatory drugs used in the treatment of HCC.

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INTRODUCTION

Liver cancer is categorized into primary and secondary liver cancer. Primary liver cancer involves hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma, and other rare cancer types. In contrast, secondary liver cancer is due to cancer cell metastasis from different body parts to the liver *via* the bloodstream[1]. Notably, HCC accounts for 95% of primary liver cancer cases and is one of the leading types and fatal liver cancer forms. HCC development is closely associated with hepatitis C virus (HCV), hepatitis B virus (HBV), and nonalcoholic fatty liver disease (NAFLD)[2]. Alcoholic fatty liver may cause alcoholic steatohepatitis (ASH), leading to progressive fibrosis and cirrhosis, and can develop into HCC[3]. All these processes leading to HCC involve a series of reactions from inflammation to cirrhosis, resulting in HCC. Therefore, inflammation is clinically significant as the initiating factor in HCC.

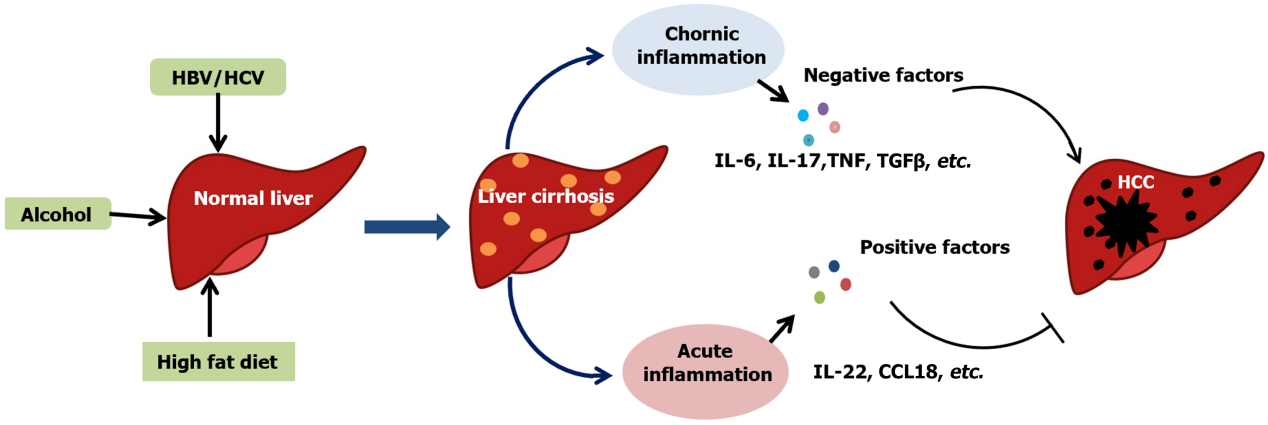
Inflammation is a defensive response of the human body against stimulation and is divided into acute and chronic inflammation. Acute or short-term hepatic inflammation is a nonfibrotic condition caused by lipopolysaccharide, hepatitis virus, and other factors, and disappears within hours or days. Chronic or long-term inflammation, driven by chronic oxidative stress, is one of the critical processes in HCC development and progression[4]. More studies are investigating the presence of inflammation in the occurrence and development of liver cancer, but its exact role remains unclear. Immune cells in the tumor microenvironment either suppress or promote tumorigenesis, participating in adaptive and innate immunity and defense mechanisms to eliminate foreign agents. Persistent chronic inflammation accelerates the growth and proliferation of tumor cells[5]. Bioactive molecules released from immune cells in the tumor microenvironment stimulate carcinogenesis programming and enhance tumor development[6]. Several inflammatory cytokines, including interleukin (IL)-22, a member of the IL-10 family[7], play a positive role in liver regeneration and the anti-inflammatory response. Other cytokines, including IL-1β and IL-17A, serve as tumor-promoting cytokines, inducing liver disease progression and hepatocarcinogenesis[8,9]. This review summarizes the recent evidence on HCC mechanisms caused by various hepatitis viruses and discusses the role of inflammatory signaling pathways in HCC progression and development (Figure 1).

DIFFERENT CAUSATIVE FACTORS IN INFLAMMATION AND HCC

Role of viral hepatitis in HCC

Viral hepatitis caused by infection with hepatitis viruses A, B, C, D, and E is a global epidemic leading to acute or chronic hepatitis, and even acute severe hepatitis related to a high mortality rate. Due to differences in the structure and features of viruses, they selectively infect the liver using various routes[10]. Approximately 80% of HCC cases are related to HBV or HCV infections, leading to cirrhosis and progressing to HCC.

Hepatitis B virus: Hepatitis B virus (HBV) can integrate its double-stranded DNA (dsDNA) into host cells to develop pregenomic RNA (pgRNA). Then, pgRNA is encapsulated into icosahedral capsids formed by the hepatitis B virus core antigen protein, meditated by polymerase action. Within the capsid, gpRNA is reverse-transcribed into single-stranded DNA (ssDNA), after which the DNA is enveloped to become infectious virions. HBV contains the gene fragments HBV X protein and HBV C protein in its genome. These gene fragments are critical regulatory proteins with crucial roles in HBV-induced HCC pathogenesis. They directly activate or inhibit the expression of hepatocyte growth-related genes, including CTbp2, HMBGA1, and CA10, affecting its transformation to HCC[11-13]. In addition to the direct effects on the host genome to attenuate stability and enhance gene mutations and chromosomal rearrangements with oncogenic or proto-oncogene expression, HBV accelerates HCC progression through multiple mechanisms. For instance, HBV promotes HCC by inducing inflammation and oxidative stress, and altering the immune cell interaction for immune evasion. Bing-Qing Zheng reported that HBsAg (surface antigen) suppressed STAT3 expression and activation in natural killer (NK) cells of chronic hepatitis B (CHB) patients by reducing the IL-21 stimulation response[14]. HBV also activates the phosphatase and tensin homolog (PTEN)/β-actin/c-Myc pathway to promote programmed cell death protein 1 expression, inhibiting T-cell activity and indirectly enhancing the immune evasion of HBV in CHB infection[15]. Furthermore, chronic HBV infection leads to CHB-induced inflammatory damage in hepatic cells due to the persistent activation of inflammatory cells and chemokines[16], causing chronic severe hepatitis or liver cancer. Overall, CHB linked



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Figure 1 The relationship between inflammation and hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; IL: Interleukin; TNF: Tumor necrosis factor; TGF: Transforming growth factor.

with HBV infection has a weak direct stimulatory role in HCC progression. However, the infection depends more on regulating various immune-related active molecules within the hepatocyte microenvironment.

Hepatitis C virus: HCV belongs to the Flaviviridae family and is an enveloped ssRNA virus. Unlike HBV infection, HCV infection mainly presents as asymptomatic chronic hepatitis, of which 20%-30% of patients progress to liver cirrhosis, and 7% suffer liver cancer[10]. As the released immune cells form a complex HCV-induced HCC tumor microenvironment, Guo-He Song performed single-cell RNA sequencing on immune cells from nontumor and HCV-associated HCC liver tissues[17]. This discovery highlighted novel macrophage and T-cell subsets, of which M2 macrophages significantly expressing CCL18 were enriched in advanced HCC patients. CCL2, CCL20, CXCL8, or CXCL10 were highly induced by the synergistic activity of HCV core protein and chemokines such as interferon (IFN)-γ and IL-1β in fibroblasts or liver sinusoidal endothelial cells (LSECs). These chemokines result in HCV-induced hepatic injury of the LSECs by recruiting leukocytes and activating hepatic stellate cells (HSCs), enabling the development and progression of fibrosis and cirrhosis [18]. CCL2 and CXCL10 are upregulated in macrophages, promoted by the HCV core protein, by interacting with the gC1qR and nuclear factor-kappaB (NF-κB) signaling pathways[19]. Tumor necrosis factor (TNF)-α, IL-1β, IL-6, IL-10, IL-18, and transforming growth factor (TGF)-β are the most relevant inflammatory cytokines associated with HBV/HCV-induced HCC *via* multiple pathways[20]. The IL-6 GC and TGF-β1 TT genotypes promoted HCC development in the HCV-infected population by altering the transcription and stability of the protein structures. These could be potential markers for the early diagnosis of HCC[21].

Role of alcoholic hepatitis in HCC

Excessive alcohol consumption can cause alcoholic liver disease (ALD), such as steatosis, ASH, fibrosis, cirrhosis, and HCC. In the liver, alcohol is metabolized using three major oxidative pathways. First, alcohol is oxidized to acetaldehyde by alcohol dehydrogenase, with NAD^+ as the cofactor[22], cytochrome P450 2E1 (CYP2E1) in the microsomal ethanol oxidizing system[23], and the heme-containing enzyme catalase[24]. Subsequently, acetaldehyde is oxidized to acetate by aldehyde dehydrogenase (ALDH). Acetaldehyde damages DNA and impairs the antioxidant defense system, decreasing antioxidant and detoxification enzymes. Adducts from acetaldehyde can disturb cellular function, promoting alcohol-induced liver injury. CYP2E1 induced by chronic alcohol intake enhances alcohol metabolism to acetaldehyde, leading to liver injury and producing reactive oxygen species (ROS)[25]. These ROS attack the hepatocyte mitochondria and reduce ALDH activity. Additionally, mutagenic etheno-DNA adducts, stimulated by CYP2E1, are essential in genetic damage and liver carcinogenesis[26]. Long-term alcohol use causes excessive CYP2E1 along with oxidative stress, producing ROS [27]. Such exposure results in structural damage, mitochondrial dysfunction, mitochondrial stress in hepatocytes, and apoptotic signal upregulation.

Long-term alcohol consumption and liver dysfunction induce alcoholic hepatitis (AH), which is linked with severe ASH and high mortality rates in the short term[28]. Excessive consumption of alcohol causes damage to the microtubule structure and dysfunction of liver cells in patients with AH, which affects the efficiency of nutrient transport. Protein adducts formed by acetaldehyde can block DNA repair and hepatocyte mitochondria, contributing to the dysfunction of oxygen utilization, collagen synthesis, and extracellular matrix accumulation, resulting in liver fibrosis, cirrhosis, and carcinogenesis[29].

Interestingly, innate immunity activation leads to carcinogenesis in two ways: it leads to alcohol-induced liver injury and results in hepatoprotection, regeneration, and anti-inflammatory reactions to decrease alcohol-induced liver damage [30]. Alcohol consumption elevates lipopolysaccharides and activates the MyD88-independent TRIF/IRF-3 pathway using Toll-like receptor 4 (TLR4), causing oxidative stress, TNF-α release, and liver damage[31]. However, TLR4 and complement factors also promote Kupffer cells to secrete protective cytokines such as IL-6 and anti-inflammatory cytokines such as IL-10. Inflammatory cytokines such as TNF-α, IL-1, and IL-6 are enhanced in the serum of ALD patients [32]. IL-10 plays a positive hepatoprotective role *via* the STAT3 signaling pathway[33]. In contrast, IL-6 and p-STAT3 are

highly expressed in HCC patients[34]. TNF-α acts as a pro-tumorigenic cytokine and activates NF-κB and c-Jun N-terminal kinase (JNK) signaling pathways in liver carcinogenesis[35]. NK cells can develop IFN-γ to attenuate liver cell regeneration and kill hepatocytes[36]. However, the function of NK and NK T cells in hepatocytes remains unexplored. IL-1β plays an essential role in the progression of inflammation, alcohol-induced liver steatosis, and liver injury[37]. IL-22 has beneficial effects on hepatic inflammation and regeneration, while F-652, an IL-22 agonist, is a promising AH treatment candidate[38]. IL-17A functions as a tumor-promoting cytokine regulating inflammatory responses and cholesterol synthesis in developing hepatic steatosis, fibrosis, and HCC in an experimental alcohol-induced mouse model [9]. Some of the inflammatory factors have various roles in different stages. If their expression can be upregulated or downregulated during a specific period, these factors could exert their unique therapeutic effects on AH to HHC.

Role of NAFLD in HCC

NAFLD is a global disease characterized by excessive fat accumulation in the liver and is not associated with excessive alcohol use. NAFLD progression occurs through several stages, such as simple steatosis, steatohepatitis, fibrosis, and cirrhosis, leading to HCC. NAFLD encompasses a group of liver diseases from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH)[39]. NAFL is a simple steatosis of liver cells without inflammation[40]. Furthermore, NAFL development is accompanied by an inflammatory response, causing NASH and liver cancer with cirrhosis[41]. NASH is characterized by the long-term accumulation of triglycerides or clearance disorders in liver cells, progressing to HCC[42]. The presence of steatosis, inflammation, and hepatocyte damage typically characterizes NASH. These are associated with a higher incidence of cirrhosis and liver cancer with NASH mortality than in NAFL[43-45]. TLR9-MyD88 signaling stimulates Kupffer cells to synthesize IL-1β, which contributes to hepatocyte damage and activates HSCs, promoting NASH development[46]. IL-33 is released during chronic hepatocellular stress to activate ILC-2 in the liver and produce IL-13, facilitating HSC activation and the onset of hepatic fibrosis[47]. Notably, the IL-33/ST2 axis has dual roles in diet-induced NASH, wherein an IL-33 supplement ameliorates hepatic steatosis but exacerbates hepatic fibrosis [48]. TNF-α promotes liver fibrosis while cooperating with TIMP-1 produced by HSCs[49]. In a recent study, IL-17A was tested at a high concentration in early-stage fibrosis with increased expression of profibrotic markers in the tissue slice culture, which revealed a significant role of IL-17A in promoting liver fibrosis in human liver tissue[50]. IL-22 treatment ameliorated CXCL1/high-fat diet-induced NASH and methionine choline-deficient diet-induced NASH *via* multiple targets, suppressing liver inflammation[51].

Others

Primary biliary cholangitis (PBC) is a chronic cholestatic liver disease with chronic and persistent bile stasis in the liver while causing cirrhosis and liver failure[52]. Some case reports show that cirrhosis is an HCC risk factor in PBC patients [53,54]. Diabetes is categorized into type 1 (T1DM) and type 2 (T2DM) diabetes. Diabetes liver fibrosis (DHF) is a chronic complication that progresses to liver disease. The main reason for DHF is to activate quiescent HSCs *via* high glucose stimulation[55]. T2DM possesses an elevated risk of advanced fibrosis in NAFL patients[56]. Clinical analysis revealed that 1 out of 20 T1DM patients and 1 out of 5 T2DM patients have elevated liver hardness (an indicator to evaluate liver fibrosis), suggesting severe or advanced liver fibrosis. Obese or T2DM patients have an increased risk of developing NASH, which can progress to cirrhosis and HCC if unchecked.

KEY INFLAMMATORY FACTORS AND HCC

Interleukin family

The IL family, with more than 40 members, was first investigated in 1976. According to the structural homology of cytokines, the IL family has seven subfamilies, including IL-1, IL-2, IL-6/IL-12, IL-10, IL-17, and chemokine α subfamilies.

IL-1 subfamily: The IL-1 subfamily includes IL-1α, IL-1β, IL-18, and IL-37[57]. Inhibition of IL-1 signaling using its agonist weakens hepatic inflammation and promotes liver regeneration, helping recovery from liver injury in AH[58,59]. In NAFLD, mice lacking IL-1α and IL-1β had inhibition of hypercholesterolemia steatosis to steatohepatitis and liver fibrosis[60]. Lack of IL-1α in Kupffer cells of mice with hypercholesterolemia weakens liver inflammation and inflammatory cytokine expression[61]. IL-1α release at different locations affects the development direction of HCC differently. Urinary excretion of IL-1α suggests an HCC-promoting effect, wherein the antitumor immune response is inhibited through myeloid-derived suppressor cells recruitment into the tumor microenvironment. Simultaneously, systemic IL-1α administration directly activates T cells to inhibit HCC development[62]. IL-1β secretion by macrophages was reduced in HBV and hepatitis D virus (HDV) infection, while IL-1β inhibited HBV and HDV replication[63]. IL-1β exerts antiviral effects by inhibiting ERK2 activation by elevating IFN-α, which inhibits HCV replication[64]. IL-1 receptor antagonists improve inflammasome-dependent ASH in mice[37]. Mice lacking the IL-1β activation gene can inhibit the development of obesity-induced NAFLD[65]. IL-1β receptor antagonists can inhibit liver fibrosis in mice, while IL-1β, a component of the NLRP3 inflammasome, can reduce liver fibrosis in NASH mice[66]. IL-1β is highly involved in hepatic lipogenesis by enhancing triglyceride accumulation and induces pathogenic liver steatosis in obesity-induced NAFLD[67]. M1 macrophages induce programmed cell death ligand 1 (PD-L1) expression in hepatoma cells *via* IL-1β signaling. This key checkpoint molecule mediates HCC immune escape[68]. IL-1β-mediated homologous box C10 overexpression enhances HCC metastasis by upregulating 3-phosphoinositol-dependent protein kinase 1 (PDPK1) and vasodilator-stimulated phosphoprotein (VASP) expressions[69].

IL-6/IL-12 subfamily: This subfamily consists of IL-6, IL-12, IL-23, IL-27, and IL-35A[70]. A case-control experimental study unraveled the potential susceptibility of IL-6 gene polymorphisms against HBV infection[71]. IL-6 regulates microRNA-125b expression in HCV infection using the STAT3 pathway, causing HCV infection onset and possibly progressing to HCC[72]. In AH, IL-6 promotes microRNA-223-rich exosome production, mitigating NAFLD-associated fibrosis[73]. Additionally, caffeine improves NAFLD with a tandem between muscle production of IL-6 and liver STAT3 activation[74]. The activation of IL-6/STAT3 signaling enhances LCSC production by hepatoma cells and resists sorafenib in hepatoma cells. This is an essential factor in inducing the occurrence, development, and metastasis of liver cancer[75]. Inhibiting IL-6/STAT3 signaling can lead to HCC cell apoptosis[76].

IL-10 subfamily: This subfamily consists of IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26[77]. In a clinical study, polymorphisms in IL-19 increased susceptibility to HBV infection in children[78]. IL-19 inhibits the progression from NAFLD to NASH *in vitro*, while its deficiency in mice leads to pro-inflammatory cytokine expression in the liver[79]. IL-22 positively affects liver inflammation and impaired hepatic regeneration in AH patients and reduces ethanol-induced liver steatohepatitis in mice[38,80]. IL-22 exerts hepatoprotective effects in NAFLD-related liver fibrosis and injury[51,81,82]. However, the role of IL-22 in viral hepatitis is controversial, wherein some studies have reported its positive effects [83], while others indicated that it promotes liver fibrosis and HCC[84,85]. IL-22 exerts pro-tumorigenic effects on hepatocytes in HCC, while IL-22 BP ameliorates liver carcinogenesis[86]. IL-22 overexpression promotes HCC progression, while metformin treatment suppresses IL-22-induced liver cell proliferation, migration, and invasion by reacting with the Hippo signaling pathway[87].

IL-17 subfamily: The IL-17 subfamily comprises IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F[88]. IL-17 expression and the methylation status of its gene promoter can enhance CHB progression[89]. Polymorphisms in the IL-17 gene are related to HCV infection in humans[90,91]. In NAFLD, IL-17 promotes M1 macrophage polarization and exacerbates the hepatic inflammatory response, accelerating NAFLD progression in mice[92]. High-fat diets lead to IL-17A expression, accelerate NAFLD progression by inhibiting fatty acid β oxidation, and promote triglyceride accumulation[93]. This prevents fibrosis in steatohepatitis in mice by inhibiting IL-17-mediated inflammation[94]. In an experimental model of alcohol-induced HCC, IL-17 promotes HCC by regulating the inflammatory response of macrophages and cholesterol synthesis in fatty hepatocytes[9]. IL-17 can also promote non-ASH and HCC[95]. In particular, IL-17A can enhance HCC invasion *via* the AKT pathway and restrict the autophagy of HCC cells by inhibiting Bcl2 degradation[96,97]. IL-17 can improve HepG2 cell proliferation *in vitro* and *in vivo* by activating the IL-2/STAT6 pathway[98].

Chemokine α subfamily: Endoplasmic reticulum stress induces IL-8 transcription and inhibits interferon reactivity in human hepatocytes to increase HBV proliferation[99]. Interferon induces IL-8 to inhibit the production of HBV surface antigen using human hepatocytes[100]. Blocking the recruitment effect of IL-8 on neutrophils can reverse ASH in mice [101,102]. In NAFLD, liver TLR2 expression is positively associated with circulating IL-8 levels. TLR2-mediated pathways are critical for NAFLD/NASH progression, and NASH progression is slower in TLR2 knockout mouse models than in wild-type mouse models[103]. HBV-induced IL-8 inhibits antitumor immunity and elevates HCC metastasis[104]. IL-8 promotes the upregulated signaling of integrin-β3 and HCC cell invasion by activating the PI3K/Akt pathway[105]. Thus, inhibiting IL-8 expression can suppress HCC growth[106,107].

TNF

TNF is a cytokine and an adipokine that plays significant roles in various cellular events, including cell proliferation, cell differentiation, and cell death. As a pro-inflammatory cytokine, TNF is actively involved in inflammation-related carcinogenesis. Gene variation in TNF is associated with increased susceptibility to HBV and HCV infection[108,109]. One study evaluated the inhibition of TNF/NF-κB signaling and macrophage M1-type polarization, suggesting a promising approach for attenuating NAFLD progression to NASH[110]. Anti-TNFR1 treatment significantly reduces liver injury and fibrosis without affecting protective TNFR2 signaling in high-fat diet-induced NAFLD[111]. Anti-TNF-α compromises HCC progression and prolongs survival time in mice by decreasing tumor cell viability[112]. TNF-α induces mesenchymal stem cells mobilization to the injured liver site to participate in the inflammatory microenvironment formation and promotes liver cancer development[113]. TNF-α-mediated extracellular Ca^{2+} influx in HCC accelerates cell apoptosis, suggesting the function of TNF-α as a tumor-killing (pro-apoptotic) cytokine[114]. In addition, TNF-α polymorphism is associated with an elevated risk of HCC[115-117]. The role of TNF-α in the development and progression of HCC requires further exploration.

CXC motif chemokine family

Hepatic stellate cell–induced CXCL1 enhances the malignant development of HCC through the MIR4435-2HG/miR-506-3p/TGFβ axis, which could be a potential target in HCC therapy[118]. Inhibiting the CXCL1-CXCR2 loop improves doxorubicin efficacy in HCC, reducing macrophage recruitment in the tumor microenvironment and restricting tumor progression[119]. CXCL2 is a tumor suppressor, and its high expression significantly enhances the overall survival rate in HCC. Exogenous expression of CXCL2 inhibits cell proliferation in HCC by causing cell cycle arrest and apoptosis[120]. CXCL3 expression is upregulated in HCC and is highly associated with poor prognosis. This promotes CD133 + CSC proliferation through Erk1/2 phosphorylation[121]. CXCL5 knockdown inhibits cell proliferation and invasion through the miR-577/NF-κB axis, while CXCL5 overexpression is a potential indicator of poor prognosis in HCC patients[122]. Circ-HOMER1 causes cell growth and HCC aggressiveness by suppressing the miR-1322 function on CXCL6[123]. The expression level of CXCL6 in HCC tissues is significantly lower than in the adjacent normal tissues[124]. Tumor-associated macrophages caused by the CXCL8/miR-17 cluster enhance tumor cell growth and metastasis in HCC[125].

CXCL10 accelerates epithelial-mesothelial transition of HCC cells through MMP-2 activation[126]. CXCL10 remodels the intrahepatic tumor microenvironment of fibrosis-related HCC, while CXCL10 depletion promotes the invasion and infiltration of immune cells in the invasive tumor margin, resulting in an antitumorigenic microenvironment[127]. CXCL11/CXCR3 can positively regulate the stemness of α2δ1+ HCC tumor-initiating cells by improving self-renewal and tumorigenic properties *via* the ERK1/2 pathway[128]. SOX4-induced CXCL12 in HCC leads to tumor-distant metastasis by regulating CXCR4 in endothelial cells and reticular fibers while shaping the tumor microenvironment and neovascularization[129]. Compared with CHB patients or healthy control subjects, serum CXCL13 is significantly higher in HCC patients, and a positive result is associated with tumor size and metastasis[130]. In a clinical study, CXCL14 mRNA expression and serum CXCL14 levels were decreased in HBV-related HCC tissues. This indicates an advanced disease stage with severe hepatitis and impaired liver function[131]. CXCL14 represses cell proliferation in HCC and expedites apoptosis by inhibiting the Akt/mTOR signaling pathway[132]. Exogenous administration of CXCL14 prohibits angiogenesis in HCC and decelerates cell proliferation, invasion, and migration[133]. Allograft inflammatory factor 1 (AIF1)-induced M2 polarization macrophages secrete CXCL16, facilitating microvascular invasion and tumor progression [134]. Upregulated expression of CXCL17 in HCC promotes tumor cell proliferation and inhibits autophagy by controlling the LKB1-AMPK pathway[135]. MiR-325-3p overexpression attenuates angiogenesis, cell proliferation, migration, and invasion in HCC by restricting the CXCL17/CXCR8 axis[136]. Thus, CXCL2, CXCL6, and CXCL14 are negatively associated with HCC development and progression, while CXCL1, CXCL3, CXCL5, CXCL8, CXCL10, CXCL11, CXCL12, CXCL13, and CXCL17 play an inverse role.

TGF-β

TGF-β is a multifunctional regulator of various processes, including angiogenesis, immunity, and cancer[137,138]. TGF-β exists as three isoforms: TGF-β1, TGF-β2, and TGF-β3. All these can interrupt different stages of HCV propagation *via* the TGF-β/SMAD signaling pathway[139]. ECM1-mediated TGF-β activation promotes liver fibrosis by initiating HSCs[140]. TGF-β1 promotes HBV/HCV-induced fibrogenesis in hepatocytes and HSCs by interacting with the OCT4/Nanog pathway[141]. TGF-β inhibition significantly suppresses high-fat diet-induced inflammation and hepatic fibrosis, ameliorating obesity-related NAFLD and NASH[142,143]. Breviscapine and corosolic acid, TGF-β inhibitors, can alleviate NASH *via* multiple pathways by decreasing hepatic lipid accumulation, inflammation, and fibrogenesis[144,145]. In HCC, high TGF-β1 expression predicted shorter survival and poor disease prognosis in HCC patients[146]. In clinical studies, treating advanced HCC patients with the TGF-βR1/ALK5 inhibitor galunisertib can reduce AFP (alpha fetoprotein) and TGF-β1 in the body and prolong survival time[147,148]. In addition, galunisertib can improve sorafenib effectiveness in HCC patients[149]. In summary, TGF-β promotes the occurrence and development of HCC *via* inflammation-mediated cancer development (Table 1).

CURRENT CLINICAL THERAPIES

There is a significant correlation between inflammation and tumors, and regulating inflammation to treat the tumor could be an effective approach. The efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) in treating tumors is evident. They can exert their anticancer effect regardless of whether administered alone or combined[150]. The therapeutic effect of NSAIDs on HCC has been demonstrated, and aspirin can decrease the risk of death from liver cancer induced by chronic liver disease[151]. Celecoxib also promotes the apoptosis of HCC cells by inhibiting Akt expression[152]. In addition, inhibiting certain inflammatory factors can inhibit HCC development. Inhibition of the NLRP3 inflammasome can hinder the growth of HCC cells and promote autophagy[153,154]. 17β-Estradiol (E2) can induce NLRP3 inflammasome activation, trigger pyroptosis, and inhibit HCC progression[155]. Furthermore, IL-6 inhibition can cause HCC cell senescence[156]. The IL-6/STAT3 pathway can enable the metastasis and proliferation of HCC. Thus, inhibiting this pathway can enhance malignant HCC progression[157,158]. Trilobolide-6-O-isobutyrate inhibited IL-6/STAT3 pathway activation to decrease HCC progression[159]. Ursodeoxycholic acid inhibited IL-8 induced ERK phosphorylation, suppressing IL-8 induced angiogenesis[160]. Neurotensin controls IL-8 expression and interferes with EMT (epithelial-mesenchymal transition)-mediated HCC invasion and migration[161]. Dicer collaborates with lenvatinib to downregulate the expression of IL-8 and inhibit HCC growth[106]. In an alcoholic hepatitis mouse model, IL-22 can improve non-ASH through multiple targets while inhibiting inflammation and anti-fibrosis. Moreover, metformin inhibits IL-22 expression, attenuating HCC cell proliferation, migration, and invasion, and promotes apoptosis[87]. Targeting IL-22 has performed well in early HCC clinical experiments, with a good safety and efficacy profile[38,162].

Notably, anti-inflammatory drugs are combined to treat HCC with beneficial therapeutic effects. Pre-clinical studies have indicated that aspirin, a nonsteroidal anti-inflammatory drug, can elevate the sensitivity to various anti-cancer drugs. These include sorafenib and doxorubicin while overcoming sorafenib resistance *in vitro* and *in vivo*[163]. Additionally, aspirin limits NF-κB activation of SLC7A11 transcription by B inhibits the growth of HCC, leading to ferroptosis[164]. However, aspirin is negatively related to the early reported incidence rate of HCC in the general population, which should be considered in the future, particularly in gastrointestinal ulcer patients[165,166]. Another cohort study discovered that using NSAIDs could decrease the risk of early HCC recurrence two years after radical hepatectomy, irrespective of the patient's age, hepatectomy range, viral hepatitis status, basic diabetes, and cirrhosis[167]. Curcumin, a traditional Chinese medicine extract, has excellent anti-inflammatory effects. Curcumin overcame lenvatinib resistance, a first-line treatment drug for unresectable advanced liver cancer, by inhibiting epidermal growth factor receptor[168]. Combining steroid anti-inflammatory drugs dexamethasone and N-acetylcysteine can be employed for post-thrombotic syndrome and post-conventional transcatheter arterial chemoembolization, which is the standard

Table 1 The key inflammatory factors in liver diseases

Disease	Promotion genes	Inhibition genes
Virus hepatitis	IL-6[71]; IL-8[101,102]; IL-17[90,91]; IL-22[84,85]; TNF[108,109]; TGF-β[139];	IL-1β[63,64]; IL-22[83]
Alcoholic hepatitis	IL-1β[37]; IL-8[101,102]	IL-6[73]; IL-22[38,80]
NAFLD	IL-1α[60,61]; IL-1β[60,67-69]; IL-8[103]; IL-17[92-94]; TNF[110-112]; TGF-β[144,145]	IL-19[79]; IL-22[51,81,82]
HCC	IL-1β[68,69]; IL-6[75,76]; IL-8[105-107]; IL-17[96-98]; IL-22[86,87]; CXCL1[118,119]; CXCL3[121]; CXCL5[122]; CXCL8[125]; CXCL10[126,127]; CXCL11[128]; CXCL12[129]; CXCL13[130]; CXCL16[134]; CXCL17[135]; CXCR3 [128]; CXCR4[129]; TGF-β[146-148]	CXCL2[120]; CXCL6[123, 124]; CXCL14[131,132]

IL: Interleukin; TNF: Tumor necrosis factor; TGF: Transforming growth factor; HCC: Hepatocellular carcinoma; NAFLD: Nonalcoholic fatty liver disease.

treatment for mid-term HCC. Only two out of 50 participants experienced mild allergic dermatitis[169,170]. Currently, only a few anti-inflammatory drugs have undergone clinical trials. More effective anti-inflammatory drugs can be applied in clinical trials of HCC by continuously enhancing fundamental experiments.

CONCLUSION

Emerging studies demonstrated that inflammation, particularly chronic inflammation, is crucial in liver deterioration. Moreover, uncontrolled inflammation is a critical factor in liver cancer development. However, at this stage, some acute inflammatory factors have the opposite effect on HCC, indicating that the role of inflammation in HCC requires more exploration regarding new regulatory factors. These factors have great development prospects for the mechanism underlying malignant HCC progression and future clinical treatment.

FOOTNOTES

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REVIEW

Cell-type specific role of autophagy in the liver and its implications in non-alcoholic fatty liver disease

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Abstract

Autophagy, a cellular degradative process, has emerged as a key regulator of cellular energy production and stress mitigation. Dysregulated autophagy is a common phenomenon observed in several human diseases, and its restoration offers curative advantage. Non-alcoholic fatty liver disease (NAFLD), more recently renamed metabolic dysfunction-associated steatotic liver disease, is a major metabolic liver disease affecting almost 30% of the world population. Unfortunately, NAFLD has no pharmacological therapies available to date. Autophagy regulates several hepatic processes including lipid metabolism, inflammation, cellular integrity and cellular plasticity in both parenchymal (hepatocytes) and non-parenchymal cells (Kupffer cells, hepatic stellate cells and sinusoidal endothelial cells) with a profound impact on NAFLD progression. Understanding cell type-specific autophagy in the liver is essential in order to develop targeted treatments for liver diseases such as NAFLD. Modulating autophagy in specific cell types can have varying effects on liver function and pathology, making it a promising area of research for liver-related disorders. This review aims to summarize our present understanding of cell-type specific effects of autophagy and their implications in developing autophagy centric therapies for NAFLD.

Key Words: Autophagy; Non-alcoholic fatty liver disease; Hepatocytes; Macrophages; Hepatic stellate cells

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Core Tip: This review presents a succinct overview of the cell-specific distinct effects of autophagy modulation on hepatic pathophysiology and its implication on the progression of non-alcoholic fatty liver disease (NAFLD). The effects of autophagy alteration on hepatocyte lipid metabolism, macrophage polarization and hepatic stellate cell plasticity are reviewed and discussed with reference to NAFLD pathobiology.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome and a risk factor for diabetes, cardiovascular ailments, and hepatocellular cancers[1-3]. It is classically defined as hepatic steatosis which has developed in individuals with no or moderate alcohol consumption. The initial clinical presentation of NAFLD involves benign steatosis that may progress to a more severe form of the disease termed non-alcoholic steatohepatitis (NASH)[4]. NASH is characterized by increased hepatocyte damage, hepatocyte ballooning, inflammation, and fibrosis[5]. Several factors including high calorie diets, sedentary lifestyle, gut-microbiome, and genetic predisposition, constitute a multiple-hit basis of the progression of benign steatosis to NASH in certain individuals[6,7]. NASH is one of the leading causes of liver transplants worldwide[5]. Presently, there are no approved drug therapies for NAFLD and NASH. As physical activity is a key determinant of metabolic control, lifestyle modifications remain the only available treatment so far[8]. Furthermore, the prevalence of NAFLD, which is currently > 30%, has increased significantly in the last ten years with a nearly 50% increase occurring between 1990-2006 to 2016-2019[2]. At the molecular level, the development of NAFLD involves pathological changes in several hepatic cells including hepatocytes, macrophages, hepatic stellate cells (HSCs), endothelial cells and cholangiocytes[9]. Intracellular changes in the cellular metabolism, mitochondrial energetics, organellar homeostasis, redox hormesis and epigenetic changes in cellular plasticity govern the tissue damage and inflammatory milieu observed during NAFLD progression[10-13].

Autophagy is a cellular quality control process which is activated in response to energy crisis and cellular stress[14-16]. Historically, the liver has been recognized as an organ with high autophagy activity and hepatocytes and Kupffer cells were the first cell types where the metabolic role of autophagy and lysosomes were discovered[17,18]. Autophagy serves as a key regulator of hepatocyte, lipid, and carbohydrate metabolism in the liver[19]. Similarly, autophagy in liver macrophages and HSCs differentially regulates their plasticity from a quiescent to activated phenotype[20]. In this review, we will describe the distinct roles of cell-type specific autophagy in hepatic physiology and its deregulation in NAFLD.

AUTOPHAGY MECHANISMS

The term autophagy means “self-digestion” and plays a pivotal role in maintaining cellular homeostasis by recycling damaged or unnecessary cellular components. Autophagy ensures cell survival and contributes to various physiological and pathological processes. To date, three types of autophagy have been described: macroautophagy, micro-autophagy, and chaperone-mediated autophagy (CMA)[21]. Autophagy involves subcellular membrane trafficking to sequester a portion of cytoplasmic constituents and organelles by a membrane-sac (termed the phagophore) to form a double-membrane structure termed the autophagosome. The autophagosome is then transported to the lysosome for bulk protein degradation (proteolysis) of the sequestered intracellular materials by the lysosomal hydrolases. The breakdown products are utilized as an internally derived source of energy. Autophagy may be adaptive or constitutive. Constitutive autophagy is a mechanism of ‘cellular housekeeping’ that involves the removal of damaged or senescent organelles and helps to preserve basal energy balance. However, adaptive autophagy is characterized by recycling of intracellular constituents (proteins, lipids, glycogens, and organelles) to fulfill energy requirements in the event of nutrient deficiency. CMA is a selective cellular process where specific proteins are targeted for degradation by lysosomes with the help of chaperone proteins.

Macro-autophagy (hereafter referred to as autophagy) is a highly orchestrated process that can be divided into several key stages: Initiation, elongation, maturation, and degradation. The coordinated activity of several regulatory components tightly regulates the process of autophagy from initiation to termination. Autophagy genes, often referred to as autophagy-related genes (*Atgs*), are a group of genes responsible for regulating and executing the autophagic process within cells[22]. More than 30 autophagy-related (*ATG*) proteins have been identified and characterized thus far. The autophagic process is initiated by a serine-threonine protein kinase, Unc-51 Like autophagy activating kinase 1 (ULK1)[23]. The mammalian target of rapamycin (mTOR) is a central regulator of cell growth and metabolism and is known to inhibit autophagy when active. In nutrient-rich conditions, mTOR is activated, preventing autophagy initiation by phosphorylating the autophagy-initiating complex, ULK1/2. This phosphorylation inhibits ULK1/2 and prevents autophagosome formation. In contrast, AMP kinase (AMPK) is a sensor of cellular energy status. When energy levels are

low (*e.g.*, during nutrient deprivation or stress), AMPK is activated. Activated AMPK phosphorylates ULK1/2, relieving the inhibition imposed by mTOR and promoting autophagy initiation. Additionally, AMPK activation further stimulates autophagy by inhibiting mTOR directly and by activating transcription factors such as transcription factor EB (TFEB), which control the expression of *Atgs* and various lysosomal genes. When activated, TFEB promotes autophagy by enhancing the production of autophagy-related proteins and lysosome biogenesis[24].

The initiation phase is primarily governed by the mTOR and AMPK pathways. The ULK1/2 complex plays a central role in autophagy initiation and is comprised of ULK1, *ATG13*, *ATG101* and *FIP200*[25]. When mTOR is inhibited or AMPK is activated in response to nutrient deprivation or stress, ULK1 is activated by phosphorylation, and in turn, phosphorylates *ATG13* and *FIP200* to initiate the process of autophagosome formation[26]. Once initiated, autophagy proceeds through the elongation and maturation stages. Key proteins like autophagy-related protein 5 (*ATG5*) and *ATG12* form complexes that contribute to the elongation (expansion) of the isolation membrane, which eventually seals to form the autophagosome, a double-membraned vesicle that engulfs cellular cargo[27]. *ATG5* is part of a complex with *ATG12* and *ATG16L1*, which is crucial for elongation of the phagophore and closure of the autophagosome. *ATG8* or lipid-conjugated microtubule-associated protein 1A/1B-light chain 3 (LC3-phosphatidylethanolamine), which is lipidated and incorporated into the autophagosomal membranes, plays a central role in the biogenesis and elongation of autophagosomes[28].

The autophagy receptor or adaptor proteins facilitate the tethering of target proteins and organelles destined for degradation on to the autophagosome. Sequestosome1, also known as p62/SQSTM1 is a cargo receptor that recognizes ubiquitinated cargo, such as damaged organelles or proteins, and targets them for selective autophagic degradation. P62 contains LC3-interacting regions to interact with LC3 on the autophagosome membrane. Once the double-membrane vesicle is formed, it travels along the microtubules to the lysosome, where the outer membrane of the autophagosome fuses with lysosomes *via* the interaction of a synaptosome complex containing STX17, SNAP29, RAB7, and VAMP8 with LAMP1 on the lysosome[28]. Inside the autolysosomes, the lysosomal enzymes enable the degradation of the cargo.

AUTOPHAGY IN NAFLD

NAFLD is characterized by the accumulation of excess fat (triglycerides) in the liver, independent from excessive alcohol consumption. Demonstration that autophagy plays a significant role in the pathogenesis of NAFLD comes from several lines of evidence described below:

ATG gene knockout mouse models

Studies performed in liver-specific autophagy gene (*ATG5* and *ATG7)* knockouts revealed a lipolytic role of autophagy, and mice deficient in either of these genes showed increased hepatic steatosis[29]. The loss of autophagy genes also increased hepatocyte susceptibility to gut endotoxin-induced injury[30]. Autophagy is also known to regulate hepatic inflammation. In this regard, hepatic macrophages also known as Kupffer cells derived from *ATG5*$^{-/-}$ mice fed with a high-fat diet (HFD), developed a pro-inflammatory phenotype resulting from macrophage polarization[31].

Studies involving pharmacological/non-pharmacological autophagy inducers in animal models of NAFLD

Preclinical experiments performed with a classical autophagy inducer, such as, rapamycin resulted in the reduction of hepatic steatosis and injury in animals fed a HFD[32]. Similarly, the administration of autophagy inducing hormones such as thyroid hormone, ghrelin, glucagon like peptide-1 and vitamin D also increased autophagy in mouse liver and reduced steatosis in animals fed high calorie diets[33-38]. In addition, several natural compounds including caffeine, epigallocatechin gallate, and resveratrol, together with several herbal extracts derived from traditional Chinese and Indian medicines, have exhibited potent pro-autophagy activity which is associated with their anti-NAFLD effect in animals[39-49]. Besides pharmacological agents, lifestyle modifications including intermittent fasting[50,51] and exercise [52-54] also induce hepatic autophagy as a means to delay and/or reduce NAFLD/NASH progression.

Analysis of liver autophagy in human NAFLD

Assessment of autophagy in the liver biopsies of patients with progressive degree of severity showed impaired autophagy characterized by reduced expression of lysosomal cathepsins, accumulation of p62 and decreased autophagy flux[55,56]. Furthermore, the impairment of autophagy strongly correlated with markers of hepatic injury and inflammation[55,56]. More recently, whole exome sequencing data has revealed pathogenic mutations in human autophagy-related genes which increases susceptibility to NAFLD development[57,58]. Notably, the defects in autophagy observed in human NAFLD are similar to that observed in murine models of NAFLD, in which an early increase in autophagic flux is followed by a late block in autophagic flux and a concomitant increase in endoplasmic reticulum (ER)-stress[56,59].

AUTOPHAGY IN HEPATOCYTES

Hepatocytes are cells of parenchymal origin, and are the metabolic hub of the liver. These are the primary functional cells of the liver and play a central role in metabolic processes, detoxification, and protein secretion. Not surprisingly, autophagy has been widely studied in these cells under physiological and pathological conditions including NAFLD. Hepatocytes rely on autophagy to remove damaged organelles, manage energy balance, and regulate lipid metabolism.

The biological effects of autophagy on hepatocytes and its modulation under NAFLD are described below.

Role of autophagy in hepatocyte lipid and carbohydrate metabolism

Hepatocytes store excess neutral lipids in the form of lipid droplets (LDs) which are composed of triacylglycerol (TAG). These TAG stores can be degraded by lipases to release free fatty acids (FFAs) as fuel for ATP production. The lipolysis of TAGs mediated by an autophagy-lysosomal pathway was termed "lipophagy" in hepatocytes undergoing starvation[29]. The sequence of events involved in lipophagy consists of the engulfment of LDs by the autophagosomes, followed by their fusion with lysosomes where lipolysis of TAG takes place. The FFAs released from the lysosomes can then be utilized for mitochondrial fat oxidation[29]. The key lipase involved in this process is known as lysosomal lipase[29]. Defects in hepatocyte lipophagy are suspected to be a major cause of early NAFLD development in humans[60-62]. In addition to lipophagy, CMA also plays a key role in the lipolysis of TAGs within hepatocytes[63]. In this regard, both LD-associated proteins perilipin 2 and perilipin 3 have been identified as CMA substrates and their degradation *via* CMA precedes lipolysis by lipophagy[63]. Additionally, lipid degradation by microautophagy termed "macrolipophagy" has been reported to occur in mouse hepatocytes supplemented with oleate, followed by nutrient starvation[64]. Lipophagy has been shown to be activated by MTORC1 inhibition[65], fibroblast growth factor-21[36], as well as by the activation of nuclear receptors including thyroid hormone receptors, peroxisome proliferator-activated receptor alpha and TFEB exhibiting anti-steatosis effects[47,66-69]. More recently, the induction of lipophagy was shown to enhance lysosomal mediated lipid exocytosis, thereby ameliorating NASH in animal models[70].

Surprisingly, autophagy and autophagy genes have also been implicated in the assembly of TAGs in hepatocytes. Reports have shown that the loss of autophagy genes such as *MAP1LC3*[71], *ATG7*[72] and *FIP200*[30] leads to decreased LD accumulation in hepatocytes (Figure 1). This opposing effect by autophagy, as described above suggests paradoxical dual roles of autophagy in LD assembly *vs* degradation which may be due to the differential effects of *ATG* genes and nutrient status in cells[73].

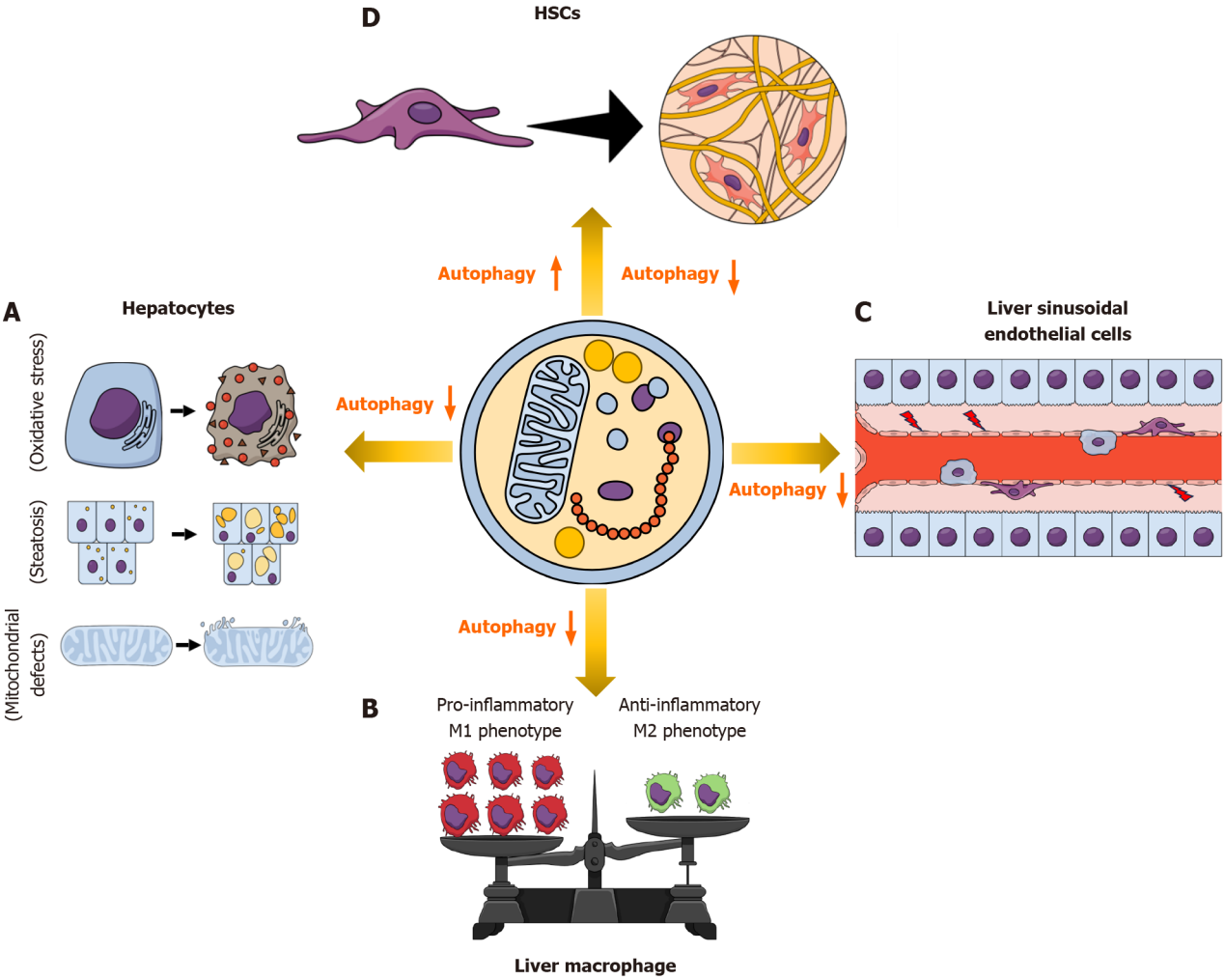
Besides its role in lipid metabolism, autophagy also plays a significant role in hepatocyte carbohydrate metabolism by regulating glycogen breakdown[74]. The lysosomal α-acid glucosidase can hydrolyze glycogen and release free glucose [75]. Excessive glycogen deposition in hepatocytes commonly coexists with hepatic injury in both patients with NAFLD [76] and those with glycogen storage disease type Ia (GSD Ia)[77]. GSD Ia is the most common glycogen storage disease. It is caused by the loss-of-function mutation of glucose-6-phosphatase, the enzyme converting glucose-6-phosphate to free glucose. Besides glycogen, GSD Ia is also characterized by excess lipid accumulation in the liver, and is now considered a fatty liver-like disease. Recently, the induction of autophagy was shown to attenuate the development of hepatic steatosis and reduce glycogen content in an animal model of GSD Ia[78]. These results, therefore, suggest an intricate interplay between hepatocyte autophagy and glycogenolysis.

Autophagy and hepatocyte lipotoxicity

Lee *et al*[79] used the term "Lipotoxicity" for the first time to describe the harmful effects of lipid species such as saturated FFAs (SFAs) and cholesterol in non-adipose organs. At the molecular level, NAFLD/NASH induced lipotoxicity in hepatocytes is characterized by increased oxidative stress, mitochondrial dysfunction, impaired unfolded protein response (UPR), pro-inflammatory cytokine production, and cell death[80,81]. Intriguingly, basal autophagy inhibition is also observed in response to SFAs such as palmitic acids[82]. Chronic SFAs administration impairs autophagosomal-lysosomal fusion, causes disruption of hepatocyte autophagy through suppression of the immune surveillance protein DDX58/Rig-1 (DExD/H box helicase) and stimulates the STING-MTORC1 pathway contributing to the autophagy inhibition reported in advanced NAFLD[65,82,83]. Therefore, restoration autophagic flux has emerged as an important strategy to counter lipotoxicity in hepatocytes[84].

In addition to being involved in macromolecular breakdown of lipids, proteins and carbohydrates, autophagy is also involved in selective removal of damaged organelles. The autophagic removal of mitochondria, known as "mitophagy" is a process of mitochondrial pruning that prevents the accumulation of damaged mitochondria resulting from increased oxidative stress[85]. Defective mitophagy has been shown to be associated with impaired mitochondrial β-oxidation and increased oxidative stress and lipoapoptosis in both animal models as well as in human NAFLD[86,87]. In hepatocytes, the accumulation of damaged mitochondria resulting from lipotoxicity, may lead to mitochondrial mediated apoptosis as well as activation of the inflammasome complex[88]. Therefore, the induction of mitophagy ensures both sustained mitochondrial energetics as well as cell survival (Figure 1). Several mechanisms have been proposed to regulate mitophagy in NAFLD[35,88-96]. Acyl coenzyme A: lysocardiolipin acyltransferase-1 expression was shown to be elevated in HFD fed mice, and its silencing restored mitophagy in isolated hepatocytes with observable improvement in mitochondrial architecture and reduced hepatic steatosis in mice[97]. Furthermore, the plant flavanol quercetin alleviates HFD-induced hepatic steatosis by activating AMPK-dependent mitophagy[98]. Furthermore, sirtuin 3 overexpression stimulates mitophagy and protects hepatic cells against palmitic acid-induced oxidative stress[99]. Mitophagy is also induced by thyroid hormone[100] through increased reactive oxygen species (ROS) production from mitochondria, the release of intracellular calcium, and activation of calcium/calmodulin-dependent protein kinase kinase and AMPK to both maintain mitochondrial fat oxidation as well as prevent further cell damage by ROS.

Autophagy also protects hepatocytes against lipotoxicity-induced oxidative stress by degrading Kelch like ECH associated protein 1 (KEAP1), which results in nuclear factor, erythroid 2 Like 2 (NRF2/NFE2L2) nuclear translocation and transcription of antioxidant genes[101]. Autophagy gene ULK1 was shown to enhance the interaction of autophagy adapter protein p62/SQSTM1 with KEAP1 which results in the autophagy-mediated degradation of KEAP1 and NRF2 mediated protection from lipotoxicity (Figure 1)[102].



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Figure 1 Cell-specific effects of autophagy modulation on liver pathology in non-alcoholic fatty liver disease. A: Hepatocytes: Loss of autophagy results in accumulation of oxidative protein and lipid adducts, triacylglycerols and defective mitochondria; B: Macrophage/Kupffer cells: Inhibition of macrophage autophagy results in increased generation of pro-inflammatory M1 polarized macrophages, which increases inflammation during non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis progression; C: Liver sinusoidal endothelial cells (LSECs): Loss of autophagy in LSECs results in cellular stress and loss of cellular integrity, resulting in increased NAFLD progression; D: Hepatic stellate cells (HSCs): The effect of autophagy on HSCs is conflicting, with some studies demonstrating its anti-fibrotic action while others support its pro-fibrotic action by regulating the transformation of quiescent HSCs into collagen-secreting myofibroblasts. HSCs: Hepatic stellate cells.

SFA-induced ER-stress and impaired UPR response also are key features associated with NAFLD progression in humans[56,103]. SFAs, cause ER stress by increasing saturated diacyl glycerolipid and saturated phospholipid accumulation in the ER, which causes persistent inositol-requiring enzyme-1α, and protein kinase RNA-like ER kinase activation in hepatocytes[104,105]. Eventually SFA-induced hepatocyte lipoapoptosis occurs owing to continuous UPR activation, which results in Jun N-terminal kinase and C/EBP Homologous Protein-mediated overexpression of proapoptotic proteins such as p53 upregulated modulator of apoptosis[106]. Autophagy serves as a key degradative mechanism for misfolded proteins in hepatocytes thus alleviating ER-stress caused by SFAs[107]. In this regard, HFD feeding was associated with increased hepatic ER stress and insulin resistance in autophagy defective animals[108]. Surprisingly, rescue experiments with *ATG7* gene overexpression dramatically relieved lipid-induced ER-stress in the mouse liver, as well as hepatic insulin sensitivity[108]. Besides degrading specific misfolded proteins, autophagy can also degrade parts of damaged ER by a process known as "ER-Phagy". Although the mechanistic basis of this process is still not very clear in hepatocytes, its role in NAFLD pathogenesis was highlighted by RNA sequencing data revealing numerous ER-phagy receptors such as ATL3, SEC62, and RTN3 which were differentially regulated in patients with NAFLD/NASH[107]. These data point towards ER-phagy playing an essential role during NASH and underscores its importance as a possible novel strategy for NASH treatment.

SFA exposure in hepatocytes triggers the NLRP3-inflammasone signaling, leading to the activation of interleukin (IL)-1 β which causes hepatocyte cell death[109-112]. The inhibition of inflammasome activation and hepatocyte pyroptosis is another way of cellular protection conferred by autophagy in hepatocytes[35].

AUTOPHAGY IN LIVER MACROPHAGES

The liver is a vital organ with diverse functions, including metabolism, detoxification, and immune regulation. Within the liver's intricate cellular landscape, Kupffer cells, the resident macrophages, are critical players in immune surveillance and tissue homeostasis. Autophagy, a conserved intracellular process, has emerged as a key regulator of Kupffer cell functions and liver physiology. Autophagy in Kupffer cells, plays a pivotal role in maintaining hepatic homeostasis, regulating inflammation, by eliminating misfolded or aggregated proteins, removing damaged organelles and invading pathogens[113].

Macrophages are highly heterogeneous immune cells, which can polarize to diverse phenotypes in response to the surrounding microenvironment[114]. During inflammation or injury, macrophage polarization determines the fate of an organ[114]. When an organ or a tissue is inflicted with an infection or injury, macrophages are first polarized to their pro-inflammatory M1 phenotype to facilitate the removal of antigens and necrotic cells by releasing pro-inflammatory cytokines. Furthermore, the M1 macrophages polarize with the M2 macrophages at the stage of repair, to secrete anti-inflammatory cytokines and suppress inflammation, which promotes tissue repair and remodeling. Autophagy regulates macrophage polarization in NAFLD[31,115,116]. Macrophage autophagy reduces chronic inflammation and lowers the progression of organ fibrosis by inhibiting M1 macrophage polarization[117] (Figure 1). Impaired macrophage autophagy increased immune response and chronic hepatic inflammation and injury in obese mice[31]. Ubiquitin-specific protease 19-induced macrophage autophagy promoted anti-inflammatory M2-like macrophage polarization[116]. Chronic liver injury results in organ scarring, termed liver fibrosis. Tissue-resident macrophages are the crucial regulators of organ fibrosis[118]. Inflammation plays a vital role and may be a cause of fibrosis[119]. As macrophage autophagy inhibits macrophage polarization to pro-inflammatory M1 type, it may be a potential target for organ fibrosis. Macrophage activation and polarization are increasingly being recognized to play an essential role in liver inflammation and fibrosis [120]. Autophagy inhibited the release of inflammatory cytokines, particularly IL-1, from hepatic macrophages and reduced HSC activation to protect against liver fibrosis in mice[121]. Also, the suppression of Atg5 showed increased liver inflammation and fibrosis *via* the enhanced mitochondrial ROS/NF-κB/IL-1α/β pathway in autophagy-deficient liver macrophages[122]. Macrophage autophagy was reported to downregulate hepatic inflammation by inhibiting inflammasome-dependent IL-1β production[123]. Spermine, a polyamine, reduced liver injury by inhibiting the pro-inflammatory response of liver-resident macrophages by inducing autophagy[124]. LC3-associated phagocytosis (LAP) inhibited inflammation and liver fibrosis by pharmacological as well as genetic interventions. Inhibition of LAP aggravated the pro-inflammatory and pro-fibrotic phenotype in the liver[125]. Autophagy is also involved in immune regulation in liver macrophages. It promotes antigen presentation and major histocompatibility complex-II expression, facilitating efficient antigen recognition by T cells. Conversely, defective autophagy can lead to exaggerated inflammatory responses[126]. Dysregulation of autophagy in Kupffer cells can have wide-ranging implications for liver diseases, making it an attractive target for future therapeutic interventions. Further research into the precise mechanisms and therapeutic potential of autophagy modulation in liver macrophages is warranted to advance our understanding of liver pathophysiology and develop novel treatment strategies.

AUTOPHAGY IN HSCs

Among several cell types that contribute to liver function and pathology, HSCs have emerged as key players in the development of liver fibrosis, a common endpoint in chronic liver diseases. Autophagy, a cellular process of self-digestion and recycling, has gained increasing attention due to its role in HSC biology and its implications in liver disease progression. Autophagy in HSCs is intricately involved in maintaining metabolic homeostasis. It ensures an efficient turnover of cellular components, provides energy during stress or activation, and helps regulate key signaling pathways. Dysregulation of autophagy in HSCs can disrupt these metabolic processes and contribute to liver fibrosis and disease progression.

Upon liver injury or inflammation, HSCs undergo activation, transforming into proliferative, fibrogenic myofibroblasts that contribute to fibrous scar formation[127]. The role of autophagy in HSC activation remains paradoxical and context specific. Studies performed in HSCs *in vitro* and *in vivo* showed the profibrotic effect of autophagy induction during transforming growth factor beta induced HSC activation[128] (Figure 1). Specifically, autophagy is proposed to induce the activation of HSCs through lipophagy, a selective type of autophagy that degrades LDs[129]. On the other hand, autophagy also plays a critical role in maintaining HSC quiescence and limiting their activation. Inhibition of autophagy in activated HSCs has been associated with increased fibrogenesis, while induction of autophagy can suppress their activation and collagen production[130] (Figure 1). Indeed, HSC autophagy attenuated liver fibrosis by inhibiting the release of extracellular vesicles[131]. Autophagy in HSCs was recently shown to induce the release of miR-29a. Inhibition of autophagy reduced miR-29a secretion and repressed fibrogenic gene expression in a mouse model of liver fibrosis and in patients with chronic hepatitis C infection[132]. These findings underscore the therapeutic potential of targeting autophagy in HSCs to mitigate liver fibrosis and, consequently, liver disease progression. Autophagy in HSCs has significant implications for liver disease. Understanding these mechanisms holds promise for developing targeted therapies to modulate HSC metabolism and mitigate liver fibrosis. The role of autophagy in maintaining HSC quiescence and limiting fibrogenesis makes it a promising target for therapeutic intervention. Pharmacological agents that regulate autophagy in HSCs are being investigated for their potential to halt or reverse liver fibrosis and alleviate the burden of liver diseases worldwide. Furthermore, strategies to enhance the specificity of these interventions to HSCs also hold promise for minimizing their off-target effects.

Autophagy in liver sinusoidal endothelial cells (LSECs)

LSECs form the first barrier of defense in the liver owing to their unique position, lining the sinusoidal lumen. Endothelial dysfunction is known to play a key role in liver injury[133]. Autophagy maintains cellular integrity, phenotype and homeostasis and can be found in various cell types, including liver endothelial cells[134]. Decreased autophagy has been observed in liver endothelial cells of patients with NASH as compared to patients with simple steatosis or those with normal liver[135]. The selective disruption of *ATG5* or *ATG7* in endothelial cells impairs the normal endothelial phenotype and favors liver injury, inflammation and fibrosis in mice exposed to prolonged HFD feeding or carbon tetrachloride[133,135] (Figure 1).

CONCLUSION

Autophagy in the liver plays key role in hepatic metabolism, immunomodulation, and cellular plasticity with profound effects on NAFLD progression. Future research should focus on better understanding the role of autophagy in inter-cellular crosstalk among various cell types of the liver and its targeting as a future therapy for NAFLD/NASH in humans. Investigating hepatocyte-specific autophagy mechanisms and their response to various stressors, such as nutrient imbalances, oxidative stress, and toxic insults, is crucial to explore the therapeutic potential of autophagy modulation in NAFLD/NASH. Understanding how autophagy affects inflammation and antigen presentation in Kupffer cells could provide insights into liver-related immune disorders and manipulating autophagy in these cells may have implications for treating conditions like liver fibrosis. Additionally, exploring how autophagy contributes to LSEC integrity, angiogenesis, and regulation of blood flow may provide a better understanding of its role in liver health and disease. Furthermore, the deduction of molecular mechanisms by which autophagy influences HSC activation and collagen production can provide insights into therapeutic strategies for liver fibrosis.

Given the dynamic sequence of involvement of different cell types and the pleiotropic effect of autophagy during NAFLD progression, an optimal therapeutic time-window for targeting autophagy should be identified. Finally, identifying biomarkers of autophagy flux in humans would be useful clinically to monitor disease progression and response to treatment. Clinical trials of autophagy modulating drugs for NAFLD/NASH treatment could provide significant therapeutic advances, particularly since there are no pharmacological treatments for this disease.

FOOTNOTES

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MINIREVIEWS

Any role for transarterial radioembolization in unresectable intrahepatic cholangiocarcinoma in the era of advanced systemic therapies?

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Abstract

Intrahepatic cholangiocarcinoma (iCCA) is recognized as the second most frequently diagnosed liver malignancy, following closely after hepatocellular carcinoma. Its incidence has seen a global upsurge in the past several years. Unfortunately, due to the lack of well-defined risk factors and limited diagnostic tools, iCCA is often diagnosed at an advanced stage, resulting in a poor prognosis. While surgery is the only potentially curative option, it is rarely feasible. Currently, there are ongoing investigations into various treatment approaches for unresectable iCCA, including conventional chemotherapies, targeted therapies, immunotherapies, and locoregional treatments. This study aims to explore the role of transarterial radioembolization (TARE) in the treatment of unresectable iCCA and provide a comprehensive review. The findings suggest that TARE is a safe and effective treatment option for unresectable iCCA, with a median overall survival (OS) of 14.9 months in the study cohort. Studies on TARE for unresectable iCCA, both as a first-line treatment (as a neo-adjuvant down-staging

strategy) and as adjuvant therapy, have reported varying median response rates (ranging from 34% to 86%) and median OS (12-16 mo). These differences can be attributed to the heterogeneity of the patient population and the limited number of participants in the studies. Most studies have identified tumor burden, portal vein involvement, and the patient's performance status as key prognostic factors. Furthermore, a phase 2 trial evaluated the combination of TARE and chemotherapy (cisplatin-gemcitabine) as a first-line therapy for locally advanced unresectable iCCA. The results showed promising outcomes, including a median OS of 22 mo and a 22% achievement in down-staging the tumor. In conclusion, TARE represents a viable treatment option for unresectable iCCA, and its combination with systemic chemotherapy has shown promising results. However, it is important to consider treatment-independent factors that can influence prognosis. Further research is necessary to identify optimal treatment combinations and predictive factors for a favorable response in iCCA patients.

Key Words: Intrahepatic cholangiocarcinoma; Transarterial radioembolization; Locoregional treatment; Overall Survival; Response rates; Neo-adjuvant therapy; Combined Therapies; Prognostic factors

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Core Tip: Intrahepatic cholangiocarcinoma (iCCA), the second most common type of liver cancer, is frequently diagnosed at an advanced stage due to limited diagnostic tools and undefined risk factors. Surgery, the potential cure, is often infeasible. Ongoing investigations into unresectable iCCA treatment include chemotherapy, targeted therapy, immunotherapy, and locoregional treatments. Transarterial radioembolization (TARE) demonstrates safety and effectiveness, with a median response rates (34%-86%) and OS (12-16 mo) varying due to patient heterogeneity. Key prognostic factors include tumor burden, portal vein involvement, and patient performance status. The median overall survival reported after TARE is of 22 mo with 22% of tumor down-staging. TARE is a viable unresectable iCCA treatment, especially when combined with systemic chemotherapy. Nonetheless, further research is needed to optimize treatment combinations and identify predictive factors for favorable responses in iCCA patients.

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INTRODUCTION

Epidemiology, clinical characteristics and treatment of cholangiocarcinoma

Cholangiocarcinoma (CCA) is a diverse group of bile duct tumors, divided into three subtypes: intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA)[1]. With increasing rates, CCA is the second most common liver cancer globally[2]. CCA rates are stable in regions with reduced alcohol-related live disease, but increasing in Europe and America due to higher alcohol intake, viral hepatitis infections, an increase in obesity rates, and the prevalence of non-alcoholic fatty liver disease (NAFLD)[1,3]. The prevalence and incidence of CCA result from a complex interplay of evolving risk factors, including chronic biliary tract inflammation and biliary stasis[1]. Risk factors include primary sclerosing cholangitis, congenital choledochal cysts, liver fluke infections, hepatolithiasis, cirrhosis, hepatitis B and C, NAFLD, obesity, asbestos exposure, diabetes, smoking, and alcohol. Some cases have no identifiable risk factors, making early diagnosis challenging[4]. Late-stage diagnosis leads to poor outcomes, and surgery is the primary curative option, aiming for R0 resection and preserving future liver remnant function. Hepatobiliary resection is preferred for iCCA and pCCA[5], while treatment approaches for dCCA are similar to pancreatic head cancer[4]. Neoadjuvant therapy is not typically recommended, but promising results have been seen in using neoadjuvant treatment as a bridge to liver transplantation [1,6]. The role of chemoradiation therapy as adjuvant treatment is uncertain, with potential benefits for pCCA and dCCA patients with R1 surgical margins. Ongoing trials explore combined cytoriductive therapy (cisplatin and gemcitabine) in an adjuvant setting[1]. Liver transplantation is considered for unresectable pCCA and iCCA cases without extrahepatic disease. Palliative treatments are the option for those ineligible for transplant[1]. Immunotherapies in CCA have limited data, and their role is under investigation, either alone or in combination with other treatments[6].

Local-regional therapies are an option for iCCA patients to manage tumor growth and complications. Limited evidence supports their use in pCCA and dCCA[7]. Common loco-regional treatments include external beam radiotherapy, trans-arterial chemoembolization (TACE), image-guided thermal ablation, trans-arterial radioembolization (TARE), and hepatic chemosynthesis. TARE has gained attention, though with limited evidence[1,6,7].

TARE techniques

TARE is a minimally invasive technique to treat liver cancer, used for both hepatocellular carcinoma (HCC) and CCA. TARE involves the delivery of small beads, called microspheres, which are coated with a radioactive material, to the tumor through the blood vessels.

The liver receives blood from two sources: The hepatic artery and the portal vein; moreover, the hepatic parenchyma has a sinusoidal cytoarchitecture. Both these aspects promote the use of intra-arterially delivered treatments, such as TACE and TARE[8]. TARE is a technique that allows the delivery of a radioactive drug directly to the tumor, minimizing systemic irradiation and preserving the health of the liver. This is achieved by introducing microspheres into the tumor's blood vessels through a catheter inserted into the femoral artery and guided to the liver using imaging techniques like angiography. These microspheres lodge in the small blood vessels that supply the tumor, leading to a reduction in blood flow to the cancer cells and ultimately causing their death. Different types of microspheres, such as Lipiodol, glass, resin, or polymer, have been used, along with various radioisotopes like Phosphorus-32 (^{32}P), Yttrium-90 (^{90}Y), Iodine-131 (^{131}I), Holmium-166 (^{166}Ho), Lutetium-177 (^{177}Lu), and Rhenium-186/188 ($^{186/188}$Re), all of which are beta emitters[8,9]. Current TARE agents use β-particle-emitting radioisotopes, although some pioneering studies are now investigating the development and use of α-particle-emitting radioisotopes[10]. Targeted alpha therapy is a highly effective treatment due to the densely ionizing track and the short path length (40-90 μm) of the emitted α-particles, resulting in a high linear energy transfer (50-30 keV/μm). Actinium-225 is one of the α-particle emitting radionuclides currently being explored for clinical applications. A recent study utilizing a mouse model demonstrated that survival rates significantly improved when using [225Ac]Ac-DOTA-TDA-Lipiodol® in comparison to control groups[10].

^{90}Y-loaded microspheres are commonly used in TARE and have proven to be a crucial tool in the treatment of primary and secondary liver tumors, with a positive safety profile[11].

^{90}Y, a radionuclide that emits beta particles, has a physical half-life duration of 64.2 hours. It does not emit gamma photons but produces secondary "bremsstrahlung" photons. Although it has a low positron emission of 32 decays per million, the maximum and mean energies of its beta particles are 2.28 MeV and 0.94 MeV, respectively, and in soft tissue, it has maximum and mean penetration depths of 11 mm and 4 mm, respectively[12]. ^{90}Y can be loaded onto either glass or ion-exchange resin microspheres, enabling the delivery of high radiation doses to tumors while sparing normal hepatic parenchyma[13,14].

Observations indicate that radioembolization is well-tolerated by patients with a good performance status, consistent with current literature[14,15].

Prior to TARE, angiography of the aorta, superior mesenteric artery, and celiac trunk is performed to assess the hepatic vascular architecture, liver contact with surrounding structures, portal vein patency, and the presence of arterio-portal shunting[16]. Non-target vessels with microspheres injection can lead to adverse events, and although coil embolization of non-target vessels was previously routine, it is no longer recommended as a standard procedure[17]. One common arterio-portal shunt is between the liver and the lung, which is accurately visualized using ^{99m}-Technetium labeled macroaggregated albumin (^{99m}Tc-MAA). It acts as a surrogate marker for ^{90}Y microspheres and is injected into the left and right hepatic arteries. After injection, any arteriovenous connections around the tumor are sharpened[17]. After ^{99m}Tc-MAA injection, to assess and quantify pulmonary shunts, known as Lung Shunt Fraction, hepatic planar scintigraphy and single-photon emission computed tomography/computed tomography (SPECT/CT) scans are subsequently conducted [18], as significant pulmonary shunts can cause late radiation lung toxicity. ^{99m}Tc-MAA SPECT/CT imaging also aids in identifying possible gastrointestinal shunts, which, if not correctable through catheter embolization, can be an absolute contraindication for treatment[19,20]. Furthermore, ^{99m}Tc-MAA SPECT/CT acquisition helps define the treatment field and calculate the required activity of ^{90}Y microspheres. When planning whole liver or selective, non-ablative or ablative TARE, it is advised to adopt a personalized method that utilizes dosimetry, grounded either in partition models or voxel-based models[21].

After microspheres injection a ^{90}Y PET/CT scan is performed to assess treatment success by visualizing tumor targeting, quantifying absorbed dose post-treatment, and predicting complications such as radioembolization-induced liver disease[22,23].

TARE is considered a safe and effective treatment option with minimal side effects, and it can be repeated as necessary. It is a valuable alternative for patients who are not suitable for surgery, and it can also be combined with other treatments such as systemic chemotherapy, immunotherapy, or other local therapies to improve outcomes in iCCA.

MATERIAL AND METHODS

A comprehensive literature search was conducted using PubMed, Embase (from 2008 to 2021), and the Cochrane Library to identify relevant studies. In order to maximize the inclusion of pertinent articles, personal knowledge of the relevant literature was utilized, the reference lists of retrieved papers were reviewed, and a manual search was performed in key journals. The search strategy employed a combination of medical subject headings (MeSH) terms and free language words to capture a wide range of relevant publications.

TARE AS A THERAPEUTIC STRATEGY IN ICCA

Locoregional treatments, including radiofrequency ablation, hepatic artery infusion, TACE, drug-eluting bead TACE, and

TARE, have been extensively studied for unresectable cholangiocarcinoma iCCA[24,25]. These treatments have shown increased median overall survival (OS) compared to traditional systemic chemotherapy, with acceptable toxicity profiles [24].

In general patients eligible for locoregional treatments should meet specific criteria, including: (1) Eastern Cooperation Oncology Group (ECOG) performance status ≤ 2; (2) Adequate laboratory tests (A neutrophil count exceeding 1.5 × 10^9/L, A platelet count over 50 × 10^9/L); (3) Adequate kidney function (creatinine level below 2.0 mg/dL); (4) Proper liver functionality (bilirubin level under 2.0 mg/dL); and (5) The ability to undergo hepatic angiography[7].

Locoregional treatments are not recommended in cases of pregnancy, breastfeeding, an expected lifespan of under three months, or in instances of clinical liver failure. TARE is typically recommended for scenarios with minimal or no spread of the tumor beyond the liver, although interpretations of this condition vary[12]. It's important to note that in patients with iCCA, lymph node metastases haven't demonstrated a detrimental effect on OS and thus shouldn't be viewed as a disqualifying factor for TARE. Nevertheless, the presence of solid organ metastases necessitates individualized treatment decisions[12,25].

TARE as an option for locoregional treatment was first described in a study by Ibrahim *et al*[26], which included 24 patients with histologically diagnosed iCCA. The median OS was 14 months, with two patients achieving downstage with resectable disease and/or liver transplantation. At follow-up imaging, the tumor response indicated partial shrinkage in 27% of cases, stable disease in 68%, and only 5% showed disease progression. In total, an objective tumor response, defined as any reduction in size, was noted in 86% of the patients[26].

Moreover, for inoperable patients treated with TARE, a systematic review by Boehm *et al*[24] reported a cumulative median OS of 13.9 months (95%CI: 9.5-18.3) and a radiologic response according to RECIST criteria of 27.4% (95%CI: 17.4%-37.5%) for complete or partial response and 54.8% (95%CI: 45.2%-56.7%) for stable disease. Another retrospective study by Gangi *et al*[15] of a cohort of patients with unresectable iCCA who underwent TARE as first- or second-line treatment showed a median OS of 12.0 months (95%CI: 8.0-15.2), with a radiologic partial response in 6.2% of patients, stable disease in 64.2%, and progressive disease in 29.6% of patients at 3 mo.

Two other studies showed encouraging results for inoperable ICCA: Pellegrinelli *et al*[27] showed a median OS of 16 mo at three months, while Robinson *et al*[14], analyzing data from the Registry for Radiation-Emitting SIR-Spheres in non-resectable Liver Tumors, reported a median OS of 14.0 mo (95%CI: 12.1-22.3) and a median progression-free survival of 5.8 months (95%CI: 4.6-7.2) for the entire cohort of patients treated with TARE. Notably, this last study demonstrated an objective radiologic response, assessed by RECIST criteria, in 34% of patients (8% complete response, 26% partial response), while 67% of patients achieved disease control[14].

TARE has been explored in combination with systemic chemotherapy to further enhance treatment success.

A recent phase-2 trial evaluated the combination of systemic chemotherapy using cisplatin and gemcitabine with TARE for unresectable iCCA[28]. The study reported a median OS of 22 mo (95%CI: 14-52 mo), with a one-year OS rate of 75% (95%CI: 62%-89%) and a two-year OS rate of 45% (95%CI: 30%-61%). The objective response, based on RECIST criteria at three months, was 39% (90%CI: 26%-53%). Additionally, the disease control rate at three months was 98% (95%CI: 89%-99%). Additionally, 22% of patients (9 patients) were able to undergo downstaging for potential surgical intervention. Although these results are promising, they still need to be confirmed through a phase-3 trial. Unfortunately, a similar trial combining cisplatin plus gemcitabine chemotherapy and TARE was prematurely halted due to insufficient patient recruitment[6]. A recent study shared findings on the combined use of TARE and CT-guided high-dose-rate interstitial brachytherapy (CT-HDRBT)[29]. A further possible approach involves an ablative method where a radioactive source, specifically Iridium 192, is inserted directly into neoplastic lesions *via* catheters under the guidance of CT imaging[30,31]. This technique overcomes size limitations and restrictions due to tumor location[32]. Among patients with CCA treated with either TARE or CT-HDRBT, the median OS was 29 mo, overall, the available evidence suggests that TARE could play a role in treating unresectable iCCA, both as a standalone therapy and when combined with systemic chemotherapy (Table 1).

Discrepancies in OS among the available studies are due to significant differences in study designs, sample size, and association with other treatment options. Therefore further studies are required to establish TARE effectiveness in randomized trial and to better investigate its potential in combination with other approaches[6].

However, the treatment of advanced-stage iCCA remains a complex task that often requires the combination of different therapeutic strategies to develop the optimal treatment approach for patients. In this context, TARE may play a role, also as observed in in real life experience[9], as a combination treatment to further increase treatment success and improve patient care (Figure 1).

Patient selection is a critical aspect of TARE for iCCA. It involves assessing the extent of the disease, extrahepatic tumor spread, liver function, and overall health. Lobar or segmental perfusion involves the selective delivery of radioactive microspheres to specific regions of the liver, making precise patient selection even more crucial. High-quality imaging, such as angiography and CT or magnetic resonance imaging (MRI) scans, is essential to identify the arterial supply to the tumor and determine the optimal catheter placement for microsphere delivery. Advanced software tools are used for treatment planning to calculate the required dose and ensure minimal radiation to healthy liver tissue.

Moreover, the optimal radiation dose and the selection of embolic agents represent important issues.

Concerning the radiation dosage, with the use of resin microspheres, the activity level of ^{90}Y to be administered is determined based on the assumption that a mean absorbed dose of 40 Gy or less to the non-tumoral liver is safe. Additionally, for iCCA, a minimum mean target-absorbed dose to the tumor of 100-120 Gy is recommended[21]. The cut-offs for calculating ^{90}Y activity change when glass microspheres are used. In this case, the mean absorbed dose for the nontumorous liver, which is considered safe, is less than 75 Gy, and the minimum mean absorbed target dose for the tumor, which is recommended to significantly increase OS, is higher than 260 Gy[12,33]. A systematic review of dosimetry after iCCA treatment shows that the mean delivered tumor dose is approximately 200 to 250 Gy for glass-

Table 1 Characteristics of the included studies evaluating transarterial radioembolization in intrahepatic cholangiocarcinoma

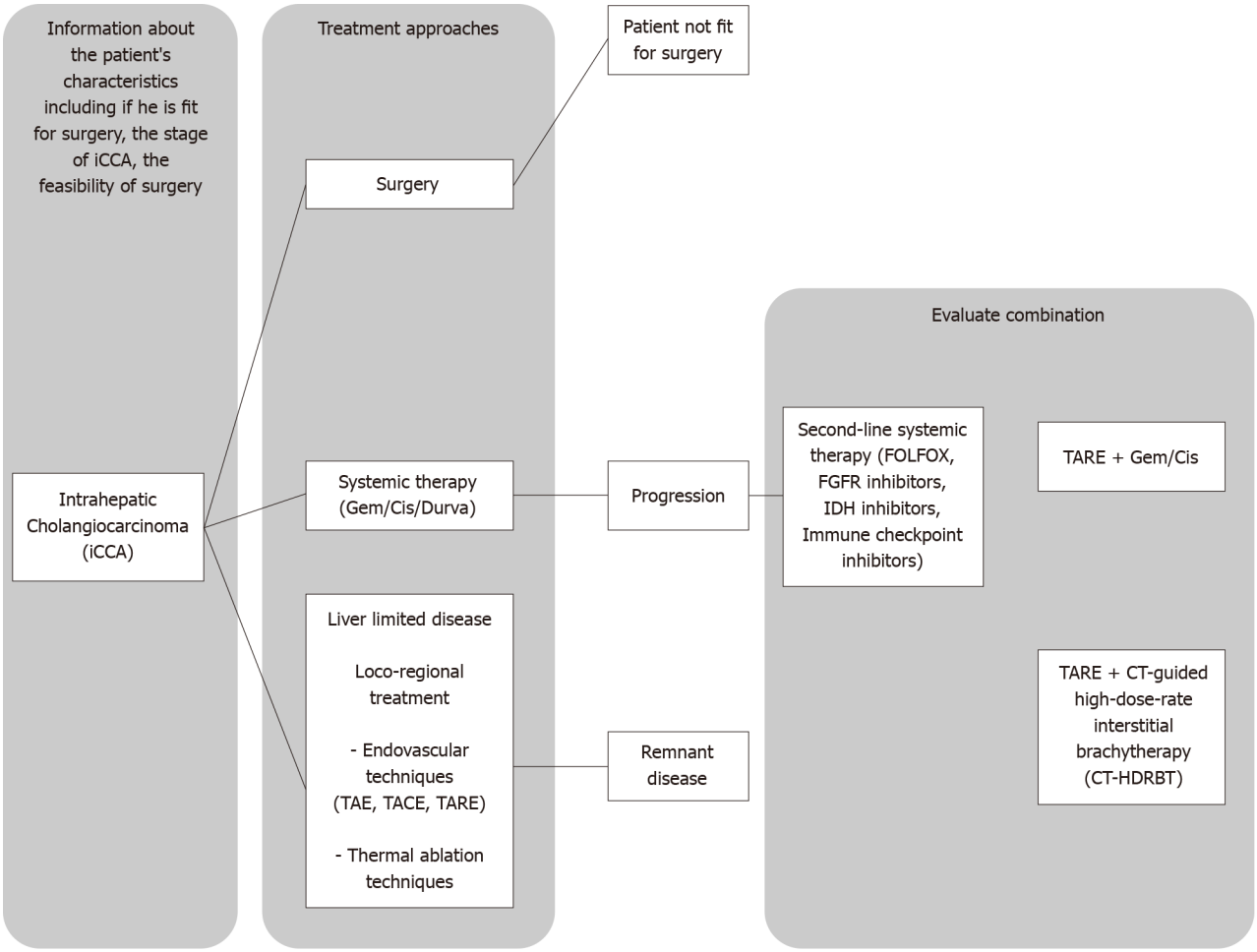
Ref.	Study design	N patients	Inclusion criteria	Technique	Median OS (mo)	R-response (%)
Ibrahim *et al* [26], 2008	Report	24	Histological diagnosis; Unresectable disease; ECOG ≤ 2; Neutrophil > 1.5 × 10^9/L; Platelet > 50 × 10^9/L; Creatinine < 2.0 mg/dL; Bilirubin < 2.0 mg/dL; Able to undergo angiography	^{90}Yttrium (^{90}Y); Radioembolization	14	86
Boehm *et al* [24], 2015	Systematic Review and Metanalysis	127	Unresectable disease; TARE treatment	^{90}Yttrium (^{90}Y); Radioembolization	13.9	27.4 (partial or complete; 54 (stable disease)
Gangi *et al* [15], 2018	Single Center; Retrospective study	85	Histological diagnosis; Unresectable disease; ECOG ≤ 2; Platelet > 50 × 10^9/L; Creatinine ≤ 2.0 mg/dL; Bilirubin ≤ 2.0 mg/dL; INR ≤ 1.5	^{90}Yttrium (^{90}Y); Radioembolization	12	6.2 (partial response); 64 (stable disease)
Pellegrinelli *et al*[27], 2021	Single center; Retrospective study	6	Unresectable disease; ECOG ≤ 2; Platelet > 50 × 10^9/L; Bilirubin < 2.0 mg/dL; Prothrombin time > 50%; Able to undergo angiography	^{90}Yttrium (^{90}Y); Radioembolization	16	NA
Robinson *et al* [14], 2022	Registry data	95	NA		14	34
Edeline *et al* [28], 2020	Phase 2; Clinical Trial	41	Unresectable disease; Never received CT; Never received intra-arterial treatment	^{90}Yttrium (^{90}Y); Radioembolization + Cisplatin and Gemcitabine	22	39
Fleckenstein *et al*[29], 2022	Single center; Retrospective study	9	Unresectable disease; At least one TARE treatment; At least one CT-HDRBT treatment	^{90}Yttrium (^{90}Y); Radioembolization + CT-HDRBT	29	NA

NA: Not available; CT: Computed tomography; CT-HDRBT: CT-guided high-dose-rate interstitial brachytherapy; R-response: Radiologic response; ECOG: Eastern Cooperation Oncology Group; OS: Overall survival; TARE: Transarterial radioembolization; INR: International normalized ratio.

based treatments and 80 to 130 Gy for resin-based treatments[34]. A recent study using glass microspheres further increased the dose for treatment of iCCA, suggesting that segmental transarterial radioembolization at > 400 Gy is an ablative approach that is feasible in terms of safety and efficacy[35].

A more subtle point, but one of potential clinical importance, is the selection of embolic agents and their infusion methods tailored to TARE in the context of iCCA. The glass microspheres, which are insoluble and infused with Y^{90}, measure between 20-30 μmol/L in diameter and possess an activity of 2500 Bq per sphere at calibration time. These microspheres are designated for use in cases of inoperable HCC and HCC with complications due to portal vein thrombosis. They have received approval from the United States Food and Drug Administration under a humanitarian exemption, which is based on their established safety and potential clinical benefits. A total count of 1.2 million microspheres generates an activity of 3 GBq (as stated in the TheraSphere® Yttrium-90 microspheres package insert, Kanata CMN. http://www.therasphere.com/physicians-package-insert/TS_PackageInsert_USA_v12.pdf). Resin microspheres are made of biocompatible resin and have a diameter ranging from 20-60 μmol/L, with an activity of 50 Bq per sphere. They contain a lower concentration of Y90 per sphere compared to glass microspheres, necessitating a larger number of spheres to administer a specific dose. This results in a higher embolic effect for the same dose delivery. To achieve 3 GBq of activity, between 40-80 million resin microspheres (each with 50 Bq) are required, in contrast to the 1.2 million needed for glass microspheres, as detailed in the SIRS-Spheres® Yttrium-90 microspheres package insert from Singapore Science Park SSM. http://www.sirtex.com/media/29845/ssl-us-10.pdf). Consequently, glass microspheres have the least embolic effect for the same prescribed activity because they are injected in much smaller numbers. The increased quantity of resin microspheres, despite the same prescribed activity level, could potentially lead to a more even distribution of the dose, resulting in a heightened biological effect, which includes both toxicity and efficacy. For resin ^{90}Y-microspheres, given the higher embolic load, no blind infusions should be performed. The microspheres are delivered slowly at a rate of no more than 5 mL/min, as rapid delivery may cause reflux. During the procedure, the radiologist must repeatedly check the position of the catheter to ensure its position and continued forward flow. In the case of glass ^{90}Y-microspheres, due to the small number of microspheres used, it's not necessary to completely saturate the entire vascular bed or to use continuous fluoroscopic guidance during infusion. Typically, a full infusion can be completed in about 5 minutes through a slow, manual injection while the patient breathes normally[12].

Another recent option is the use of microspheres containing holmium-166 (^{166}Ho), which are now available in Europe as an alternative to ^{90}Y microspheres[12,36]. ^{166}Ho offers advantages with its shorter half-life (26.8 h), high-energy beta and gamma radiation, and MRI-friendly properties[37]. Treatment planning uses the same microspheres as radioembolization, eliminating the need for ^{99m}Tc MAA. Clinical evidence supports the efficacy and safety of 166Ho in unresectable cancer[37]. Based on the latest usage guidelines, it's permissible for the average dose absorbed by the treated volume to surpass 60 Gy, provided that the average dose absorbed by the entire liver remains below 60 Gy. This approach aims to strike a balance between effective treatment and the safety of the liver[38].



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Figure 1 Simplified algorithm for therapies in managing advanced-stage intrahepatic cholangiocarcinoma. iCCA: Intrahepatic cholangiocarcinoma; TAE: Trans-arterial embolization; TACE: Trans-arterial chemoembolization; TARE: Transarterial radioembolization; FGFR: Fibroblast Growth Factor Receptor FOLFOX: FOL: Folinic acid (leucovorin), F: Fluorouracil (5-FU), OX: Oxaliplatin; IDH: Isocitrate Dehydrogenase; CT: Computed tomography; CT-HDRBT: CT-guided high-dose-rate interstitial brachytherapy.

TOXICITIES

TARE for iCCA has demonstrated a favorable safety profile overall (Table 2). Ibrahim *et al*[26] reported a low incidence of severe adverse events (grade 3 or 4) in their study, with 17% of patients experiencing grade 3 albumin toxicities and 4% experiencing grade 3 bilirubin toxicities. Mild to moderate (grade 1 or 2) clinical toxicities were more common and included fatigue (75% of patients), transient abdominal pain (38%), vomiting (13%), anorexia (8%), and nausea (4%). Moderate to severe clinical toxicities were infrequent, with one patient (4%) developing a gastroduodenal ulcer, three patients (14%) developing ascites, and two patients (9%) developing pleural effusion. Two patients died within 30 d after the procedure. A subsequent review and meta-analysis by Boehm *et al*[24] found that TARE was associated with minor treatment-related complications such as minor pain, fatigue, nausea/vomiting, and fever, while major complications were rare, occurring in less than 1 event per patient. Liver-related complications varied widely, including increases in liver enzyme levels, the development of hepatic abscesses, and cases of liver failure. Overall, grade 3 or 4 toxicities were low following TARE.

Pellegrinelli *et al*[27] reported a low incidence of complications and side effects in their study of TARE. Out of 70 patients, 12 developed grade 1 side effects such as nausea, abdominal pain, fever, and vascular-like pseudoaneurysm. Only two patients encountered severe (grade 3) side effects: one suffered from radiation-induced cholecystitis due to unintended accumulation of ^{90}Y microsphere, while the other developed angiocholitis. No hepatic dysfunction or deaths related to TARE were observed[27]. Similarly, other studies, including those by Robinson *et al*[14] and Gangi *et al*[15] reported no mortality associated with TARE. Biochemical toxicities were generally mild to moderate, with 3%-10% of patients experiencing grade 3 biochemical toxicities. Constitutional toxicities were mostly grade 1 or 2, such as abdominal pain, fatigue, nausea, and vomiting, with rare occurrences of grade 3 or 4 constitutional toxicities. The toxicities observed in the study by Fleckenstein *et al*[29] were also mild to moderate, with few adverse events reported. When TARE was combined with systemic chemotherapy in a study by Fleckenstein *et al*[29], the toxic effects observed were primarily due to chemotherapy, with prevalent hematologic toxic effects of grade 3 or higher. The authors suggested a possible association between TARE and chemotherapy in the development of these hematologic toxic effects. However, in patients

Table 2 Transarterial radioembolization induced toxicities

Paper	Mild to moderate (grade 1-2)		Severe (grade 3-4)	
	Biochemical (%)	Constitutional (%)	Biochemical (%)	Constitutional (%)
Ibrahim *et al*[26], 2008	-	Fatigue (75); Abdominal pain (38); vomiting (13); anorexia (8)	Albumin (17); Bilirubin (4)	Gastroduodenal Ulcer (4); Ascites (14); Pleural effusion (9)
Gangi *et al*[15], 2018	(53)	Fatigue (42.3); Abdominal Pain (18.8); Weight loss (7.1); Ascites (5.9)	Bilirubin and Alkaline phosphatase elevation (9)	Liver abscess (2)
Pellegrinelli *et al*[27], 2021	-	-	-	Cholecystitis and angiocholitis (2.85)
Robinson *et al*[14], 2022	-	-	Bilirubin (10.5), Albumin (2.6), AST increase (7.8), ALT increase (5.2)	Abdominal pain, Cholecystitis (4.1)

AST: Aspartate transaminase; ALT: Alanine transaminase.

with cirrhosis, the number of hepatic toxicities was high. Liver toxic effects such as ascites, altered liver function tests, cholangitis, and acute cholecystitis were more common in cirrhotic patients. Among cirrhotic patients treated with TARE without chemotherapy, liver failure occurred in 75% of cases (9 out of 12 patients), including non-reversible cases, compared to 17% in patients without cirrhosis (all reversible cases). It was concluded that the combination of chemotherapy and TARE should be avoided in patients with cirrhosis, while liver toxicities in patients without cirrhosis were manageable, and no irreversible liver toxic effects were observed[28].

The researchers determined that combining chemotherapy with TARE is not advisable for patients who have cirrhosis. On the contrary, liver toxicities was passable in patients without cirrhosis, and no irreversible liver toxic effect was seen.

PROGNOSTIC FACTORS

Several studies have investigated both tumor-independent and tumor-dependent factors that may influence the prognosis of patients undergoing TARE for iCCA. The initial study by Ibrahim *et al*[26] showed that ECOG performance status had an impact on OS, with grade 0 having a significantly higher median OS compared to grade 1 or 2. On the other hand, tumor-dependent factors such as previous systemic chemotherapy, portal vein invasion, and infiltrative tumor morphology were associated with a poorer prognosis. These factors indicate a more advanced or aggressive disease, suggesting that TARE may be a viable treatment option for unresectable iCCA without portal vein involvement and/or infiltrative behavior.

Similarly, Gangi *et al*[15] found a correlation between ECOG performance status score and median OS. Patients treated with TARE who had ECOG performance status scores of 0 and 1 had a median OS of 18.5 mo, while those with a score of 2 had a median OS of 5.5 mo (P = 0.0012). Additionally, patients with symptoms or signs of liver failure, indicated by low serum albumin levels, low international normalized ratio, and elevated aspartate aminotransferase, had lower OS. Poorly differentiated tumor histology was also associated with lower OS, as previously described by Ibrahim *et al*[26]. The relationship between metastatic disease and prognosis remains more controversial[14,15].

In contrast, Edeline *et al*[28] did not find any correlation between ECOG performance status and median OS in their study. Similarly, Pellegrinelli *et al*[27] did not identify any tumor-independent factors that correlated with patients' median OS.

CONCLUSION

To summarize, the available literature suggests that TARE may have a role in the treatment of iCCA, either as a standalone treatment or in combination with systemic chemotherapy. However, further randomized trials and investigations of combination therapies are necessary to establish its efficacy and confirm its safety. TARE for iCCA is generally considered to have a favorable safety profile, with low rates of severe adverse events. However, caution should be exercised when combining TARE with systemic chemotherapy, particularly in patients with underlying cirrhosis, as they may be at a higher risk of liver toxic effects. Concomitant use of chemotherapy and TARE should be avoided in these patients. It is important to note that advancements in systemic therapies have resulted in improved prognosis for iCCA patients. Therefore, the role of TARE should be assessed within the context of these newer treatment options. Further randomized studies are needed to determine the optimal patient population, treatment regimen, and long-term outcomes associated with TARE in iCCA.

FOOTNOTES

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Study of liver cirrhosis over twenty consecutive years in adults in Southern China

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Abstract

BACKGROUND

Liver cirrhosis (LC) is a prevalent and severe disease in China. The burden of LC is changing with widespread vaccination of hepatitis B virus (HBV) and antiviral therapy. However, the recent transition in etiologies and clinical features of LC cases requiring hospitalization is unclear.

AIM

To identify the transition in etiologies and clinical characteristics of hospitalized LC patients in Southern China.

METHODS

In this retrospective, cross-sectional study we included LC inpatients admitted between January 2001 and December 2020. Medical data indicating etiological diagnosis and LC complications, and demographic, laboratory, and imaging data were collected from our hospital-based dataset. The etiologies of LC were mainly determined according to the discharge diagnosis, and upper gastrointestinal bleeding, ascites, hepatic encephalopathy, spontaneous bacterial peritonitis, hepatocellular carcinoma (HCC), portal vein thrombosis, hepatorenal syndrome, and acute-on-chronic liver failure (ACLF) were considered LC-related complications in our study. Changing trends in the etiologies and clinical characteristics were investigated using logistic regression, and temporal trends in proportions of separated years were investigated using the Cochran-Armitage test. In-hospital prognosis and risk factors associated with in-hospital mortality were also invest-

igated.

RESULTS

A total of 33143 patients were included in the study [mean (SD) age, 51.7 (11.9) years], and 82.2% were males. The mean age of the study population increased from 51.0 years in 2001-2010 to 52.0 years in 2011-2020 ($P < 0.001$), and the proportion of female patients increased from 16.7% in 2001-2010 to 18.2% in 2011-2020 ($P = 0.003$). LC patients in the decompensated stage at diagnosis decreased from 68.1% in 2001-2010 to 64.6% in 2011-2020 ($P < 0.001$), and the median score of model for end-stage liver disease also decreased from 14.0 to 11.0 ($P < 0.001$). HBV remained the major etiology of LC (75.0%) and the dominant cause of viral hepatitis-LC (94.5%) during the study period. However, the proportion of HBV-LC decreased from 82.4% in 2001-2005 to 74.2% in 2016-2020, and the proportion of viral hepatitis-LC decreased from 85.2% in 2001-2005 to 78.1% in 2016-2020 (both P for trend < 0.001). Meanwhile, the proportions of LC caused by alcoholic liver disease, autoimmune hepatitis and mixed etiology increased by 2.5%, 0.8% and 4.5%, respectively (all P for trend < 0.001). In-hospital mortality was stable at 1.0% in 2011-2020, whereas HCC and ACLF manifested the highest increases in prevalence among all LC complications (35.8% to 41.0% and 5.7% to 12.4%, respectively) and were associated with 6-fold and 4-fold increased risks of mortality (odds ratios: 6.03 and 4.22, respectively).

CONCLUSION

LC inpatients have experienced changes in age distribution and etiologies of cirrhosis over the last 20 years in Southern China. HCC and ACLF are associated with the highest risk of in-hospital mortality among LC complications.

Key Words: Liver cirrhosis; Epidemiology; Etiology; Upper gastrointestinal bleeding; Hepatocellular carcinoma; In-hospital mortality

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Core Tip: The etiologies and clinical characteristics of liver cirrhosis (LC) in hospitalized patients changed during the 2001-2020 period in Southern China. The mean age and female proportion have increased but the disease severity at LC diagnosis has alleviated over time. The proportion of hepatitis B virus-LC has decreased, while proportions of LC caused by alcoholic liver disease, autoimmune hepatitis and mixed etiology have increased gradually. Among all complications of LC, hepatocellular carcinoma and acute-on-chronic liver failure manifest the highest increase in prevalence and are associated with the highest risk of in-hospital mortality.

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INTRODUCTION

Liver cirrhosis (LC) is the end stage of progressive liver fibrosis and causes more than 1.3 million deaths worldwide annually, whereas the underlying etiologies, including hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, and alcohol intake, negatively impact 1.5 billion people[1-3]. China accounts for a large proportion of the regional burden of LC, with an estimated 7 million cirrhotic patients, and the annual incident cases are approximately 220000[4,5].

Because of widespread HBV vaccination and the development of potent antiviral treatment for HBV and HCV, the LC mortality rate has greatly decreased from 20.0 per 100000 person-years in the 1980s to 15.8 per 100000 person-years in the 2010s globally[2]. However, there have been 10%-20% increases in LC incidence from 2000 to 2015 in North America, Europe, and East Asia[4,5]. Meanwhile, hospital-based studies from North America and Japan found that the proportion of viral hepatitis LC decreased, whereas non-viral etiologies, including LC caused by alcoholic liver disease (ALD), metabolic dysfunction-associated steatotic liver disease (MASLD) and autoimmune hepatitis (AIH), increased over the years[6,7]. Previous studies in China also reported similar changing trends[8,9], but the recent transition in etiologies of LC patients is unclear. Continuous work investigating the trend of newly diagnosed LC is urgent and critical to facilitate clinical practice and evidence-based policy-making. Furthermore, most previous studies defined 'cirrhosis' as LC and chronic liver diseases together, whereas data addressing clinically diagnosed LC cases, especially cases requiring hospitalization with detailed clinical features, were scant[1,2]. In this context, we conducted this study to investigate changing trends in etiologies, clinical features and in-hospital prognosis of LC inpatients. Moreover, we compared the recent data to our previous study to explore the etiological evolution of LC over 20 years[8].

MATERIALS AND METHODS

Study design

We performed this retrospective cross-sectional study based on data extracted from the electronic dataset of medical records of our hospital, which is a university-affiliated, tertiary medical center and specialized liver center in Southern China. The diagnosis of LC was based on the discharging diagnosis code established according to the International Classification of Diseases, tenth revision. Hospitalization records with a diagnostic code of cirrhosis (K70, K71, K74) or discharge diagnosis of LC admitted from Jan 2011 to Dec 2020 were extracted. In clinical practice, LC diagnosis is established according to clinical manifestations of impaired liver function and/or portal hypertension in combination with typical biochemical and radiological findings or liver biopsies[7,10]. Patients were included if they were aged 18 years or older at the time of diagnosis. To ensure that these were incident cases, we only collected the first admission record during the study period, and patients with a diagnosis of cirrhosis within the prior 5 years before the first admission were excluded. Moreover, we excluded patients who had no definite etiologies at diagnosis and did not undergo necessary laboratory tests to confirm etiologies. The patient selection flowchart is shown in Figure 1. This study was approved by the institutional review board of our hospital and conducted in accordance with the Declaration of Helsinki as revised in 2013. Informed consent was waived because we used deidentified retrospective data. This article follows the Strengthening the Reporting of Observational Studies in Epidemiology reporting guidelines[11].

Classification of etiologies and LC-related complications

The etiologies of LC were mainly determined according to the discharge diagnosis in the medical record. If the etiology was not confirmed in the discharge diagnosis, further screening of the collected data was performed to confirm the etiology according to the criteria elaborated in our previous work[8]. Generally, viral hepatitis was diagnosed according to universally accepted serological criteria, including positivity of HBs antigen for HBV infection and positivity of HCV antibodies or HCV RNA for HCV infection. The diagnosis of ALD was based on established criteria[8,12]. Considering other non-viral etiologies, several classifications require attention as follows: (1) LC caused by primary biliary cholangitis, primary sclerosing cholangitis or biliary cirrhosis secondary to biliary tract obstruction was classified as cholestatic LC; (2) LC caused by Wilson's disease, hemochromatosis, MASLD, glycogen storage disease, or amyloidosis was classified as genetic or metabolic LC; and (3) LC caused by more than one etiology was classified as LC of mixed etiology, except for HBV-HCV overlap LC, which was classified as HBV + HCV LC[7,8].

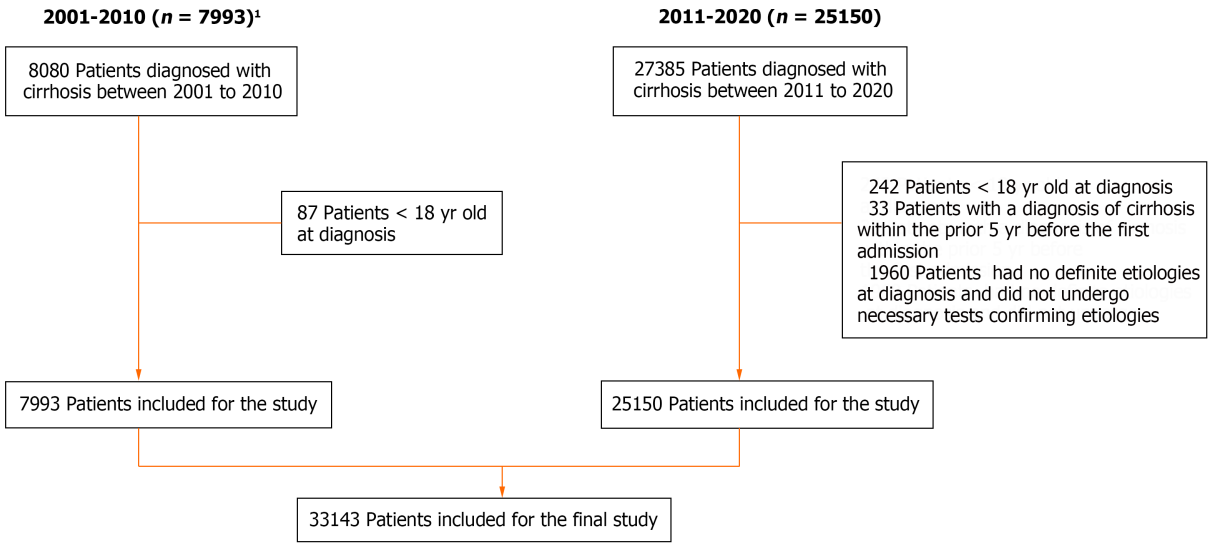
Upper gastrointestinal bleeding (UGIB), variceal bleeding, ascites, hepatic encephalopathy (HE), spontaneous bacterial peritonitis (SBP), hepatocellular carcinoma (HCC), portal vein thrombosis (PVT), hepatorenal syndrome (HRS), and acute-on-chronic liver failure (ACLF) were considered LC-related complications in our study. UGIB and variceal bleeding were diagnosed according to discharge diagnosis, or symptoms of hematemesis or melena, in combination with esophageal varices/gastric varices in the diagnosis or procedure code for band ligation, injective sclerotherapy or tissue glue injection[10]. Ascites was diagnosed according to discharge diagnosis, procedure code for paracentesis, or corresponding imaging findings. HE, HCC and PVT were diagnosed according to discharge diagnosis or corresponding imaging findings. ACLF was diagnosed according to discharge diagnosis, or acute aggravation of bilirubinemia and coagulation dysfunction on the basis of chronic liver disease per Chinese guideline[13]. SBP and HRS were diagnosed based on discharge diagnosis[6]. Liver decompensation was defined as the presence of any one of ascites, variceal bleeding, HE, or jaundice[14].

Data collection

Demographic, clinical and laboratory data at the index admission were extracted along with imaging reports of abdominal ultrasound, computerized tomography or magnetic resonance. Moreover, variables associated with etiologies and complications were also collected. Outcome variables, including deaths and liver transplantation (LT) during hospitalization and intensive care unit (ICU) admission, were only collected during the 2011-2020 period. Data from adult patients in our previous study[8] were collected to delineate the evolution of etiologies, clinical features, and complications of LC from 2001 to 2020 (Figure 1).

Statistical analysis

Categorical variables were described using counts and percentages, and comparisons were performed with the chi-square test or Fisher's exact test when appropriate. Quantitative variables were described using means ± SD or medians and interquartile range (IQR), and Student's *t* test or Mann-Whitney U test were performed as applicable. The trend of etiological proportion every 5 years was investigated using ordered logistic regression, and temporal trends in proportions of separated years were assessed using the Cochran-Armitage test. Moving average analysis and univariable linear regressions were performed to investigate trends in the mean ages of the study population, and the coefficient of determination (R square) was used to measure the strength of the correlation between mean ages and diagnosis years. Logistic regressions were used to explore risk factors for in-hospital mortality. Two-tailed *P* values of < 0.05 were considered statistically significant. All data were analyzed using R statistics version 4.2.0 (R Core Team, Vienna, Austria).



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Figure 1 Flowchart of the study population selection. A total of 33143 patients were finally included in the study out of 35465 patients diagnosed with liver cirrhosis from 2001 to 2020. [1]Detailed patient selection was elaborated in our previous study[8].

RESULTS

Overall characteristics of the study population

A total of 33143 patients were included in the study from Jan 2001 to Dec 2020 (7993 patients in 2001-2010 and 25150 patients in 2011-2020). Baseline characteristics are shown in Table 1. The mean age at diagnosis was 51.7 years, and 82.2% of patients were men. The median (IQR) model for end-stage liver disease (MELD) score was 12 (8.0, 18.0), and 31.7% of patients had MELD scores of 15 or above. As many as 65.5% of patients were in the decompensated stage, and the most prevalent complications were ascites (50.3%), HCC (36.7%) and UGIB (14.3%). During the 2011-2020 period, 6.8% of patients were referred from the emergency room, and 54.0% of patients were reimbursed by medical insurance for hospitalization (Supplementary Table 1).

Transition in baseline characteristics

The mean age of LC inpatients increased from 51.0 years in 2001-2010 to 52.0 years in 2011-2020 ($P < 0.001$) (Table 1). Three-year moving average analysis showed that the increasing trend was continuous, and the value increased linearly from 50.6 years in 2001-2003 to 52.6 years in 2018-2020 ($R^2 = 0.764$, Figure 2). The proportion of female patients increased from 16.7% in 2001-2010 to 18.2% in 2011-2020 ($P = 0.003$). During the 2001-2020 period, the proportion of patients in the decompensated stage decreased from 68.1% to 64.6% ($P < 0.001$), and the median MELD score also decreased from 14.0 to 11.0 ($P < 0.001$) (Table 1). Meanwhile, HCC and UGIB increased by nearly 10% and over 2%, respectively (both $P < 0.001$). During the 2011-2020 period, the proportion of patients with ACLF and patients referred from the emergency department increased dramatically from 5.7% to 12.4% and 5.1% to 8.1%, respectively (both $P < 0.001$) (Supplementary Table 1).

Etiology distribution and transition in etiology percentages over 20 years

The top three causes of LC were viral hepatitis (79.3%), ALD (7.1%) and mixed etiology (7.6%) in the entire population, and this pattern of constitution did not change over the 20 years (Table 2). HBV cirrhosis accounted for 75.0% of the entire population, and 94.5% of the patients had viral hepatitis. HCV and HBV + HCV etiologies accounted for 3.6% and 0.8%, respectively. The etiology distribution is presented in Table 2.

To determine the transition in etiology over the 20-year study period, we divided the study population into four groups, which were defined as 2001 to 2005, 2006 to 2010, 2011 to 2015 and 2016 to 2020. As shown in Table 2, proportions of the top three etiologies changed dramatically over the years. Viral hepatitis decreased from 85.2% to 78.1%, and ALD and mixed etiology increased by 2.5% and 4.5%, respectively (all P for trend < 0.001). Meanwhile, AIH and cholestatic etiology steadily increased from 0.4% to 1.2% (P for trend < 0.001) and 1.0% to 1.8% (P for trend = 0.018), respectively. As the major cause of viral hepatitis LC, HBV cirrhosis also decreased from 82.4% to 74.2% during the period (P for trend < 0.001). When comparing the proportions of separated years, the Cochran-Armitage test for P trend (CAP) found no statistical significance (CAP values > 0.05) (Table 2). Temporal trends of the major etiologies are illustrated in Figure 3. When comparing the proportion of different age groups (≤ 40 years as the young-age group, 41-65 years as the middle-age group, and > 65 years as the old-age group) in different etiologies, the proportion of young-aged patients gradually decreased from 21.2% in 2001 to 15.7% in 2020, whereas the proportion of middle-aged patients and old-aged patients increased from 65.6% to 69.6% and 13.3% to 14.7%, respectively (Figure 4A). Similar patterns of trends were also observed when restricting the population to HBV LC (Figure 4B). CAP found no statistical significance when comparing the proportions of separated years (CAP values > 0.05).

Table 1 Transition in characteristics of the study population over 20 years

Characteristic	Total population, *n* = 33143	2001-2010, *n* = 7993	2011-2020, *n* = 25150	*P* value
Age (yr, mean ± SD)	51.7 (11.9)	51.0 (12.3)	52.0 (11.8)	< 0.001
Gender (male, %)	27237 (82.2)	6658 (83.3)	20579 (81.8)	0.003
Laboratory test (mean ± SD)				
Albumin (g/L)	34.1 (6.3)	33.4 (6.4)	34.2 (6.2)	< 0.001
Bilirubin (umol/L)	87.7 (139.4)	98.3 (148.9)	85.4 (137.1)	< 0.001
Prothrombin INR	1.45 (0.65)	1.53 (0.65)	1.44 (0.65)	< 0.001
MELD score (Median, IQR)	12.0 (8.0,18.0)	14.0 (10.0, 19.0)	11.0 (8.0,18.0)	< 0.001
Decompensation[1] (*n*,%)	21701 (65.5)	5443 (68.1)	16258 (64.6)	< 0.001
Complications				
UGIB	4729 (14.3)	998 (12.5)	3731 (14.8)	< 0.001
Ascites	16656 (50.3)	4444 (55.6)	12212 (48.6)	< 0.001
HE	1901 (5.7)	535 (6.7)	1366 (5.4)	0.007
HCC	12159 (36.7)	2392 (29.9)	9767 (38.8)	< 0.001
PVT	2449 (7.4)	403 (5.0)	2046 (8.1)	< 0.001
HRS	713 (2.2)	291 (3.6)	422 (1.7)	< 0.001

[1]Presence of any one of ascites, variceal bleeding, hepatic encephalopathy, or jaundice (total bilirubin > 51.3 umol/L). INR: International normalized ratio; MELD: Model for end-stage liver disease; IQR: Interquartile range; UGIB: Upper gastrointestinal bleeding; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma; PVT: Portal vein thrombosis; HRS: Hepatic renal syndrome.

Table 2 Transition in etiological proportions over 20 years, *n* (%)

Etiology	2001-2005, *n* = 2870	2006-2010, *n* = 5123	2011-2015, *n* = 10538	2016-2020, *n* = 14612	*P* value[1]	*P* value[2]
Viral hepatitis	2444 (85.2)	4046 (79.0)	8385 (79.6)	11418 (78.1)	< 0.001	0.780
HBV	2366 (82.4)	3852 (75.2)	7788 (73.9)	10835 (74.2)	< 0.001	0.747
HCV	66 (2.3)	159 (3.1)	484 (4.6)	474 (3.2)	0.951	0.912
HBV + HCV	12 (0.4)	35 (0.7)	113 (1.1)	109 (0.7)	0.685	0.932
Alcohol	155 (5.4)	304 (5.9)	753 (7.1)	1155 (7.9)	< 0.001	0.869
Autoimmune hepatitis	12 (0.4)	41 (0.8)	87 (0.8)	170 (1.2)	< 0.001	0.875
Cholestatic	29 (1.0)	92 (1.8)	154 (1.5)	266 (1.8)	0.005	0.925
Genetic or Metabolic	21 (0.7)	62 (1.2)	105 (1.0)	191 (1.3)	0.019	0.923
Mixed etiology	102 (3.6)	374 (7.3)	864 (8.2)	1188 (8.1)	< 0.001	0.771
Cryptogenic	74 (2.6)	153 (3.0)	70 (0.7)	36 (0.2)	< 0.001	0.746

[1]Determined using ordered logistic regression model by comparing proportions of the separated four 5-yr groups.
[2]Determined using Cochran-Armitage test by comparing proportions of separated years from 2001 to 2020. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

In-hospital prognosis and risk factors for in-hospital mortality over the 2011-2020 period

A total of 264 (1.0%) patients died during the index hospitalization. Over 80% of deaths were caused by liver-related diseases, including 107 cases (40.5%) of liver failure, 63 cases (23.9%) of HCC, and 27 cases (10.2%) of UGIB. The in-hospital mortality was stable at approximately 1% during the 2011-2020 period (P = 0.372). Meanwhile, 242 patients (1.0%) received LT, and the rate of LT increased by 3.0% during the last ten years (P < 0.001). A total of 1010 patients (4.0%) were admitted to the ICU, and the ICU admission rate increased from 2.7% to 5.0% during the last ten years (P < 0.001) (Supplementary Table 2). In multivariable regression, as shown in Table 3, transferring from the emergency department resulted in a nearly 3-fold increased risk of in-hospital mortality (adjusted OR: 2.97, 95%CI: 2.01-4.38). MELD scores of 15 or above and older age also increased the risk of mortality (OR: 3.55, 95%CI: 2.24-5.64, and OR: 1.90, 95%CI:

Table 3 Risk factors of in-hospital mortality from 2011 to 2020

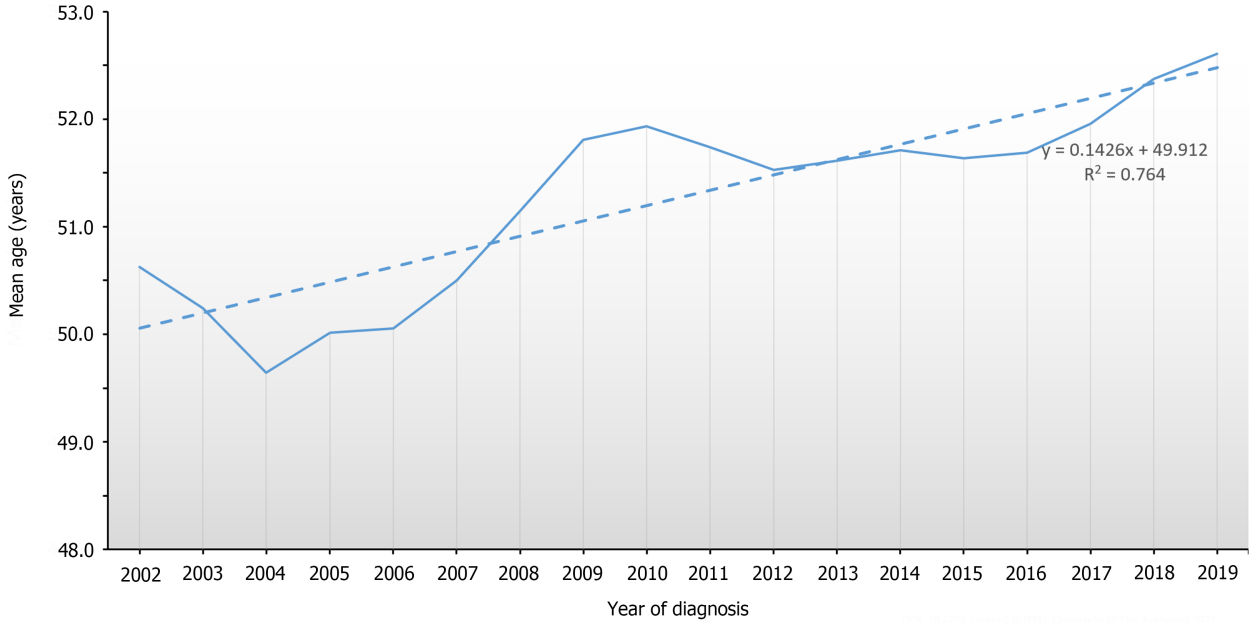
Characteristics, *n* = 25150	Unadjusted		Adjusted[1]	
	OR	*P* value	OR (95%CI)	*P* value
Age[2]	1.49	0.011	1.90 (1.28-2.80)	0.001
Gender	1.25	0.230	0.93 (0.58-1.49)	0.761
Etiology				
Viral hepatitis	1.10	0.583	--	--
Alcohol	0.89	0.721	--	--
Other etiologies	Reference	--	--	--
Referring site				
Emergency	6.11 (4.67-7.99)	< 0.001	2.97 (2.01-4.38)	< 0.001
Clinic	Reference	--	Reference	--
Insurance status[3]	1.09 (0.84-1.41)	0.558		
MELD[4]	6.85 (5.15-9.09)	< 0.001	3.55 (2.24-5.64)	< 0.001
Liver transplantation	1.22 (0.57-2.63)	0.764	--	--
Residence				
Rural area	3.40 (0.84-13.73)	0.070	3.33 (0.81-13.73)	0.096
Urban area	Reference	--	Reference	--
Complications				
Ascites	2.70 (2.06-3.53)	< 0.001	1.74 (1.18-2.56)	0.005
UGIB	2.13 (1.62-2.81)	< 0.001	2.35 (1.59-3.46)	< 0.001
HE	10.63 (8.22-13.74)	< 0.001	3.27 (2.20-4.84)	< 0.001
HCC	1.42 (1.15-1.81)	0.005	6.03 (4.07-8.94)	< 0.001
SBP	4.06 (3.15-5.23)	< 0.001	1.34 (0.92-1.96)	0.123
ACLF	8.60 (6.73-11.00)	< 0.001	4.22 (2.70-6.59)	< 0.001

[1]Adjusted by all variables with *P* value < 0.10 and age, gender in univariate logistic analysis. Multivariate logistic analysis is performed.
[2]≥ 65 years *vs* < 65 yr.
[3]Medical insurance vs self-paying.
[4]Model for end-stage liver disease (MELD) score ≥ 15 points *vs* MELD score < 15 points. OR: Odds ratio; MELD: Model for end-stage liver disease; UGIB: Upper gastrointestinal bleeding; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma; SBP: Spontaneous bacterial peritonitis; ACLF: Acute-on-chronic liver failure.

1.28-2.80, respectively). LC complications associated with increased mortality were ascites (OR: 1.74, 95%CI: 1.18-2.56), UGIB (OR: 2.35, 95%CI: 1.59-3.46), HCC (OR: 6.03, 95%CI: 4.07-8.94), ACLF (OR: 4.22, 95%CI: 2.70-6.59), and HE (OR: 3.27, 95%CI: 2.20-4.84). Meanwhile, male gender, LC etiology, medical insurance, LT, and residential areas of patients were not associated with in-hospital mortality (Table 3).

DISCUSSION

This large sample cross-sectional study showed the transition in etiologies and characteristics of newly diagnosed LC in our medical center during the past two decades. We found that HBV remained the most prevalent cause of LC throughout the study period, but the percentage decreased gradually, especially in people younger than 40 years old. Meanwhile, LC cases caused by ALD and AIH are increasing over time. Regarding clinical characteristics, LC patients are aging and less likely to be decompensated at diagnosis. Moreover, we found that the in-hospital mortality rate was relatively low, and emergency referral, a higher MELD score and LC complications were associated with in-hospital mortality. Since a considerable proportion (52%) of patients are from regions of Southern China other than Guangdong Province, our single-center-based findings may suggest the changing trend of LC etiologies and clinical features in entire Southern China[8].



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Figure 2 Trend in the mean age of the study population in 2001-2020. The three-year moving averages in ages were calculated and shown. The mean age of cirrhotic patients increased continuously over the 20-year study period, with a high coefficient of determination in linear regression (R^2 = 0.764). The three-year moving averages were plotted on the central year, as the year of diagnosis in the horizontal axis (*e.g.* 2002 represented 2001-2003, and 2019 represented 2018-2020).

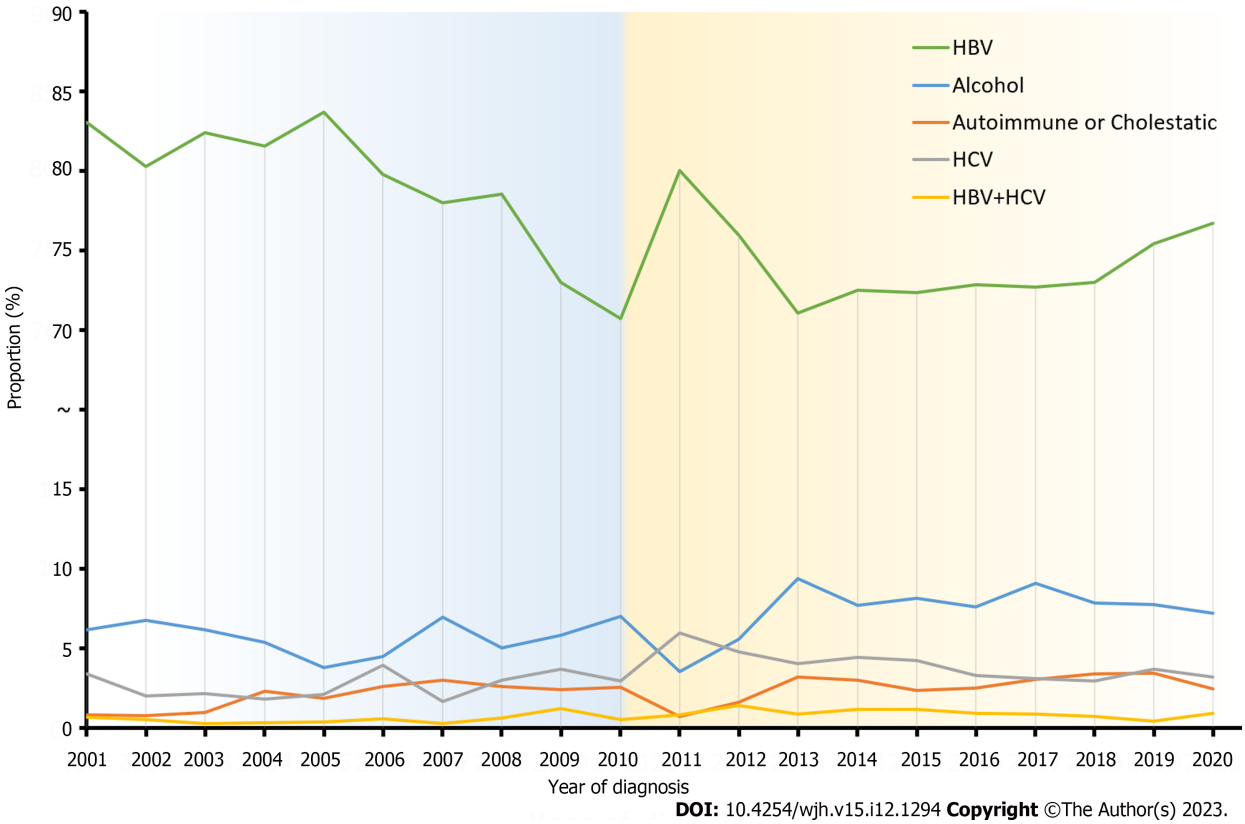
Etiologies

The viral hepatitis-predominant pattern in etiology in our study is consistent with a global report (42% HBV infection and 21% HCV infection in LC) and some recent studies from other East Asian regions[15,16]. Meanwhile, the > 70% HBV contribution indicated that China is still a highly endemic area of HBV infection. However, the continuous decreases in the proportion of patients with HBV cirrhosis measured every 5-year period in our study reflected an overall descending trend of HBV contribution to LC, which was in line with recent studies from other regions of China[9,17]. The descending proportion should be attributed to the widespread HBV vaccination and the subsequent declining prevalence of HBV in China[4]. Contrast to our results, there was a slight increase of 5.5% in HBV-related LC incidence in China (7.7/100000 in 2000 *vs*. 8.1/100000 in 2015) according to Global Burden of Disease 2015 study data[5]. However, the same study reported a 38% and 8.8% increase in the incidence of alcoholic-related LC and non-viral non-alcoholic LC, respectively, which would actually result in a relative decrease in the proportion of HBV-related LC when added up. Furthermore, HBV-related LC data in the above study included cases of HBV overlapping with other etiologies, whereas our study defined HBV-LC as mono HBV infection. Considering the difference in etiology definition, our study is supposed to be more accurate to reflect the trend of HBV LC. Given the low prevalence of HBV carriers of only 1.0% among children born after 1999 in China, newly diagnosed cases of HBV LC are expected to decline continuously in the future[18].

Our data showed a continuous increase in the alcoholic LC proportion during the last two decades, while the percentage increased from 5.7% in the 2000s to 7.6% in the 2010s. Alcohol-use disorder is one of the main causes of liver disease-related mortality and accounts for approximately 50% of LC cases worldwide[19]. In China, ALD has become the second leading cause of end-stage liver disease and possesses the highest percentage change in age-standardized incidence among all LC etiologies[5,20]. Significant increases in admissions and the proportion of alcoholic cirrhosis have recently been reported in LC inpatients, and our data are consistent with the data from Northern China[9,17]. Regarding the increasing trend in alcohol consumption over the last three decades in China[21], alcoholic LC cases are expected to continue rising in the future. Our data support the allocation of more resources to reduce the burden of alcohol use disorder.

Clinical characteristics

It is interesting to note that our data showed an increasing trend in the age of LC patients; in addition, a slight but significant increase in the female proportion was also observed (1.5% increase, P = 0.003). Our findings are consistent with a recent nationwide survey from Japan showing a mean age nearly 2 years older when comparing patients from 2015-2017 to patients from 2008-2010 (68.2 years *vs*. 66.4 years)[7]. Moreover, a hospital-based study from Southwest China reported a nonsignificant increase in the mean age of LC patients from 52.3 years in 2003 to 52.7 years in 2013[22]. Plausible explanations are as follows: first, decreased HBV incidence in young adults and improved survival of LC patients, which resulted in an increased proportion of older patients and more patients admitted at an older age[6]; second, increased incidence of non-viral LC cases, especially alcoholic and autoimmune liver diseases, who were older at diagnosis compared to HBV LC (Supplementary Table 3). Population-based studies in North America have shown decreasing mortality rates of LC, whether for in-hospital mortality or for 1-year mortality, and the benefits may largely be



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Figure 3 Temporal trends of viral hepatitis and other major etiologies in 2001-2020. The proportion of hepatitis B virus-liver cirrhosis (HBV-LC) decreased dramatically during the 2001-2010 period but fluctuated and decreased slightly during the 2011-2020 period. Meanwhile, proportions of alcoholic-LC and autoimmune or cholestatic-LC have increased during the 20-year period. Hepatitis C virus (HCV)-LC and (HBV + HCV)-LC have not shown explicit trends. The Cochran-Armitage test for *P* trend found no statistical significance ($P > 0.05$) when comparing proportions in separated years ($P > 0.05$). HBV: Hepatitis B virus; HCV: Hepatitis C virus.

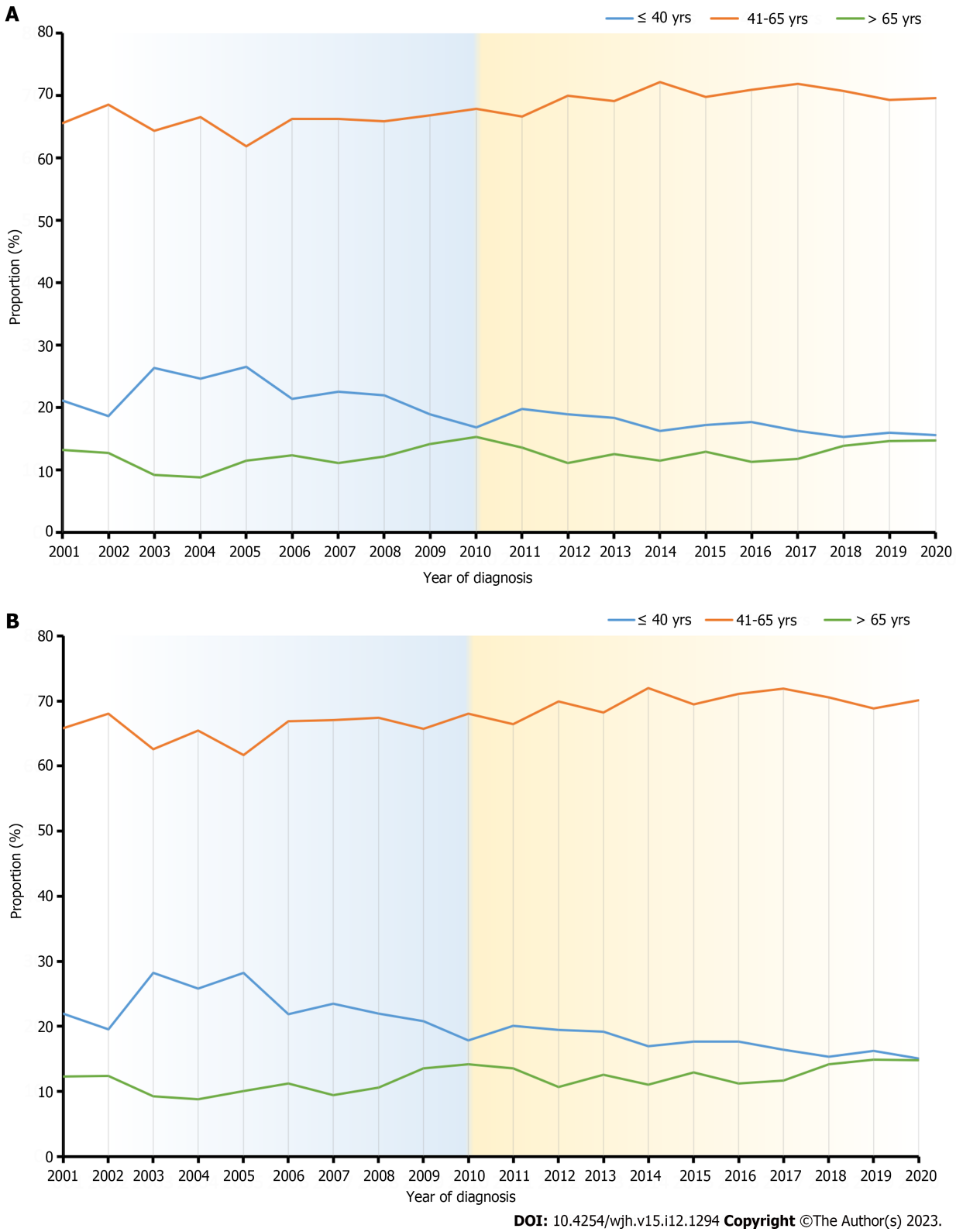
due to improved care[23]. For hospitalized patients with cirrhosis, timely endoscopic treatment and paracentesis and available LT and intensive care have been linked to improved survival[10,24,25]. On the other hand, the increased female proportion can be explained by the decreased proportion of viral hepatitis LC, which has more male patients, and the increased proportion of non-viral LC, especially for AIH LC, which affects more female patients. Moreover, improved health resources to women may also contribute to the increasing female proportion (Supplementary Table 1).

Another notable finding is that the decreasing trend in the HBV proportion is most prominent in patients ≤ 40 years, while the proportions of both young LC patients and young HBV-LC patients are decreasing significantly (Figure 4). Our data are likely to be the outcome of a low prevalence of HBV infection in young adults in China and are also in line with observations from North America[6]. Importantly, the transition has implications for the future because viral hepatitis, especially CHB, is highly associated with liver decompensation and liver cancer[26,27]. Reducing new diagnoses in young people may greatly reduce the burden of end-stage liver disease and liver cancer in the future.

In-hospital mortality and risk factors

Apart from illustrating the transition of etiologies and clinical features of LC, we also investigated in-hospital outcomes and associated risk factors. The overall in-hospital mortality was 1.0% in our study, which is similar to that in nationwide and regional studies in China[25,28]. In contrast to a decline of 17.6% in the mortality rate of cirrhosis and chronic liver disease in China from 1990 to 2016, we did not observe a descending trend in in-hospital mortality in our study[20]. However, considering the dramatic increase in liver decompensation, LC complications, and ICU admissions in our study population, which indicates an increase in disease severity over time, the observed stable in-hospital mortality is reasonable. In-hospital mortality is considered a hard endpoint of inpatient prognosis and varies across countries. In the 2010s, the reported mortality rates were 5.4% to 7.6% in the United States, 9.1% in Korea, and 11.6% in Spain[23,29-31]. The discrepancy between data from other hospitals and ours may be due to differences in patient selection and criteria for patient admission. Moreover, well-equipped service of intensive care and increased LTs may also contribute to improving patient prognosis[10,23]. Our results are of clinical significance to report satisfactory inpatient care in Southern China.

Reported risk factors for in-hospital mortality included older age, HE, HCC, gastrointestinal bleeding, and sepsis from previous studies[29,31], and our data supported the observation by finding that all major LC complications are associated with poor outcomes. In addition, we emphasized that HCC and ACLF were associated with the highest risk of in-hospital mortality (OR 6.0 and 4.6, respectively) and increased faster than other complications (Supplementary Table 1). Our data are consistent with the increasing trend of HCC and ACLF in China[1,32]. With the rising burden of HCC and ACLF, more attention should focus on early diagnosis and timely treatment of the two severe complications[33,34].



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Figure 4 Temporal trends in different age groups of the study population. A: In the whole population, the proportion of patients ≤ 40 years was decreasing (21.2% in 2001 to 15.7% in 2020), and those aged 41-65 years and > 65 years were increasing (65.6% in 2001 to 69.6% in 2020, and 13.3% in 2001 to 14.7% in 2020, respectively) during the 20-year study period; B: In patients with hepatitis B virus-liver cirrhosis, the proportion ≤ 40 years decreased (22.0% in 2001 to 15.1% in 2020), and those aged 41-65 years and > 65 years increased (65.8% in 2001 to 70.1% in 2020, and 12.3% in 2001 to 14.8% in 2020, respectively) during the 20-year study period.

Strengths and limitations

In this study, we revealed the evolution of etiologies and clinical characteristics of hospitalized LC patients over the last 20 years in a large-sample dataset. Meanwhile, we filled the knowledge gap to reveal in-hospital mortality and associated risk factors, which were scarcely reported in China previously. However, there are several limitations to this study. First, as a retrospective study, although we searched cases using both diagnosis codes and literal discharge diagnosis, there are still possibilities of missing cases of LC due to the lack of LC-indicative diagnosis in the original database. Moreover, the retrospective design in HBV endemic region may also result in the underestimation of MASLD, and therefore this study cannot reveal the trend of MASLD-derived LC. Second, different from manual data extraction in the 2001-2010 dataset, data extraction in 2011-2020 is automatic from a single admission note, whereas there are no subsequent data to supplement the diagnosis when the primary etiology cannot be confirmed. The consequent case exclusion due to uncertain etiologies may explain the turbulence in temporal trends in the 2011-2020 period, especially the variance in the proportion of HBV LC between 2010 and 2011. Third, the cross-sectional study did not include follow-up data after patient discharge, so the actual mortality rate over time is unclear. Future studies focusing on trend of prognosis of LC are highly needed. Fourth, the epidemiological indications based on our single-center study are limited, and care should be taken when applying our findings to other countries or regions.

CONCLUSION

In conclusion, our study showed a decreased ratio of viral hepatitis LC, especially HBV LC, and an increased ratio of alcoholic and AIH LC in real-world inpatients over the past two decades. In-hospital mortality has been low over the last 10 years, and HCC and ACLF are the strongest risk factors for mortality. Further multicenter studies are needed to reveal the actual incidence of LC in China, and more attention should be given to the rising burden of HCC and ACLF in LC patients.

ARTICLE HIGHLIGHTS

Research background

Liver cirrhosis (LC) is the end stage of chronic liver disease and is associated with significant morbidity, mortality and healthcare utilization. China accounts for a large proportion of the regional burden of LC. Thanks to widespread hepatitis B virus (HBV) vaccination and the potent antiviral treatment for HBV and hepatitis C virus, the LC mortality has greatly decreased.

Research motivation

In the context of changing burden of LC, the recent transition in etiologies and clinical features of LC is unclear in China.

Research objectives

Our main objective was to identify the transition in etiologies and clinical characteristics of hospitalized LC patients in Southern China. Furthermore, in-hospital prognosis and associated risk factors were also investigated.

Research methods

We included LC inpatients admitted from 2001 to 2020 in this retrospective, cross-sectional study. The etiologies of LC were mainly determined according to the discharge diagnosis, and upper gastrointestinal bleeding, ascites, hepatic encephalopathy, spontaneous bacterial peritonitis, hepatocellular carcinoma (HCC), portal vein thrombosis, hepatorenal syndrome, and acute-on-chronic liver failure (ACLF) were considered LC-related complications. Changing trends in the etiologies and clinical characteristics were investigated using logistic regression, and temporal trends in proportions of separated years were investigated using the Cochran-Armitage test.

Research results

The study included a total of 33143 patients and the mean age increased from 51.0 years in 2001-2010 to 52.0 years in 2011-2020 ($P < 0.001$). In the meantime, proportion of decompensated LC and the score of model for end-stage liver disease decreased. During the study period, HBV remained the major etiology of LC (75.0%), but the proportion of HBV-LC decreased from 82.4% in 2001-2005 to 74.2% in 2016-2020 (P for trend < 0.001). Meanwhile, the proportions of LC caused by alcoholic liver disease and autoimmune hepatitis both increased slightly (both P for trend < 0.001). In-hospital mortality was low, and HCC and ACLF were associated with 6-fold and 4-fold increased risks of mortality.

Research conclusions

Our study showed a decreased ratio of HBV LC, and an increased ratio of alcoholic and autoimmune hepatitis LC in real-world inpatients over the past two decades. HCC and ACLF were identified as the strongest risk factors for in-hospital mortality.

Research perspectives

Our large sample cross-sectional study showed the transition in etiologies and characteristics of newly diagnosed LC in our medical center over the past twenty years and the findings may reflect the changing trend of LC in entire Southern China. Future multicenter studies are needed to reveal the changing incidence of LC in China, and more attention should be given to the rising burden of HCC and ACLF in LC patients.

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FOOTNOTES

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Author contributions: Wang X, Luo JN, Wu XY and Wu B planned and designed the study; Wang X, Luo JN, Wu XY and Zhang QX collected the data; Wang X and Luo JN performed the data analysis; Wang X and Luo JN drafted the manuscript; Wu B critically revised the manuscript; all authors have approved the submitted manuscript. Wang X and Luo JN contributed equally to this work as co-first authors. Wang X and Luo JN are designated as co-first authors due to their equal and substantial contributions to the study conception, design, data acquisition, and data analysis, as well as manuscript preparation and editing, each playing pivotal roles in ensuring the integrity and quality of the research.

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ORIGINAL ARTICLE

Retrospective Study

Peri-operative score for elderly patients with resectable hepatocellular carcinoma

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Abstract

BACKGROUND

Liver resection is the mainstay for a curative treatment for patients with resectable hepatocellular carcinoma (HCC), also in elderly population. Despite this, the evaluation of patient condition, liver function and extent of disease remains a demanding process with the aim to reduce postoperative morbidity and mortality.

AIM

To identify new perioperative risk factors that could be associated with higher 90- and 180-d mortality in elderly patients eligible for liver resection for HCC considering traditional perioperative risk scores and to develop a risk score.

METHODS

A multicentric, retrospective study was performed by reviewing the medical records of patients aged 70 years or older who electively underwent liver resection for HCC; several independent variables correlated with death from all causes at 90 and 180 d were studied. The coefficients of Cox regression proportional-hazards model for six-month mortality were rounded to the nearest integer to assign risk factors' weights and derive the scoring algorithm.

RESULTS

Multivariate analysis found variables (American Society of Anesthesiology score, high rate of comorbidities, Mayo end stage liver disease score and size of biggest lesion) that had independent correlations with increased 90- and 180-d mortality. A clinical risk score was developed with survival profiles.

CONCLUSION

This score can aid in stratifying this population in order to assess who can benefit from surgical treatment in terms of postoperative mortality.

Key Words: Hepatocellular carcinoma; Score; Laparoscopy; Surgical resection; Elderly patients; Multivariate analysis

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Core Tip: To support the decision-making process in elderly patient with resectable hepatocellular carcinoma (HCC) and understand who can benefit from surgical treatment in terms of postoperative mortality, we analyzed data from 11 hepato-biliary centers during a 10-years period. A multivariate analysis was performed to find variables (American Society of Anesthesiology score, high rate of comorbidities, Mayo end stage liver disease score and size of biggest lesion) that had independent correlations with increased 90- and 180-d mortality. The evaluation of elderly patients who underwent liver resection for HCC need to be supported by any form of possible analysis of risk.

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INTRODUCTION

The life expectancy of the population has increased in recent years, and this led to an increased rate of malignant disease in elderly population[1,2]. Hepatocellular carcinoma (HCC) became even more frequent in elderly population[3,4].

According to current guidelines liver resection, ablation and liver transplant are still the mainstay treatments for HCC.

Liver resection presented better overall and disease-free survival than other curative treatments[5]. Despite this, liver resection presented a significant risk postoperative morbidity and mortality.

The approach of liver disease in elderly population needed of an accurate stratification of patients at risk, with the involvement of multidisciplinary preoperative assessment.

The aim of our study was to analyze a population of elderly patients who underwent liver resection for HCC, to investigate the possible presence of risk predictors of postoperative mortality at 90 and 180 d.

MATERIALS AND METHODS

Study Design

A multicentric, retrospective cohort study was carried out by reviewing the medical records of patients aged > 70 years or over undergoing liver resection for HCC from January 2009 to January 2019. We evaluated all preoperative independent variables linked with patients (demographics data), with lesion (number and size, calculated on the preoperative imaging) and preoperative clinical assessment in eligible patients. The primary endpoint was to define 90 d and 180 d mortality rate. The second one was to explore the association among variables and post operative mortality rate.

Statistical Analysis

All analyses were conducted using STATA software, version 16 (Stata-Corp LP, College Station, Tex). Data are reported as means (standard deviations) for continuous variables or numbers (percentages) of patients for categorical variables. Six-month follow-up was chosen to analyze at least 20 fatal events after the surgery. Associations between baseline pre-operative variables with six-month mortality were evaluated using a univariate Cox proportional-hazards model. A score point system was derived from the multivariable Cox proportional-hazards model including univariate predictors with $P < 0.05$. For a dichotomous risk factor, the estimated regression coefficient was rounded to the nearest integer. For a non-dichotomous risk factor, continuous or discrete, the estimated regression coefficient was multiplied by observed values, rounded to the nearest integer and rescaled to assign zero points to the lowest risk-category. Hazard ratios (HRs) with their 95%CI were reported. The discriminative ability of the models was assessed using the Harrell's concordance index (C-index). Patients were stratified into three groups of risk by the estimated six-month mortality probability (low-risk < 5%, mid-risk 5%-10%, and high-risk > 10%). The cumulative mortality was displayed using Kaplan-Meier estimates with comparison between curves based on the Log-Rank statistic. The score was internally validated by resampling 1000 bootstrap replications. The bias was calculated as the difference between estimation and the mean of the bootstrap sample. Theoretical profiles were constructed by combining variables of the final model as well as a risk score for death in the period. The cut off of 6 mo as final follow up has been chosen to obtain an appropriate number of events, but its significance was validated at 3 mo. A P value < 0.05 was considered statistically significant.

RESULTS

A total of 429 patients, who underwent liver resection for HCC were included (Table 1). The majority of patients were male (n = 319, 74.3%, and 110 females, 25.7%), aged ≥ 70 years (mean of 75.3 ± 4.1 years); 20 deaths (4.7%) occurred up to 180 d after surgery, as shown in Table 1.

Two hundred fifty-seven patients, 60% presented an American Society of Anesthesiology (ASA) score III-IV, and the median range of Mayo end stage liver disease (MELD) score was 7 (7.4 ± 2.1). Roughly one third of patients was affected by more of 2 comorbidities (n = 142, 33.1%). Most patients presented a single, unilobar lesion (n = 421, 98%). Most of patients underwent to a minor hepatectomy, while only 54 patients (13.1%) underwent to a major hepatectomy, according to Brisbane classification.

The overall survival curve calculated by the Kaplan–Meier estimator is shown in Figure 1. The ASA score, MELD score, the presence of Comorbidities > 2 and the size of the biggest lesion presented in the univariate analysis an HR greater than 1, as shown in Table 1. They are used as predictor factors in the multivariate analysis (Table 2). Table 3 showed a score system which provides a balanced weight for each variable. Combining the four variables we obtained different profiles of patients with a different preoperative risk, based on personal score, groupable in a low-risk (< 5% at 6 mo), mid-risk (5%-10% at 6 mo) and high-risk class (> 10% at 6 mo) (Table 4 and Figure 2).

Figure 2 showed the curves of six-month mortality probability, according to the different profile created on various score. The rate of mortality probability significantly increased from patients with score 2 to patients with score 6: Patients with a score ≥ 2 presented a 5.7% of mortality, patients with a score ≥ 3 presented a 7%, patients with a score ≥ 4 showed a 9.3% of mortality, patients with a score ≥ 5 showed a 13.6%, patients with a score ≥ 6 presented 22.9% of mortality.

We performed an Internal validation using a bootstrapping technique with 1000 resamples, the derived score point system had good discrimination as 0.803 of the Harrell C-Index (bootstrap 95%CI 0.741-0.875). The bias of the estimated risk assigned to 1 point of the score, as the difference between coefficient estimation in the derivation model (0.875) and the mean of the bootstrap sample (0.888), it was negligible (-0.013).

Table 1 Characteristics of samples used to study the variables and deaths 180 d after surgery, *n* (%)

	All		Alive at 180 d		Death at 180 d		HR	*P* value
	N	*n* = 429	*N*	*n* = 409	*N*	*n* = 20		
Age, yr	429	75.3 ± 4.1	409	75.3 ± 4.1	20	76.9 ± 4.9	1.52 (0.94-2.47)	0.086
Male	429	319 (74.4)	409	306 (74.8)	20	13 (65.0)	0.61 (0.24-1.54)	0.296
BMI	429	26.9 ± 3.5	409	26.9 ± 3.6	20	26.9 ± 0.9	0.97 (0.52-1.82)	0.921
ASA score	429	2.60 ± 0.50	409	2.59 ± 0.50	20	2.90 ± 0.31	4.49 (1.47-13.74)	0.008
Comorbidity > 2	429	142 (33.1)	409	129 (31.5)	20	13 (65.0)	3.92 (1.56-9.82)	0.004
HBV	429	80 (18.6)	409	80 (19.6)	20	0 (0.0)	-	-
HCV	429	217 (50.6)	409	210 (51.3)	20	7 (35.0)	0.51 (0.2-1.28)	0.151
ALD	429	60 (14.0)	409	56 (13.7)	20	4 (20.0)	1.58 (0.53-4.72)	0.415
Others	429	72 (16.8)	409	63 (15.4)	20	9 (45.0)	4.3 (1.78-10.37)	0.001
F4 cirrhosis	429	178 (41.5)	409	173 (42.3)	20	5 (25.0)	0.46 (0.17-1.27)	0.134
CHILD A	429	370 (86.2)	409	353 (86.3)	20	17 (85.0)	0.92 (0.27-3.13)	0.891
MELD score	429	7.4 ± 2.1	409	7.4 ± 2.0	20	8.9 ± 2.7	1.25 (1.09-1.44)	0.001
Albumin	382	3.80 ± 0.60	367	3.80 ± 0.60	15	3.71 ± 0.77	0.75 (0.33-1.73)	0.504
Bilirubin	424	1.05 ± 0.64	404	1.05 ± 0.64	20	0.99 ± 0.54	0.81 (0.37-1.76)	0.587
Creatinin	425	1.03 ± 0.36	405	1.02 ± 0.36	20	1.17 ± 0.42	2.55 (0.9-7.2)	0.077
INR	422	1.20 ± 0.23	402	1.20 ± 0.23	20	1.17 ± 0.28	0.51 (0.07-3.8)	0.508
AST	424	68 ± 61	405	69 ± 61	19	48 ± 41	0.92 (0.85-1)	0.045
ALT	201	51 ± 80	182	52 ± 82	19	41 ± 48	0.98 (0.91-1.05)	0.540
GGT	192	145 ± 218	174	145 ± 218	18	146 ± 228	1 (0.98-1.02)	0.958
Platelets	425	191 ± 92	406	191 ± 92	19	177 ± 85	0.98 (0.93-1.04)	0.509
Number of lesions	429	1.09 ± 0.30	409	1.09 ± 0.31	20	1.05 ± 0.22	0.54 (0.08-3.77)	0.531
Size of biggest lesion (mm)	429	33 ± 10	409	32 ± 10	20	37 ± 13	1.26 (1.01-1.58)	0.043
Bilobar lesion	429	8 (1.9)	409	8 (2.0)	20	0 (0.0)	-	-
Preop treatment	429	53 (12.4)	409	50 (12.2)	20	3 (15.0)	1.24 (0.36-4.23)	0.732
Major HTC	429	56 (13.1)	409	55 (13.4)	20	1 (5.0)	0.34 (0.05-2.56)	0.297

BMI: Body mass index; ASA: American Society of Anesthesiology; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; Major HTC: Hepatectomy; ALD: Alcoholic liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MELD: Mayo end liver disease score; INR: International normalized ratio; HR: Hazard ratio.

Table 2 Multivariate analysis

	Beta	HR	*P* value
ASA score	1.189	3.28 (1.04-10.34)	0.042
Comorbidity 2	1.071	2.92 (1.14-7.45)	0.025
MELD	0.202	1.22 (1.06-1.41)	0.005
Size of largest lesion	0.046	1.05 (1.01-1.09)	0.034
C-index = 0.807	-	-	-

ASA: American Society of Anesthesiology; MELD: Mayo end liver disease; HR: Hazard ratio.

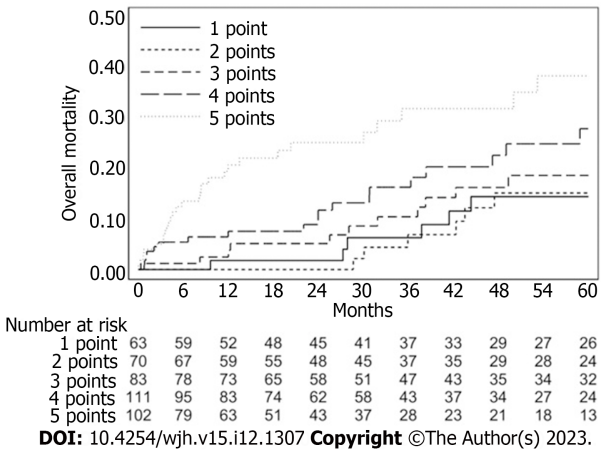
Table 3 Score point system

	Values	Points
ASA score	1	0
	2	0
	3	2
	4	3
Comorbidity > 2	Yes	1
MELD	< 8	0
	8-12	1
	> 12	2
Size of largest lesion (mm)	≤ 10	0
	10-32	1
	> 32	2
	Max score	8

ASA: American Society of Anesthesiology; MELD: Mayo end liver disease.

Table 4 Stratification of mortality risk on preoperative score

Score	Number and prevalence, *n* (%)	Three-month mortality, %	Six-month mortality, %
≥ 2 *vs* ≤ 1	366 (85.3) *vs* 63 (14.7)	3.3 *vs* 0.0	5.7 *vs* 0.0
≥ 3 *vs* ≤ 2	296 (69.0) *vs* 133 (31.0)	4.1 *vs* 0.0	7.0 *vs* 0.0
≥ 4 *vs* ≤ 3	213 (49.7) *vs* 216 (50.3)	5.3 *vs* 0.5	9.3 *vs* 0.5
≥ 5 *vs* ≤ 4	102 (23.8) *vs* 327 (76.2)	5.0 *vs* 2.2	13.6 *vs* 2.2
≥ 6 *vs* ≤ 5	28 (6.5) *vs* 401 (93.5)	11.3 *vs* 2.3	22.9 *vs* 3.6

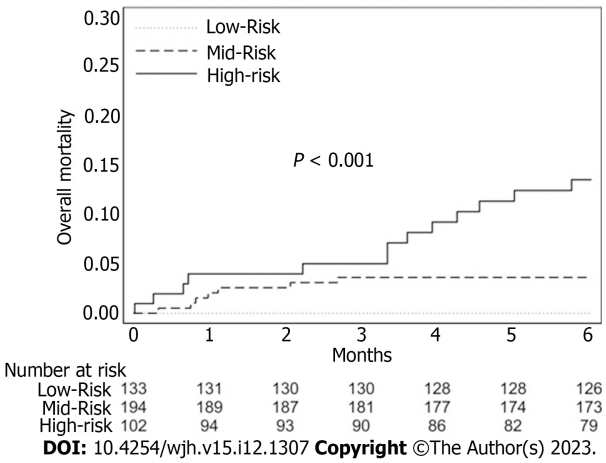


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Figure 1 Overall mortality.

DISCUSSION

The present study observed a population of elderly patients (≥ 70 years) who underwent liver resection for HCC, and it showed that a simple preoperative score, resulting from the evaluation of presence and degree of ASA score, MELD score, the presence of more than 2 comorbidities and the size of the biggest lesion, can predict 90 d and 180 d mortality rate.



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Figure 2 Profile risk of six-month mortality probability.

The process of 'aging society' resulted in an increasing rate of surgical oncological elderly patients and it made necessary to provide an accurate preoperative assessment to optimize the choice of the best possible treatment. Liver resection represented the treatment of choice for resectable HCC, even in elderly population[6-9]. Age itself should not be a contraindication to liver resection in treatment of HCC, but this population needed a more accurate selection and preoperative evaluation of benefits and drawback.

The assessment of liver function needed to be linked with the identification of modifiable and not modifiable risk factors to improve surgical outcomes. There were several predictive of 30 d mortality after liver resection for HCC[10-13]. MELD score was often considered a significant parameter, as well in our study where this score was ranged in 3 degrees with a different impact on final sum. Conversely Lee *et al*[14] in a nationwide cohort study recognized the Platelet-Albumin-Bilirubin score had an higher sensitivity and specificity than MELD or Albumin-Bilirubin score[15].

With the aim to better explore the concept of 'frailty' in this population also the ASA score gained more relevance. In our results an ASA score of 1-2 or 3-4 can weight in a different significantly way on the final score and so have impact on the post operative mortality probability. Not only the evaluation of the degree of pathological physical state, but also the presence of more of 2 comorbidities resulted significant as risk predictor in our score. The limit was represented by not knowing the type of comorbidity which made impossible to optimize the stratification. Preoperative evaluation of the physiological age could be more useful in predicting risk of postoperative morbidity and mortality than chronological age [16,17], but several external validation of comprehensive score are needed.

As previously reported the size of largest tumor was a useful factor to predict prognostic outcomes after liver resection for HCC[13,18,19]. Also our results showed in univariate and multivariate analysis how an increasing size could be a risk factor on postoperative mortality. In the setting of liver disease almost completely represented by a single nodule of HCC, a size > 32 mm could impact on postoperative mortality risk as a MELD score > 12. The idea of the importance of morphological tumor data was yet explored by Mazzaferro[18] with 'Metroticket paradigm' before, and 'Up to7 criteria' after, more useful in the context of liver transplantation, but it had represented the substrate for comprehensive measures as reported by Tokumitsu *et al*[12] with its NxS score which provide a cut off value of tumor burden to predict the prognosis following hepatectomies for HCC[12]. Despite this, prognosis of HCC was more complex than other solid tumors because it depended not only from tumor burden but also from liver function reserve.

ASA score, MELD score, the presence of more than 2 comorbidities and the size of the lesion were all non-modifiable factor. Our work underlined how the process of decision making could be delicate in elderly patients with HCC. The association of evaluation of liver (functional and oncological) disease and the physiological age of patients needed to be assessed before surgery[19-20] to better stratifying patients at risk and to implement preoperative and postoperative programs of rehabilitation which could bridge the gap of physiopathological state[21].

However, this study had some limitations. First of all, because of its retrospective nature, there was a possibility of an unavoidable selection bias. Secondly, the surgical procedures included were laparoscopic and open approach without considering their different impact on the postoperative outcomes. In addition, our aim was to evaluate 90 and 180 d mortality but another key point was represented by postoperative complications and their correlations with preoperative and intraoperative data. This could be the focus for future works.

CONCLUSION

In conclusion, our score resulted from granular evaluation of possible risk factors for the postoperative mortality at 90 d and 180 d in elderly patients resected for HCC.

It would be a simple and useful tool to provide a better cognition of patients who could benefit of liver resection and to improve 180 d mortality.

ARTICLE HIGHLIGHTS

Research background

Liver resection represented one of the mainstay treatment for hepatocellular carcinoma (HCC). The approach of liver disease in elderly population needed of an accurate stratification of patients at risk, with the involvement of multidisciplinary preoperative assessment.

Research motivation

Liver resection is burdened by a variable rate of postoperative morbidity and mortality. Elderly patients represented more often the major rate of patients who underwent liver resection for HCC. This aspect makes mandatory an accurate preoperative assessment and a specific evaluation of potential postoperative risk.

Research objectives

The aim of our study was to analyze a population of elderly patients who underwent liver resection for HCC, to investigate the possible presence of risk predictors of postoperative mortality at 90 and 180 d.

Research methods

Associations between baseline pre-operative variables with six-month mortality were evaluated using a unit-variate Cox proportional-hazards model. A score point system was derived from the multi-variable Cox proportional-hazards model.

Research results

The American Society of Anesthesiology (ASA) score, Mayo end stage liver disease score, the presence of comorbidities > 2 and the size of the biggest lesion are included in the stratification of the score. Combining the four variables we obtained different profiles of patients with a different preoperative risk at 6 mo: Low-risk < 5%, mid-risk 5%-10% and high-risk class > 10%.

Research conclusions

This score can aid in stratifying this population in order to assess who can benefit from surgical treatment in terms of postoperative mortality.

Research perspectives

Randomized controlled studies are needed to better explore these results.

FOOTNOTES

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Institutional review board statement: This study does not require approval from the hospital ethics committee.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Observational Study

Serum omentin-1 is correlated with the severity of liver disease in patients with chronic hepatitis C

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Abstract

BACKGROUND

Patients with chronic hepatitis C virus (HCV) infection have increased serum omentin-1. Omentin-1 is an anti-inflammatory adipokine, and higher levels may be a direct effect of HCV infection. Successful elimination of HCV by direct acting antivirals almost normalized circulating levels of various molecules with a role in inflammation.

AIM

To evaluate the effect of HCV infection on serum omentin-1, serum omentin-1 levels of HCV patients were measured before therapy and at 12 wk after therapy end. Associations of serum omentin-1 with parameters of inflammation and liver function were explored at both time points. Serum omentin-1 levels of patients with and without liver cirrhosis, which was defined by ultrasound or the fibrosis-4 (FIB-4) score, were compared.

METHODS

Serum omentin-1 levels were measured by enzyme-linked immunosorbent assay in 84 chronic HCV patients before therapy and at 12 wk after therapy end where sustained virological response 12 (SVR12) was achieved in all patients. Serum omentin-1 of 14 non-infected controls was measured in parallel.

RESULTS

In patients with chronic HCV, serum omentin-1 levels were not related to viral load or viral genotype. HCV patients with liver steatosis and HCV patients with

diabetes had serum omentin-1 levels comparable to patients not suffering from these conditions. Serum omentin-1 levels at SVR12 were similar in comparison to pretreatment levels. In addition, serum levels did not differ between HCV-infected patients and non-infected controls. Serum omentin-1 levels did not correlate with leukocyte count or C-reactive protein. Positive correlations of serum omentin-1 with bilirubin and the model for end-stage liver disease score (MELD) were detected before therapy and at SVR12 in the whole cohort. Bilirubin and the MELD score also positively correlated with serum omentin-1 levels in the subgroup of patients with ultrasound diagnosed liver cirrhosis before therapy. At SVR12, serum omentin-1 levels of patients with liver cirrhosis negatively correlated with albumin. Before therapy start, patients with high FIB-4 scores had increased serum omentin-1 in comparison to patients with a low score. Serum omentin-1 levels of patients with liver cirrhosis defined by ultrasound were increased at baseline and at SVR12.

CONCLUSION

Present study showed that liver cirrhosis, but not HCV infection per se, is related to elevated serum omentin-1 levels.

Key Words: Direct acting antivirals; Hepatitis C; Liver cirrhosis; Adipokine

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Core Tip: Omentin-1 is an adipokine well described for its anti-inflammatory and insulin-sensitizing functions. Aim of this study was to identify the effect of hepatitis C virus (HCV) infection and liver cirrhosis on serum omentin-1 levels. This study showed that circulating omentin-1 levels of HCV infected patients and non-infected healthy controls were similar. Accordingly, serum omentin-1 levels did not change upon effective elimination of the virus by direct acting antiviral therapy. Serum omentin-1 was not associated with diabetes or C-reactive protein in the HCV cohort. Patients with liver cirrhosis had increased serum omentin-1 levels before treatment and at sustained virological response 12 in comparison to HCV patients without liver cirrhosis. This analysis shows that increased serum omentin-1 levels of chronic HCV patients with liver cirrhosis persist after viral elimination. Serum omentin-1 has no role in the favourable metabolic outcomes of HCV eradication.

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INTRODUCTION

Chronic infection with hepatitis C virus (HCV) causes systemic and hepatic inflammation, which drive the development and progression of liver fibrosis[1,2]. Highly effective elimination of HCV by direct acting antivirals (DAAs) rapidly improves inflammation and circulating levels of various cytokines and chemokines decline[3-5]. Interestingly, levels of pro- as well as anti-inflammatory cytokines were reduced shortly after start of DAA therapy[3-6]. Regression of liver fibrosis after HCV cure is possible, and complete reversal is expected to take several years[7]. Non-invasive tests for scoring of liver fibrosis are sonographic shear-wave elastography and transient elastography, and improvement of these measures at sustained virological response 12 (SVR12) or SVR24 is correlated with the resolution of liver inflammation[4,8]. The fibrosis-4 (FIB-4) score is calculated from age, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as platelet number, and was found accurate to assess advanced liver fibrosis in patients with HCV[9]. ALT and AST levels rapidly decline after start of DAA therapy, and lower FIB-4 scores at SVR12 are attributable to reduced hepatic inflammation[4,10].

Omentin-1 was first described as intelectin-1, a protein expressed in the intestine, which recognizes complex carbohydrates of bacterial cell walls. Later on, omentin-1 was shown to be highly expressed by stromal vascular cells of visceral adipose tissues. Omentin-1 protects from oxidative stress and exerts anti-inflammatory effects in macrophages, endothelial cells and adipocytes[11]. Omentin-1 serum levels are decreased in obesity and negatively correlate with fasting insulin levels[12]. There is convincing evidence that low serum omentin-1 levels in obesity contribute to impaired insulin signalling and insulin resistance[12-14].

Insulin resistance in chronic HCV infection is common, but the underlying mechanisms are not fully understood. HCV-infected patients with elevated aminotransferase levels also had increased serum omentin-1. In this cohort, HCV patients with type 2 diabetes had lower serum omentin-1, which was negatively correlated with the homeostasis model assessment of insulin resistance (HOMA-IR) index and fasting insulin levels[15]. Serum omentin-1 concentrations of HCV patients with liver cirrhosis stratified by a HOMA-IR index below and above 2.5 were, however, similar[16]. Insulin resistance is associated with liver steatosis, which was not related to altered serum omentin-1 levels. Moreover, there was

no correlation of serum omentin-1 levels with liver inflammation grades[16,17].

However, patients with alcoholic liver cirrhosis and patients with HCV-related liver cirrhosis had increased serum omentin-1 levels in comparison to healthy controls[17,18]. No significant differences in omentin-1 serum levels were found regarding the severity of liver cirrhosis assessed by the model of end stage liver disease (MELD) score or Child-Pugh score[18,19]. Accordingly, another study could not observe associations of serum omentin-1 levels with histologically scored fibrosis stages in patients with HCV[17]. In contrast, it was also reported that HCV patients with advanced liver fibrosis, which was histologically scored, had higher serum omentin-1 levels in comparison to those patients with a lower score[16].

Current studies suggest that serum omentin-1 is increased in chronic HCV, but whether this is caused by viral infection or related to liver disease severity has not been clarified to date. Aim of this study was to examine the effect of efficient virus elimination by DAA therapy on serum omentin-1 levels and to describe associations of serum omentin-1 levels with measures of liver disease severity.

MATERIALS AND METHODS

Study cohort

This prospective study included patients with chronic HCV from September 1, 2014 to February 27, 2017, and was conducted at the Department of Internal Medicine I at the University Hospital Regensburg. Patients with an indication for therapy with DAAs (drug combinations used were sofosbuvir/daclatasvir, sofosbuvir/ledipasvir, sofosbuvir/velpatasvir, glecaprevir/pibrentasvir, or elbasvir/grazoprevir) according to recent guidelines participated in the study [20]. The patients were older than 18 years, and had not been treated for HCV before. Patients with decompensated liver cirrhosis, hepatitis B virus or human immunodeficiency virus co-infection were excluded. The 14 controls were patients not infected with HCV, and without any severe diseases such as liver cirrhosis. This group included eight females and six males. Age was 63 (38-87) years and body mass index (BMI) was 26.9 (17.8-43.4) kg/m^2, and both measures were similar to the patient cohort ($P > 0.05$ for all).

The study protocol was approved by the ethical committee of the University Hospital of Regensburg (14-101-0049, date of approval May 22, 2014) and was performed according to the updated guidelines of good clinical practice and updated Declaration of Helsinki. Informed consent was obtained from all patients. Laboratory measures were provided by the Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg.

Ultrasound examination and calculation of fibrosis-4 score

Cirrhosis diagnosis by ultrasound relied on nodular liver surface, small liver size, and coarse liver parenchyma[21]. The FIB-4 score is an accepted non-invasive fibrosis score[22]. Cut-off values for the FIB4-score were: fibrosis > 3.25, no fibrosis: < 1.3 for patients < 65 years, and < 2 for patients older than 65 years[23].

Omentin-1 enzyme-linked immunosorbent assay

Serum aliquots were stored at -80 °C and thawed immediately before being used. Serum was used undiluted for omentin-1 measurement with the Human Intelectin-1/Omentin DuoSet enzyme-linked immunosorbent assay (R&D Systems; Catalog #: DY4254-05; Wiesbaden-Nordenstadt, Germany).

Measurement of IL-6 and IL-10

Levels of the cytokines interleukin (IL)-6 and IL-10 were quantified using Multiplex Luminex® Assays (Merck, KGaA, Germany) as described before[4].

Statistical analysis

Data are shown as boxplots, which represent the minimum value, the maximum value, the median, the first quartile and the third quartile. Small circles and asterisks above the boxes are outliers. Data in tables are given as median value, and the minimum value and the maximum value are listed in brackets. The non-parametric Mann-Whitney U test and Kruskal-Wallis test were used for comparison of two or more independent groups, respectively (SPSS Statistics 26.0 program). Students' *t*-test (Ms Excel) was used for analysis of paired data and Spearman correlation for correlation analysis (SPSS Statistics 26.0 program). A value of $P < 0.05$ was regarded significant.

RESULTS

Association of serum omentin-1 levels with age, body mass index, gender, liver steatosis and diabetes in patients with chronic HCV infection

Eighty-four patients with chronic HCV infection (Table 1) and 14 non-infected controls were included in the study. Serum omentin-1 levels of controls and HCV patients were similar (Figure 1A). In the HCV cohort, serum omentin-1 levels of females and males were comparable (Figure 1B). Associations of serum omentin-1 with the BMI (r = -0.082, P = 0.486) or age (r = 0.092, P = 0.408) were not identified. The 15 diabetic HCV patients had similar omentin-1 serum levels in comparison to the non-diabetic HCV patients (Figure 1C). Ten of the patients with diabetes had liver cirrhosis, as was

Table 1 Gender distribution, age, body mass index, model for end stage liver disease score, and laboratory parameters of the 84 patients before therapy start

Parameter	Before therapy	Patients at SVR12
Gender (F/M)	37/47	36/42
BMI kg/m^2	26.5 (18.4-40.4)	26.8 (18.4-40.4)
Age yr	56 (24-82)	57 (24-82)
MELD score	7 (6-19)	7 (6-19)
ALT U/L	70 (22-247)	26 (6-135)[c]
AST U/L	64 (14-1230)	23 (10-1390), *n* = 76[c]
Bilirubin mg/dL	1.0 (1.0-4.3)	1.0 (1.0-2.8)
Albumin g/L	37.3 (19.0–45.5), *n* = 83	39.1 (26.1-47.7)[c]
INR	1.1 (1.0-2.3)	1.1 (1.0-2.8)
Creatinine mg/dL	1.0 (1.0-1.3)	1.0 (1.0-1.4)
GFR ml/min	98 (47-161)	94 (52–129)
Leukocyte number/L	6.1 (2.2-12.3)	6.2 (1.9-38.6), *n* = 77
CRP mg/L	2.9 (2.9-29.9)	2.9 (2.9-20.4)
PCT ng/mL	0.08 (0.01–11.02), *n* = 83	0.04 (0.01-0.15)[c]
Platelet number/nL	157 (38-364)	171 (38-312), *n* = 77
HDL mg/dL	52 (19-103), *n* = 78	51 (23-100), *n* = 71
LDL mg/dL	91 (31-204), *n* = 78	117 (38-243), *n* = 70[c]

[c]*P* < 0.001.

Data of the 78 patients where serum was available at SVR12 are also listed. In the case that laboratory values were not obtained of all patients, the number (*n*) of patients of whom these measures were known is given. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CRP: C-reactive protein; GFR: Glomerular filtration rate; HDL: High density lipoprotein, INR: International normalized ratio; LDL: Low-density lipoprotein; MELD: Model for end stage liver disease; PCT: Procalcitonin.

diagnosed by ultrasound imaging. In the cirrhosis group diabetic and non-diabetic patients had comparable serum omentin-1 levels (P = 1.00). Thirty-eight patients of the HCV cohort had liver steatosis but serum omentin-1 did not differ from patients without liver steatosis (Figure 1D).

Associations of serum omentin-1 with viral load and genotype in patients with HCV

Serum omentin-1 was not related to viral load (r = -0.100, P = 0.364). HCV genotypes were 1a (22 patients), 1b (39 patients) and 3a (15 patients), and rare genotypes (8 patients) were assigned to one group. Serum omentin-1 levels did not vary between HCV genotypes (Figure 1E).

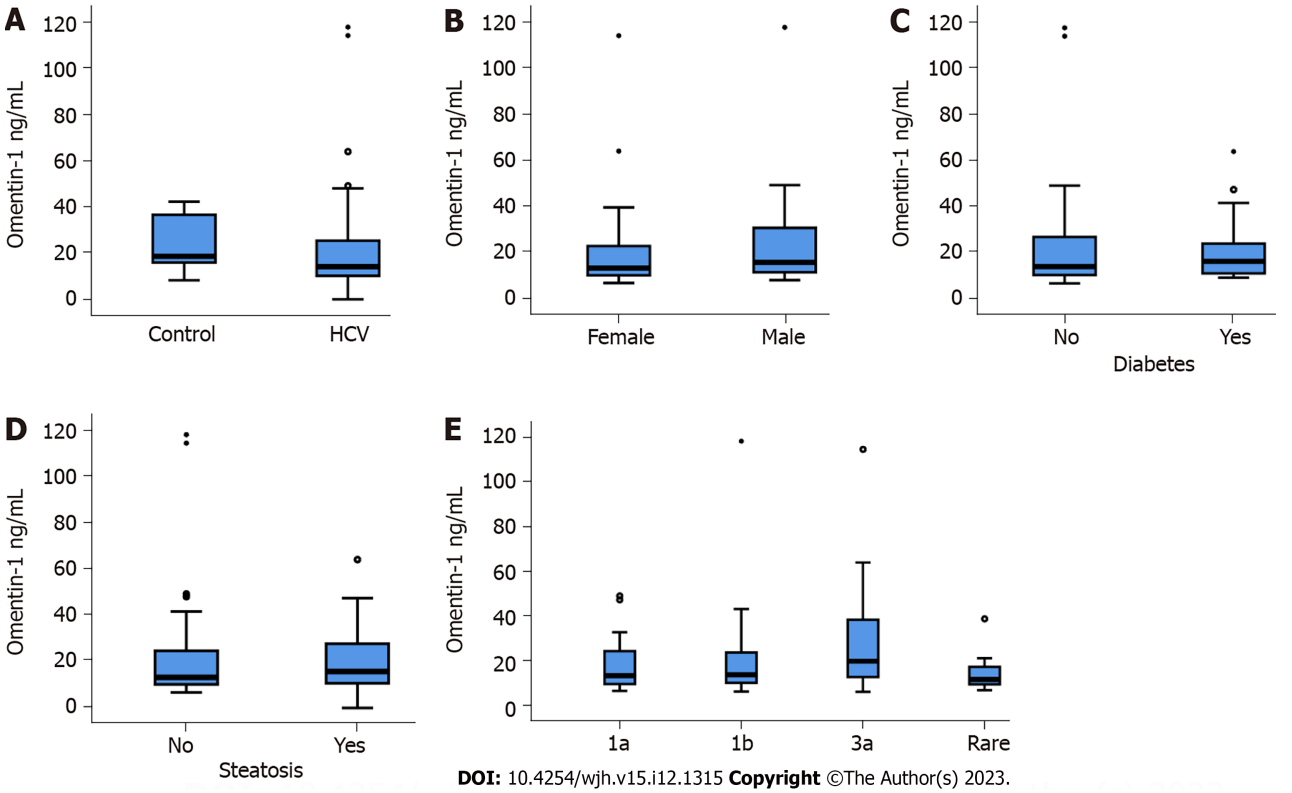
Correlations of serum omentin-1 levels with measures of inflammation and liver function before and after DAA treatment

In the whole cohort, serum omentin-1 levels positively correlated with the MELD score (r = 0.428, P < 0.001), bilirubin (r = 0.435, P < 0.001), international normalized ratio (INR) (r = 0.380, P < 0.001) and procalcitonin (r = 0.365, P = 0.001), and negatively with platelet number (r = -0.303, P = 0.005), albumin (r = -0.297, P = 0.006) and low density lipoprotein (LDL) (r = -0.426, P < 0.001). Partial correlation of serum omentin-1 with LDL controlled for the MELD score was not significant (P = 0.075). Serum omentin-1 levels did not correlate with creatinine (r = 0.176, P = 0.107) or glomerular filtration rate (r = -0.042, P = 0.705).

DAA therapy cleared HCV effectively and viral load was significantly reduced at four weeks after therapy start and at SVR12 (P < 0.001 in comparison to pre-treatment levels)[24]. At SVR12, ALT, AST, ferritin and procalcitonin were declined, and albumin and LDL were increased in contrast to levels before therapy (Table 1). Serum omentin-1 levels did not change during therapy (Figure 2).

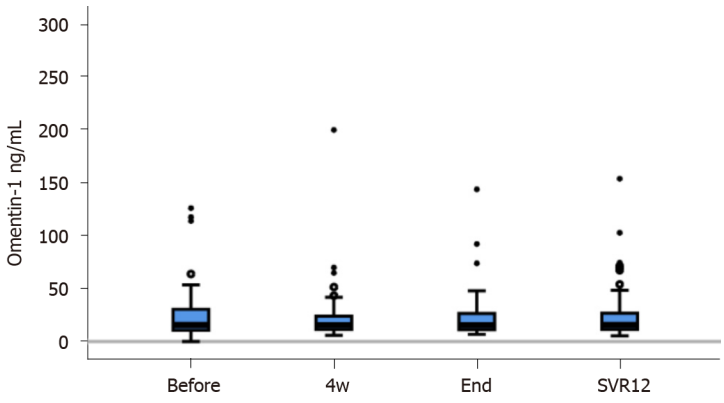
Associations between serum omentin-1 and most of the clinical markers of liver disease severity persisted at SVR12. Serum omentin-1 levels correlated with the MELD score (r = 0.314, P = 0.005), bilirubin (r = 0.435, P < 0.001), albumin (r = -0.255, P = 0.025), INR (r = 0.293, P = 0.009) and LDL (r = -0.265, P = 0.027). Partial correlation of omentin-1 and LDL corrected for the MELD score was not significant (P = 0.625).

Serum omentin-1 did not correlate with creatinine (r = 0.047, P = 0.685) or glomerular filtration rate (r = -0.112, P = 0.334).



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Figure 1 Serum omentin-1 levels of controls and patients with chronic hepatitis C virus. A: Serum omentin-1 Levels of 14 controls and 84 patients with chronic hepatitis C virus (HCV); B: Serum omentin-1 levels of 37 female and 47 male HCV patients; C: Serum omentin-1 levels of 15 patients with and 69 patients without diabetes; D: Serum omentin-1 levels of 38 patients with and 46 patients without steatosis; E: Serum omentin-1 levels of patients categorized for HCV genotype (Rare group signifies 8 patients with genotypes other than 1a, 1b, and 3a). The small circles or asterisks above the boxes mark outliers.



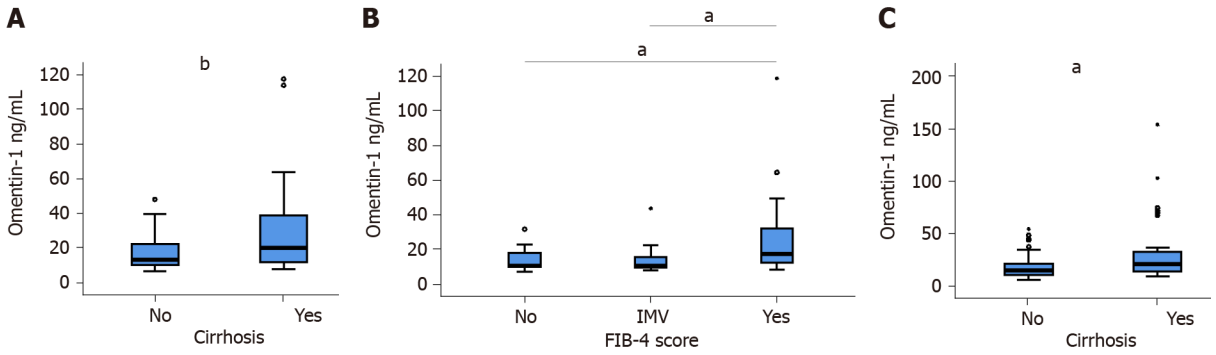
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Figure 2 Serum omentin-1 levels during direct acting antiviral therapy. Omentin-1 serum levels before therapy start (Before), at 4 wk after therapy start (4w), at therapy end (End) and at sustained virological response 12 (SVR12). Small circles or asterisks above the boxes mark outliers.

Serum omentin-1 did not correlate with leukocyte count (r = -0.205, P = 0.061), C-reactive protein (r = 0.117, P = 0.290), IL-6 (0.022, P = 0.113) or IL-10 (r = -0.138, P = 0.218) levels before DAA therapy. At SVR12 no significant associations between serum omentin-1 with leukocyte count (r = -0.093, P = 0.423), C-reactive protein (r = -0.098, P = 0.394), IL-6 (r = 0.067, P = 0.578) or IL-10 (r = 0.225, P = 0.058) were found.

Serum omentin-1 in relation to non-invasive measures of liver fibrosis

Before therapy, the 31 patients with liver cirrhosis diagnosed by ultrasound examination had higher omentin-1 (Figure 3A). The FIB-4 index for non-invasive scoring of liver fibrosis indicated higher omentin-1 serum levels of patients with advanced liver fibrosis in comparison to patients with low and intermediate scores (Figure 3B). Positive correlations with the MELD score (r = 0.375, P = 0.031) and bilirubin (r = 0.481, P < 0.005), and a negative correlation with LDL (r = -0.626, P < 0.001) were observed in the sub-cohort of the 31 patients with ultrasound diagnosed liver cirrhosis before therapy.



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Figure 3 Serum omentin-1 in relation to liver fibrosis. A: Serum omentin-1 levels of hepatitis C virus (HCV) patients with liver cirrhosis and non-cirrhotic patients, which was diagnosed by ultrasound before therapy; B: Serum omentin-1 levels of HCV patients categorized according to the fibrosis-4 score (26 patients no fibrosis = No; 18 patients had intermediate values = IMV, 40 patients had fibrosis = Yes) before therapy; C: Serum omentin-1 levels of HCV patients without and with liver cirrhosis, which was diagnosed by ultrasound, at sustained virological response 12 (SVR12). Small circles and stars above the boxes mark outliers. [a]*P* < 0.05, [b]*P* < 0.01.

AT SVR12, in the subgroup of patients with ultrasound diagnosed liver cirrhosis, a negative correlation of albumin and serum omentin-1 was detected (r = -0.436, P = 0.018). Serum omentin-1 levels of patients with cirrhosis were higher in contrast to HCV patients without cirrhosis at SVR12 (Figure 3C).

DISCUSSION

Here, we have shown that serum omentin-1 is increased in HCV patients with advanced liver fibrosis. HCV infection per se did not affect serum omentin-1 levels, which were not related to viral titer or genotype, did not decline upon efficient elimination of HCV and were similar among HCV patients and non-infected controls.

In our previous study we have measured serum omentin-1 levels of patients with liver cirrhosis[18], and the 37 patients with alcoholic liver cirrhosis and the 3 patients with HCV related cirrhosis had similar levels (P = 0.693). Though the number of patients with HCV caused cirrhosis was small, this finding is in accordance with the assumption that HCV infection has no effect on serum omentin-1 levels.

Omentin-1 is best described for its role as an insulin-sensitizer[11,12]. Serum omentin-1 was, however, similar between diabetic and non-diabetic HCV patients in accordance with previous studies. It has been shown before that patients with liver cirrhosis and diabetes had similar omentin-1 levels in comparison to non-diabetic cirrhosis patients[19]. In addition, chronic HCV patients with a high HOMA-IR index had omentin-1 levels comparable to patients with a low index[16]. In apparently healthy cohorts, serum omentin-1 correlated negatively with the HOMA-IR index[14,25], and this association seems to be lost in chronic HCV.

HCV infection can promote diabetes and liver steatosis[26]. Metabolic dysfunction–associated fatty liver disease (MAFLD) is relatively common in the general population, and accordingly, is abundant in patients with HCV[27]. MAFLD is associated with insulin resistance, and reduced levels of circulating omentin-1[11,28]. In the HCV cohort, serum omentin-1 levels of patients with hepatic steatosis did not change. The diagnosis of MAFLD in patients with HCV infection is, however, challenging[27] and virus-induced fatty liver was not discriminated from metabolic liver steatosis in our study. Therefore, we can only conclude that liver steatosis either caused by HCV and/or by metabolic dysfunction is not related to changed serum omentin-1 levels.

HCV is classified into eight genotypes and more than 80 subtypes[29]. Most prevalent in Europe is HCV genotype 1 and the subtypes 1a and 1b[30], and 61 of our patients were infected by 1a or 1b. The second most prevalent genotype in our study cohort was 3a. HCV genotypes differ in their clinical features, and insulin resistance and liver steatosis are more common in genotype 1 and 3 infection[31]. Serum omentin-1 levels of patients infected with genotype 1a, 1b or 3a were, however, similar.

In accordance with previous data[16], serum omentin-1 levels were not associated with viral load. Highly efficient elimination of HCV by DAA treatment had no effect on the circulating levels of omentin-1. This indicates that hepatic and systemic inflammation caused by HCV infection[4] do not change serum omentin-1 levels. In line with this suggestion the current analysis showed that serum omentin-1 levels of HCV patients and controls were similar.

HCV patients with advanced liver fibrosis had increased serum omentin-1 levels when liver fibrosis was assessed by the non-invasive FIB-4 score or diagnosed by ultrasound. Higher concentrations of omentin-1 have been described in HCV patients with liver cirrhosis in comparison to healthy controls[17,18]. Current findings suggest that severe liver disease and not viral infection is the cause of increased serum omentin-1 levels.

Serum omentin-1 levels did, however not increase with higher Child-Pugh scores and did not correlate with the MELD score in patients with mostly alcoholic liver cirrhosis[18]. In our HCV patients with liver cirrhosis, serum omentin-1 levels did not correlate with the MELD score at SVR12. At this time, there was an association between serum omentin-1 and albumin, a biomarker for impaired hepatic function. Small cirrhosis cohorts and relatively weak associations between

serum omentin-1 levels and laboratory measures of liver disease may prevent the observation of significant associations. Future research has to identify the causes for higher serum omentin-1 levels in patients with advanced liver fibrosis.

LDL of patients with liver cirrhosis is reduced[32-34], and negative correlation of serum omentin-1 levels with LDL is in this context. Negative associations of serum omentin-1 and LDL have been reported in healthy controls[35] and this finding is in line with the beneficial metabolic effects of this adipokine[11]. In HCV infection and at SVR12 the protective effects of omentin-1 against dyslipidemia could not be observed.

Increased serum omentin-1 levels of patients with advanced liver disease may originate from visceral adipose tissues with high omentin-1 expression[11]. It has been also suggested that impaired hepatic clearance of serum omentin-1 by the damaged liver contributes to higher serum levels[18]. Serum omentin-1 levels of patients with chronic HCV were not correlated with the hepatic mRNA expression of omentin-1[17], indicating that omentin-1 synthesis in the liver plays a minor role for systemic omentin-1 levels. Further study is needed to clarify the origin of higher serum omentin-1 in patients with liver cirrhosis.

Patients with end-stage renal disease had elevated serum omentin-1[36] indicating renal excretion of omentin-1. In the current analysis, which included patients with normal and slightly reduced glomerular filtration rates, associations between serum omentin-1 levels and markers for kidney function such as creatinine or glomerular filtration rates were not observed [37] This shows that minor renal dysfunction has no role for higher serum omentin-1 levels in HCV patients with liver cirrhosis.

Induction of omentin-1 levels of patients with liver cirrhosis is in agreement with studies on serum adiponectin levels, an anti-inflammatory adipokine functioning as an insulin sensitizer, whose circulating levels also decline in obesity[38, 39]. Serum adiponectin levels of patients with HCV, hepatitis B virus and MAFLD related liver cirrhosis were increased [40]. In these patient cohorts, circulating levels of adiponectin were not associated with BMI or markers of insulin sensitivity[41]. These findings suggest that adiponectin does not exert its protective roles in liver cirrhosis as seems to be the case for omentin-1.

A limitation of this study is that Child-Pugh scores were not included. Early guidelines commenting on treatment in patients with decompensated liver cirrhosis, who have Child-Pugh B or C, did not recommend the use of DAAs for these patients[42]. Our cohort was recruited shortly after these drugs were approved, and patients with decompensated cirrhosis were excluded. The Child-Pugh score of our cohort was not determined but from the clinical data of our patients we assume that most of them had Child-Pugh score A.

CONCLUSION

In summary, the current analysis showed that higher serum omentin-1 levels in chronic HCV infection is not a marker of metabolic health but rather indicates advanced liver fibrosis.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) infection can cause severe liver damage. Chronic HCV infection can be cured with direct acting antiviral therapy, which also decreases insulin resistance. Omentin-1 is regarded a beneficial adipokine and improves insulin resistance. Serum omentin-1 levels are increased in HCV infection. The effect of viral cure on serum omentin-1 levels has not been analysed as far as we know. Moreover, data regarding associations of omentin-1 with liver injury are discordant.

Research motivation

From a pathophysiological standpoint, it can be important to evaluate associations of serum omentin-1 levels with HCV infection and liver disease severity.

Research objectives

The objective of this study was to assess the effect of HCV clearance on serum omentin-1 levels, and to evaluate associations of serum omentin-1 levels with clinical markers of inflammation, liver steatosis, diabetes and liver dysfunction.

Research methods

This observational study included 84 patients with chronic HCV, and collected serum before therapy, at 4 wk after therapy start, at therapy end and at sustained viological response 12 (SVR12). Serum omentin-1 was measured by enzyme-linked immunosorbent assay. Serum omentin-1 levels of 14 controls were also determined.

Research results

The study found evidence of increased serum omentin-1 levels in patients with liver cirrhosis. HCV elimination did not change serum omentin-1 levels, suggesting that viral infection has no effect on serum omentin-1.

Research conclusions

Effective elimination of HCV is associated with favourable metabolic outcomes. This study indicates that omentin-1 has no role herein. Serum omentin-1 of HCV patients with liver cirrhosis is increased at baseline and SVR12, and may have a role in liver cirrhosis pathogenesis.

Research perspectives

Evaluation whether increased serum omentin-1 is just a marker of impaired liver function or contributes to liver damage.

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FOOTNOTES

Author contributions: Peschel G, Weigand K and Buechler C were the guarantors and designed the study; Peschel G, Weigand K and Grimm J participated in the acquisition of the serum samples; Buechler C participated in the analysis, and interpretation of the data, and drafted the initial manuscript; Peschel G, Weigand K, Grimm J, Müller M and Buechler C revised the article critically for important intellectual content.

Institutional review board statement: The study protocol was approved by the ethical committee of the University Hospital of Regensburg (14-101-0049, date of approval: May 22, 2014).

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

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

Data sharing statement: No additional data are available.

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

Prospective Study

High incidence of periodontitis in patients with ascitic decompensated cirrhosis

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Abstract

BACKGROUND

Periodontitis has been associated with various liver diseases. However, the relevance of periodontitis in the progression of decompensated cirrhosis remains inconclusive. In particular, it is unclear whether the common periodontitis pathogens, *Porphyromonas gingivalis* (*P. gingivalis*) and *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*), can be detected not only in the oral mucosa but also in ascites and stool.

AIM

To investigate the significance of periodontitis, *P. gingivalis*, and *A. actinomycetemcomitans* in cirrhosis patients with ascitic decompensation.

METHODS

This prospective study was conducted at the University Hospital Hamburg-

Eppendorf, a tertiary center in Northern Germany. A cohort of 27 patients with ascitic decompensated liver cirrhosis underwent dental examinations to assess the association between periodontitis and various clinical parameters of cirrhosis, as well as patient outcomes. PCR was used to test gingival samples, ascites, and stool for the presence of *P. gingivalis* and *A. actinomycetemcomitans*. Gingival samples were collected by probing the deepest gum pocket of a sextant and wiping them on a cotton swab.

RESULTS

Periodontitis was diagnosed in 22 out of 27 (82%) ascite patients, which is significantly more common than in a control cohort of 100 unselected patients (59%, $P = 0.04$). *P. gingivalis* was detected in the gingiva of six patients, and one of them also had *P. gingivalis* in their stool. However, *P. gingivalis* was not found in the ascites of any patient. Five out of six patients with *P. gingivalis* had periodontitis (83%). *A. actinomycetemcomitans* was not detected in any sample. Patients without periodontitis had a significantly higher mortality rate compared to those with periodontitis, and survival (Kaplan-Meier analysis) was longer in patients with periodontitis ($P = 0.02$). Transplant-free survival was also more common in patients with periodontitis compared to those without (63% *vs* 0%, $P = 0.02$).

CONCLUSION

Decompensated cirrhotic patients frequently suffer from periodontitis. However, there was no evidence of the translocation of *P. gingivalis* or *A. actinomycetemcomitans* into ascites. The survival of cirrhotic patients with periodontitis was not reduced.

Key Words: Cirrhosis; Ascites; Decompensation; Periodontitis; Survival; Gingiva

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Core Tip: In this prospective cohort study, we aimed to assess the prevalence of periodontitis and the potential dissemination of classical periodontitis pathogens into ascites among 27 cirrhotic patients experiencing ascitic decompensation. We also compared this group with 100 unselected patients from a dental practice. Our findings revealed that decompensated cirrhotic patients often experience periodontitis. However, we did not observe any evidence of the translocation of *Porphyromonas gingivalis* or *Actinobacillus actinomycetemcomitans* into ascites. Furthermore, the presence of periodontitis did not appear to have a detrimental effect on the survival of cirrhotic patients.

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INTRODUCTION

Cirrhosis, the final stage of chronic liver disease, is a very serious condition associated with a significantly reduced life expectancy[1]. One of its most threatening complications is spontaneous bacterial peritonitis (SBP)[1,2]. It is assumed that bacteria from the intestinal flora migrate into the abdominal cavity and multiply in the ascites. In contrast, secondary bacterial peritonitis in patients with portal hypertension is caused by an abdominal source of infection, such as an abscess or perforation. Secondary bacterial peritonitis is much less common than SBP, accounting for approximately 15% of all peritonitis cases. It carries a 20% risk of mortality[3].

SBP is defined as the presence of more than 250 polymorphonuclear cells, specifically neutrophil granulocytes, per mm^3 in ascites[4]. Gram-negative bacteria, commonly found in the intestine, are the primary pathogens associated with SBP. The extent to which colonization of the oral flora contributes to the occurrence of SBP and whether bacteria from the oral cavity can enter the ascites through the intestinal tract and migration remain unclear.

Periodontitis, an inflammation of the gingiva, has garnered increasing interest over the past two decades. The presence of periodontitis has been linked to systemic inflammation. Animal experiments in mice have demonstrated a connection between periodontitis and the development of liver fibrosis[5].

Porphyromonas gingivalis (*P. gingivalis*), one of the most significant periodontal pathogens, is believed to enter the bloodstream through the oral mucosa, potentially leading to the release of various cytokines. Apart from direct translocation of *P. gingivalis* into the bloodstream through damaged gingiva with reduced barrier function, this bacterium can also easily move from the oral cavity to the intestine. It can be envisioned that the disruption of the intestinal microbiota composition by orally derived *P. gingivalis* may contribute to the gut-liver axis and the pathogenesis of SBP[6,7]. However, this aspect has not been thoroughly investigated.

The relevance of periodontitis has been studied in various patient cohorts with liver disease, including those with cirrhosis. A 1995 Vienna study involving 97 cirrhotic patients, including 64 with alcoholic cirrhosis and 33 with non-alcoholic cirrhosis, revealed that alcohol-dependent cirrhosis, but not cirrhosis in general, was associated with reduced oral hygiene ($P < 0.01$), decreased dental care ($P < 0.001$), the presence of periodontitis, and the need for dental treatment ($P < 0.001$)[8]. A study conducted in the United States prospectively examined whether periodontitis treatment influenced the oral-gut-hepatic axis in cirrhosis patients[9]. In this study, the impact of periodontal therapy was assessed in 26 cirrhotic patients receiving treatment compared to 24 cirrhotic patients without periodontitis therapy. Over an observational period of 30 d, the treated cirrhosis group exhibited improvements in dysbiosis in stool and saliva, as well as reductions in endotoxin and lipopolysaccharide-binding protein levels.

Additionally, both periodontitis and cirrhosis have the potential to trigger an inflammatory response and generate inflammatory mediators, which may enable them to mutually influence each other. In several patient cohorts, individuals with cirrhosis have been observed to exhibit poorer periodontal clinical parameters compared to those without cirrhosis [6].

Some studies have suggested a potential connection between periodontitis and liver diseases, including cirrhosis. It is theorized that the inflammatory responses associated with periodontitis may contribute to the development of liver diseases like cirrhosis. Chronic inflammation in the body can result in liver cell damage and expedite the progression of liver diseases.

However, it is crucial to emphasize that further research is required to fully comprehend and clarify the relationship between periodontitis and liver diseases such as cirrhosis.

These observations naturally raise the question of whether *P. gingivalis* also contributes to the development of SBP and whether this pathogen can be detected in ascites.

To investigate whether *P. gingivalis* and *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*), bacteria typically associated with periodontitis, can be detected in ascites, we conducted a prospective study examining oral mucosal samples, ascites specimens and stool specimens for the presence of *P. gingivalis*. Mucosal samples were collected by penetrating the deepest gum pocket of a sextant and swabbing the area, followed by PCR testing. Additionally, we explored whether dental factors such as the detection of these bacteria, the presence of periodontitis, the number of teeth, and other variables were associated with the outcomes of patients with end-stage liver cirrhosis.

MATERIALS AND METHODS

Patients

In this prospective study, we invited all adult cirrhosis patients who required paracentesis at the University Hospital Hamburg-Eppendorf in Hamburg, Germany, between March 2021 and July 2021 to participate. This cohort comprises both inpatients and outpatients experiencing ascitic decompensation. Twenty-seven patients agreed to participate, provided written informed consent, and were subsequently enrolled in this study. Among these participants, 27 were afflicted with ascitic decompensation attributable to liver cirrhosis. The diagnosis of cirrhosis had previously been established based on clinical criteria in conjunction with liver elastography results and biopsies.

To assess patient follow-up, we conducted a review of medical records in January 2023. It is important to note that there were no interventions by the investigators between the time of study inclusion and this evaluation. Given that all patients were well-documented cases of end-stage liver cirrhosis under the care of our university hospital, we had access to comprehensive records maintained by the treating physicians.

An intraoral examination, encompassing a full mouth assessment, including the assessment of bleeding on probing, was conducted by a proficient dental medicine student (Ashouri MM under the supervision of the head of the Department of Periodontics, Preventive and Restorative Dentistry (author Beikler T). This comprehensive intraoral examination encompassed the evaluation of dental status, including the number of teeth, mucosal health, and oral hygiene, as measured by the sulcus bleeding index. The grading of periodontal disease was carried out in accordance with the guidelines and recommendations established by the European Federation of Periodontology/Oral Reconstruction and Rehabilitation[10].

All patients underwent a standardized interview and completed a questionnaire, which inquired about various parameters, including the frequency of their dental visits and their smoking habits.

To assess the prevalence of periodontitis in a control group unaffected by cirrhosis, we included 100 unselected patients from a typical dental practice as our control cohort. These patients were retrospectively studied and anonymized to protect their privacy. The control group comprised individuals who had undergone standardized periodontitis screening at a regular dental practice in Hamburg. Basic demographic information, such as age, sex, and periodontal status, was retrospectively analyzed for these anonymized patients in accordance with our local regulations and ethical guidelines. It is worth noting that this control cohort has been previously referenced in a prior publication[11].

Examinations

P. gingivalis was identified using species-specific PCR as previously described[12]. A probe was inserted into the deepest gum pocket of a sextant and then wiped onto a cotton swab, which was subsequently tested by PCR. Laboratory data and baseline patient characteristics, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin levels, age, clinical attachment loss – the most critical parameter for assessing periodontal tissue loss due to periodontal disease – the number of teeth, smoking status, and the patients' interest in dental medicine, were all recorded.

Statistical analysis

Continuous variables with a non-normal distribution were presented as the median and interquartile range. The distribution of these parameters among different groups was compared using the Mann-Whitney U-test. Categorical variables were expressed as numbers (%) and compared using Fisher's exact test. A significance level of $P < 0.05$ was used to determine statistical significance. Statistical analyses were conducted using SPSS, version 21.0 (IBM Corp., Armonk, NY, United States).

Ethical statement

This prospective study underwent review and received approval from the Ethics Committee of the Medical Council of Hamburg (PV-4081 and MC-368/18). The study adhered to the guidelines set forth in the Declaration of Helsinki. The retrospective analysis of the control cohort was conducted with complete anonymization, eliminating the need for further clarification or formal ethics committee approval in accordance with local laws and regulations.

RESULTS

In 22 out of 27 (82%) ascites patients, periodontitis was diagnosed. Characteristics of patients with periodontitis, in comparison with those without it, are presented in Table 1.

The age of the total cohort ranged from 37 to 76 years, with a median age of 57 years. Out of the total cohort, 15 individuals (56%) were male. Among the patients, nine (33%) had diabetes, and four (15%) were taking anticoagulants. Furthermore, 16 patients (59%) were diagnosed with alcoholic cirrhosis, five with non-alcoholic steatohepatitis (NASH) cirrhosis (19%), three (11%) had re-cirrhosis following liver transplantation, and one patient each (4%) had primary biliary cholangitis, hepatitis B virus (HBV), or hepatitis C Virus infections. Additionally, six patients had a Transjugular Intrahepatic portosystemic Shunt, and two patients had hepatocellular carcinoma (two with alcoholic liver cirrhosis, one with HBV, one with NASH). Notably, one of the patients with re-cirrhosis after transplantation had NASH as the underlying disease, another had primary sclerosing cholangitis, and one had alcoholic cirrhosis.

In order to assess the incidence of periodontitis in the cirrhosis cohort compared to the baseline incidence in the general population, we analyzed a retrospective cohort comprising 100 patients from a dental practice in Hamburg. This particular cohort has been previously described. Out of these subjects, 47 were male (47%), and their ages ranged from 17 to 89 years, with a median age of 51 years. Among these patients, 59 (59%) had periodontitis, a significantly lower rate than observed in the cirrhosis cohort, where the incidence was 82% ($P = 0.04$).

A notable finding was that a greater number of patients without periodontitis experienced mortality compared to those with periodontitis. Survival analysis using Kaplan-Meier methods indicated that patients with periodontitis had a longer survival duration compared to those without ($P = 0.02$, Figure 1). Additionally, transplant-free survival was more common among patients with periodontitis as opposed to those without (Table 1).

Among the patients, two had a complete set of teeth with no missing teeth (0/32), one patient had one missing tooth, two patients had three missing teeth, one patient had five missing teeth, six patients had six missing teeth, while one patient each had seven, eight, 13, 14, or 16 missing teeth. Additionally, three patients had ten missing teeth, two patients had 12 missing teeth, four patients had partial prostheses, and five patients were completely toothless and had total prostheses. Importantly, no association was found between the number of teeth and survival.

AST, ALT, bilirubin, and model for end-stage liver disease (MELD) scores showed no significant differences between patients with and without periodontitis (Table 1). Nonetheless, it is worth noting that there was a tendency towards a higher MELD score in patients without periodontitis, as depicted in Figure 2.

DISCUSSION

This study revealed a remarkably elevated incidence of periodontitis among cirrhotic patients (82%) when compared to healthy controls (59%, $P = 0.04$). This underscores the significance of regular dental check-ups for individuals with cirrhosis. Hepatologists should inquire explicitly about the dental care habits of their cirrhotic patients during medical history assessments and, if necessary, advocate for regular dental visits.

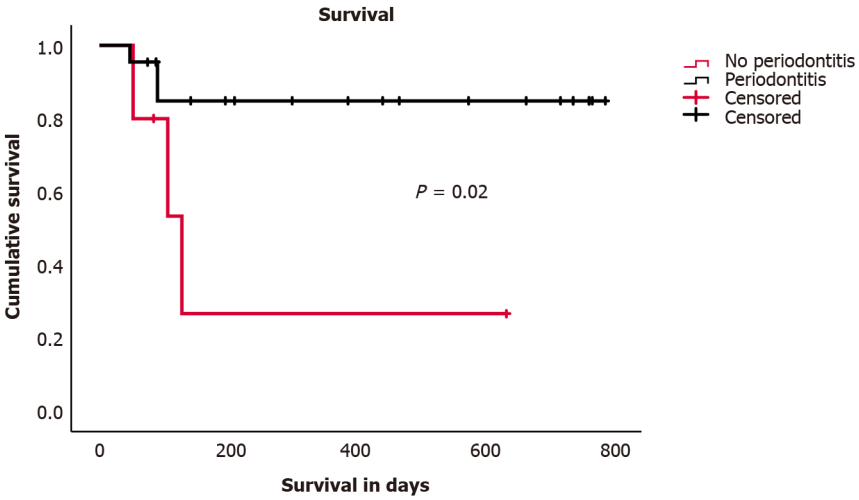
The identification of a substantial prevalence of periodontitis aligns with findings from a Danish study involving 262 cirrhotic patients. In this particular investigation, 46% ($n = 66$) exhibited severe periodontitis, 39% ($n = 55$) demonstrated moderate periodontitis, and only 15% ($n = 22$) displayed no or mild periodontitis[13]. Consequently, it is unequivocal that cirrhotic patients constitute a high-risk group for the development of periodontitis.

However, a far more critical question arises: whether periodontitis is correlated with reduced survival or transplant-free survival. In this regard, our study revealed that 63% of patients with periodontitis (12/22) survived without requiring transplantation, compared to 0% (0/5) of patients without periodontitis (Table 1, $P = 0.02$). Additionally, overall survival was superior among patients with periodontitis when compared to those without (Table 1, $P < 0.05$, Figure 1). Furthermore, there was a tendency toward higher MELD-score values in the five patients without periodontitis in contrast to the 22 patients with periodontitis (Figure 2). Hence, our preliminary study does not indicate worsened graft-free survival or a trend toward more severe liver damage (MELD score) in patients with periodontitis; in fact, it suggests slightly better outcomes. These findings diverge from a previously published study from Denmark[14]. In that study involving 184 cirrhotic patients, 44% had severe periodontitis, and unlike our study, there was a poorer survival

Table 1 Comparison of patients with and withour periodontitis

		Total cohort (*n* = 27)		*P* value
		Periodontitis (*n* = 22, 81.50%)	No periodontitis (*n* = 5, 28.50%)	
Male		12 (55%)	3 (60%)	1
Age, mean in years (range)		56 (37-76)	59 (56-68)	0.61
Smoker		17 (77%)	2 (40%)	0.13
Diabetes		8 (36%)	1 (20%)	0.62
Good oral hygiene		0 (0%)	3 (60%)	**< 0.01**
Outcome	Alive without transplantation	14 (63%)	0 (0%)	**0.02**
	Transplantation	5 (23%)	2 (40%)	0.61
	Deceased	3 (14%)	3 (60%)	**<0.05**
Motivated for oral hygiene (exact question was: "are you interested in oral hygiene")		12 (55%)	5 (100)%	0.11
MELD score, mean (range)		20 (15-25)	15 (7-22)	0.055
AST U/ml, mean (range)		75 (52-114)	109 (9-652)	0.83
ALT U/ml, mean (range)		66 (28-175)	50 (9-340)	0.15
Bilirubin mg/dL, mean (range)		6.0 (1.4-10.9)	3.3 (0.4-8.3)	0.17

P values determined by Mann-Whitney-test (age, aspartate aminotransferase, alanine aminotransferas, bilirubin, model for end-stage liver disease) and Chi-Square test (all other variables). AST: Aspartate aminotransferase; ALT: Alanine aminotransferas; MELD: Model for end-stage liver disease.

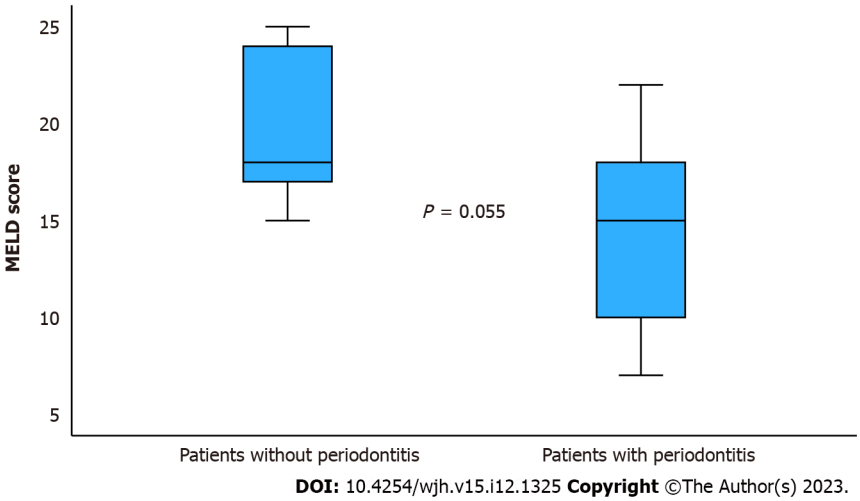


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Figure 1 Survival of patients with and without periodontitis (Kaplan-Meier analysis).

associated with the presence of severe periodontitis. The reasons behind this discrepancy and the tendency in our study toward better survival and lower MELD scores in cirrhotic patients with periodontitis remain unclear. However, it is crucial not to overinterpret this aspect, given the small sample size of five patients without periodontitis in our pilot study. Larger cohorts are needed to validate these findings, as less than 20% of the cirrhotic patients in our study did not have periodontitis.

A prior study established a link between the severity of NASH and the presence of periodontitis[11], a finding of particular relevance here. From a pathophysiological perspective, it is conceivable that the gingival entry point in periodontitis patients serves as a gateway for bacteria to enter the bloodstream, triggering cytokine release and inflammation. The intriguing aspect is that this inflammation did not appear to negatively impact the survival of our end-stage cirrhosis patients with ascites. Instead, our study revealed that patients without periodontitis had a less favorable survival outcome, which necessitates further investigation through large-scale studies.



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Figure 2 Model for end-stage liver disease score in patients (Mann-Whitney test, *P* = 0.055: Not significant). MELD: Model for end-stage liver disease.

Our pilot study holds considerable validity, being based on a well-defined and thoroughly characterized cohort of 27 patients. Nevertheless, larger cohorts are imperative for further exploration of this question. The limitations of our study encompass not only the relatively small patient sample but also the single-center study design and the varied nature of the inquiries we pursued. We simultaneously investigated the prevalence of periodontitis in decompensated cirrhosis patients, the potential translocation of *P. gingivalis* and *A. actinomyctemcomitans* into ascites, and the association between periodontitis and survival in these patients. We successfully addressed two of these three questions, specifically the frequency of periodontitis in decompensated cirrhotic patients and the potential bacterial translocation into ascites. However, to elucidate the third question concerning the impact of periodontitis on survival, larger cohorts are indispensable. Additionally, future studies should also prospectively examine whether gingival status and bacterial colonization change over time.

It is especially pertinent in this context to consider whether periodontal therapy can potentially enhance the survival prospects of end-stage liver cirrhosis patients. A recent review article unequivocally demonstrated that periodontal therapy could exert a beneficial influence on the progression of NASH[15]. It is worth exploring to what extent this therapeutic approach might also be applicable to cirrhotic patients. Additionally, a recent study conducted in the United States, involving 442 cirrhosis patients, revealed a significant association between poor oral health and 3-month hospitalizations, irrespective of portal hypertensive complications, minimal hepatic encephalopathy, or frailty[16].

Nonetheless, the primary objective of our study was to investigate whether classical periodontitis pathogens, namely *P. gingivalis* and *A. actinomyctemcomitans*, could be detected in decompensated cirrhotic patients through translocation into the ascites. However, we did not observe such translocation in our study.

CONCLUSION

Based on our small pilot study, it appears that these two bacteria may not play significant roles in the development of ascites or potentially in the occurrence of SBP. This particular question had not been explored previously. While our study did not confirm the hypothesis that these microorganisms could enter ascites from the gingival reservoir in decompensated cirrhotic patients, this finding remains noteworthy because it provides conclusive clarification on the matter.

ARTICLE HIGHLIGHTS

Research background

This pilot study examines the prevalence of periodontitis in cirrhotic patients experiencing ascite decompensation.

Research motivation

Previous studies have not investigated whether bacteria from the oral mucosa associated with periodontitis can be translocated into the ascites of cirrhotic patients.

Research objectives

To investigate the significance of periodontitis in cirrhotic patients with ascites.

Research methods

This is a prospective cohort study. The oral hygiene and dental status of 27 patients with cirrhosis and ascites decompensation were documented. The prevalence of periodontitis in these patients was compared to that of 100 unselected patients from a standard dental practice. Samples from ascites and gingiva were tested for *Porphyromonas gingivalis* (*P. gingivalis*) and *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) using PCR.

Research results

Periodontitis was diagnosed in 22 out of 27 patients (82%) with ascites. This rate is significantly higher than in the control group of 100 unselected patients, where the rate was 59% (P = 0.04). *P. gingivalis* was identified in the gingiva of six patients and concurrently in the stool of one patient. However, *P. gingivalis* was not found in the ascites of any patient. Of the patients who tested positive for *P. gingivalis*, 83% (five out of six) suffered from periodontitis. *A. actinomycetemcomitans* was not detected in any of the samples. Significantly, a greater number of patients without periodontitis passed away compared to those with periodontitis, and the survival rate (as determined by the Kaplan-Meier analysis) was longer for patients with periodontitis (P = 0.02). Transplant-free survival was observed more often in patients with periodontitis than those without (63% *vs* 0%, P = 0.02).

Research conclusions

Periodontitis is common in cirrhotic patients with ascites.

Research perspectives

Hepatologists should recommend regular dental visits for cirrhotic patients. Future studies should assess whether this recommendation improves dental health and reduces the incidence of periodontitis.

FOOTNOTES

Author contributions: Pischke S and Ashouri MM contributed equally to this work; Pischke S, Ahouri MM, Peters U, Shiprov A, Schulze Zur Wiesch J, Sterneck M, Fischer F, Hüberner P, Mader M, Fischer L, Fründt T, Aarabi G and Beikler T designed this study and wrote the manuscript.

Institutional review board statement: This prospective study was reviewed and approved by the Ethics Committee of the Medical Council of Hamburg (PV-4081 and MC-368/18). The study was performed according to the recommendations of the Declaration of Helsinki. The retrospective analysis of the control cohort was completely anonymized and therefore did not require any clarification or formal ethics committee approval according to local laws and regulations.

Conflict-of-interest statement: All the Authors have no conflict of interest related to the manuscript.

Data sharing statement: No additional data are available.

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CASE REPORT

Drug induced autoimmune hepatitis: An unfortunate case of herbal toxicity from Skullcap supplement: A case report

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Abstract

BACKGROUND

The surge in traditional herbal dietary supplement (HDS) popularity has led to increased drug-induced liver injuries (DILI). Despite lacking evidence of efficacy and being prohibited from making medical claims, their acceptance has risen over sevenfold in the last two decades, with roughly 25% of United States (US) adults using these supplements monthly. An estimated 23000 emergency room visits annually in the US are linked to HDS side effects. NIH-funded research suggests HDS contribute to 7-20% of DILI cases, with similar trends in Europe—Spain reporting 2% and Iceland up to 16%. Patients with acute liver failure from HDS undergo liver transplantation more frequently than those from prescription medicines. Here we describe a case of drug-induced autoimmune hepatitis due to Skullcap supplements, this association appears to be the first documented instance in literature.

CASE SUMMARY

A middle-aged Caucasian woman, previously healthy, presented with sudden jaundice. Four months earlier, her liver enzymes were normal. She mentioned recent use of Skullcap mushroom supplements. Tests for chronic liver disease were negative. The first liver biopsy indicated severe resolving drug-induced liver injury. Despite treatment, she was readmitted due to worsening jaundice. Follow-up tests raised concerns about autoimmune hepatitis. A subsequent biopsy confirmed this diagnosis. The patient responded as expected to stopping the medication with improvement in liver enzymes.

CONCLUSION

This scenario highlights an uncommon instance of DILI caused by Skullcap supplements. It's crucial for hepatologists to recognize this connection due to the increasing prevalence of herbal supplements.

Key Words: Drug induced liver injury; drug induced autoimmune hepatitis; herbal

supplement; Skullcap; Case report

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Core Tip: This case report highlights a rare presentation of drug induced liver injury from Skullcap supplement usage. While Chinese Skullcap (Scutellaria baicalensis) has been associated with a mixed hepatocellular and cholestatic picture of drug-induced liver injuries, the association of North American Skullcap is not as robust. Here we present a case of drug induced autoimmune hepatitis from North American Skullcap supplement use. Hepatologists must be aware of this association of rare histopathological presentation.

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INTRODUCTION

The rise in popularity of traditional herbal dietary supplements (HDS) has caused an increase in incidence of drug induced liver injury (DILI). Herbs and botanicals along with their extracts and metabolites fall under the umbrella term "dietary supplements" in the United States[1]. Even though these agents generally lack proof of efficacy and that their manufacturers are not permitted to make medical claims, their acceptance in the society has increased over 7-fold in the last two decades[2]. Approximately, 50% of the adult population in the United States admits to having used a "dietary supplement" in the last month[3]. It is estimated that there are 23000 emergency room visits in the United States each year are secondary to the side effects of HDS[4]. The true incidence of liver injury secondary in the United States to HDS is difficult to estimate. The NIH funded Drug Induced Liver Injury Network estimates that approximately 7%-20% all cases of DILI are secondary to HDS[5]. Which is akin to the data from Europe, with Spain reporting 2% of all DILI cases as secondary to HDS and Iceland reporting the rate as high as 16%[6,7]. In addition, patients presenting with acute liver failure secondary to HDS have been found to undergo liver transplantation more frequently than those with DILI from prescription medicines, (56.1 *vs* 31.9%, $P < 0.005$)[8]. As such, awareness about the side effects of these medications and a comprehensive understanding of their outcomes is paramount. Here we describe a case of drug induced autoimmune hepatitis (DIAIH) resulting from Skullcap supplements. To our understanding, this is the first such association described in literature

CASE PRESENTATION

Chief complaints

New onset Jaundice.

History of present illness

A 62-year-old Caucasian female, with Sjogren's disease presented with generalized fatigue, arthralgias, pruritus and new onset jaundice. She denied any prior history of liver disease, alcohol intake, intravenous or intranasal drug use, blood transfusions or needlestick injuries. She did however endorse taking Skullcap supplements over for 1-2 months due to long standing history of anxiety and insomnia.

History of past illness

Sjogren's syndrome; Anxiety; Insomnia.

Personal and family history

Nonsmoker, No alcohol or illicit Drug use. No gastrointestinal (GI) related malignancy or Liver disease in the family.

Physical examination

General: Age appropriate female in no distress. HEENT: Atraumatic, normocephalic; scleral icterus present, moist mucous membranes. Cyclic vomiting syndrome: S1,S2+ RRR. Lungs: Symmetric chest rise seen. Abdomen: Soft, non-distended, non-tender, baroreceptor sensitivity present, no palpable hepatomegaly appreciated. Extremities: No edema or clubbing. Skin: Jaundiced. Neuro: Alert, Awake, oriented × 3. No gross neurological deficits appreciated.

Laboratory examinations

The initial laboratory results on presentation that were pertinent: International normalized ratio (INR) 2.4, Alkaline Phosphatase (Alk Phos) 164 IU/L, aspartate transaminase (AST) 1091 IU/L, alanine transaminase (ALT) 980 IU/L, total bilirubin (T bili) 9.5 mg/dL. The R factor on initial calculation was 20.5, indicative of a primary hepatocellular injury pattern. Immunoglobulin G was elevated at 2573 mg/dL. Testing for Viral Hepatitis including - Hepatitis A, B, and C, cytomegalovirus, Epstein-Barr virus, and herpes simplex virus were all negative including serology and quantitative testing. ANA was positive given her history of Sjogren's disease. Her baseline AST, ALT, Alk phos and bilirubin were within normal range 4 months before presentation.

Imaging examinations

Magnetic resonance cholangiopancreatography done at admission did not show any signs obstruction or primary hepatic pathology.

Case Summary

Given her prior history of autoimmune disease and new onset hypergammaglobulinemia in the context of suspected DILI, a liver biopsy was pursued. Her initial liver biopsy revealed resolving centrilobular necrosis with predominant eosinophilic inflammation. Over the next 72 h, the liver enzymes showed a strong downward trend. As such, the patient was discharged with outpatient follow-up. However, the patient was re-admitted a month later with worsening jaundice, acute kidney injury, and new onset ascites concerning for subacute liver failure. Her LFTs were Alk Phos 619 IU/L, AST 1222 IU/L, ALT 540 IU/L, and T bili 6.6 mg/dL. Infectious workup was unremarkable. In the next few days, the transaminases showed a downtrend. However, the T bili continued to rise, reaching its peak at 11.8 gm/dl. Paracentesis showed a serum ascites albumin gradient of 1.4 which was consistent with portal hypertension. A repeat liver biopsy was pursued which revealed extensive plasma cells consistent with new onset autoimmune hepatitis resulting from previous DILI (Figure 1). Unfortunately, the patient's course was complicated by the development of spontaneous bacterial peritonitis, GI bleed and acute tubular necrosis necessitating dialysis. As such, she was never challenged with steroids. Due to her worsening status, she was listed for a simultaneous liver kidney transplantation. However, the patient finally did improve following a long and protracted course with resolution of jaundice but remained on hemodialysis.

FINAL DIAGNOSIS

DILI causing autoimmune Hepatitis.

TREATMENT

Cessation of culprit drug.

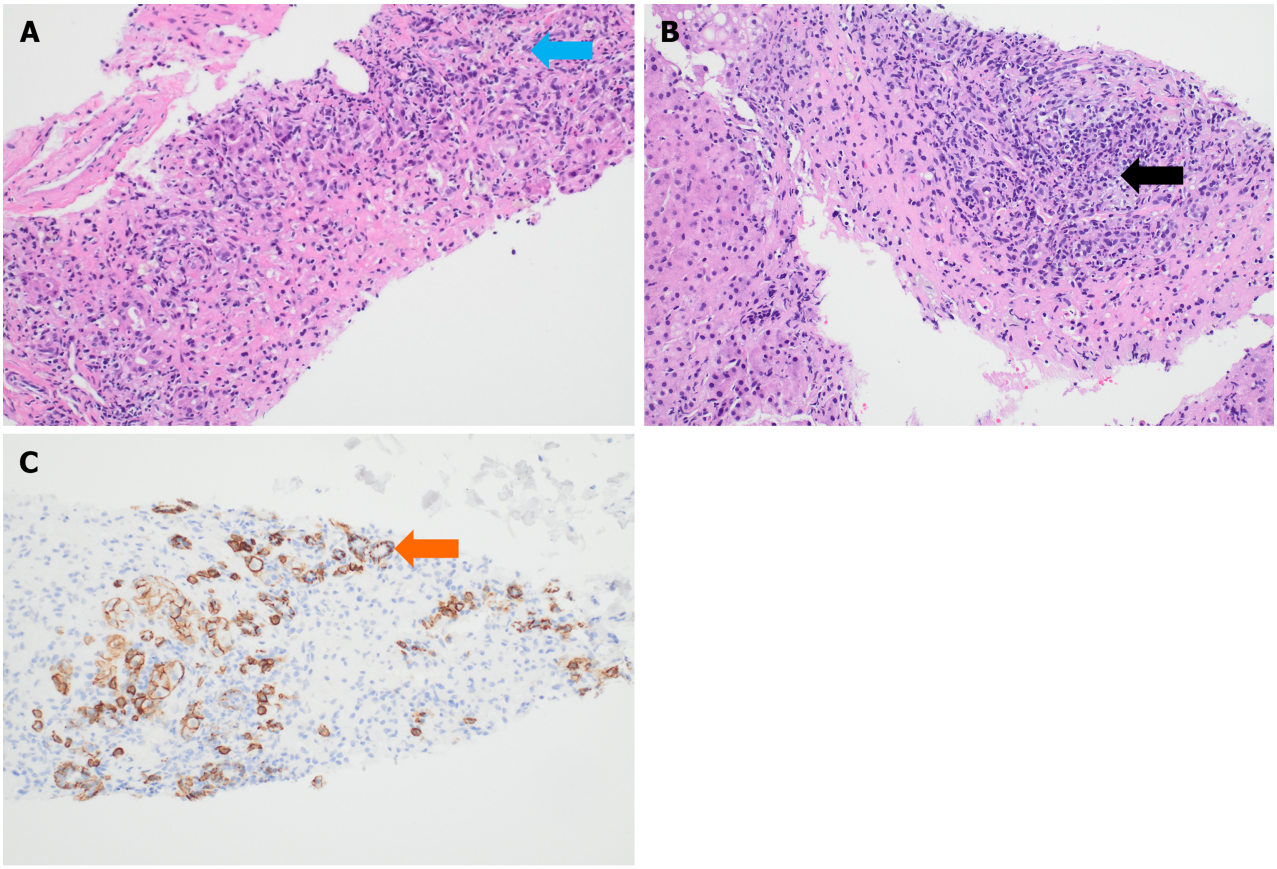
OUTCOME AND FOLLOW-UP

Resolution of Jaundice.

DISCUSSION

The last three decades have heralded an increased use of herbal medicinal products with up to 25% of the adult American population admitting to their use at some point[9]. These products are not regulated by the Food and Drug Administration and their side effect profile remains largely unknown. Skullcap is a plant native to North America (*Scutellaria lateriflora*), which has been used for centuries to treat anxiety, digestive disorders, and menstrual disorders. Skullcap extracts contain large quantities of flavonoids like scutellarin and baicalin which account for its sedative and antispasmodic activities[10]. However, Skullcap has been associated with a mixed hepatocellular and cholestatic pattern of liver injury. Additionally, majority of cases of DILI from Skullcap are attributed to Chinese Skullcap (*Scutellaria baicalensis)*, while the association of North American Skullcap with DILI is not as robust[11]. In our case, as is evident form the biopsy and the corresponding serology, the patient did have DIAIH.

Castiella *et al*[12] have postulated a-5-fold classification for drug induced autoimmune liver disease. Type 1, autoimmune hepatitis (AIH) with DILI: Reactivation of pre-existing AIH after the introduction of a new drug. Type 2, Drug induced Autoimmune Hepatitis (DIAIH): New onset AIH resulting from DILI. This results from an immune mediated reaction in a genetically primed individual, resulting in the necessitation of immunosuppressive treatment. Type 3, Immune Mediated DILI (IMDILI): acute or chronic liver injury that resolves upon cessation of the drug. DILI is often accompanied by a myriad of other features including fever, eosinophilia, lymphadenopathy, and rash. Individuals usually respond well to treatment and achieve sustained remission without relapse. Type 4, mixed autoimmune type: Mixed features of DI-AIH and IM-DILI. Individuals exhibit a full response to treatment, but assessing relapse is hindered



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Figure 1 Histopathology. A: Initial biopsy showing confluent centrilobular necrosis with interspersed eosinophils (blue arrow); B: Resolving centrilobular necrosis with plasma cells (black arrow); C: Immunohistochemical staining of plasma cells with CD 138 (orange arrow).

for non-hepatological reasons. Lastly, type 5 encompasses individuals with DILI and positive autoimmune antibodies. The significance of these antibodies remains uncertain.

CONCLUSION

This case outlines a rare presentation of DILI from Skullcap supplements. Hepatologists must be aware of this association as the popularity of herbal supplements continues to rise.

FOOTNOTES

Author contributions: Thakral N, Konjeti VR, and Salama F were involved in patient care and drafting of the manuscript.

Informed consent statement: Our institution does not require separate consent for case reports.

Conflict-of-interest statement: There are no conflicts of interest associated with our report.

CARE Checklist (2016) statement: The authors have read the CARE Checklist (2016), and the manuscript was prepared and revised according to the CARE Checklist (2016).

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CASE REPORT

Hepatic pseudotumor associated with *Strongyloides* infection: A case report

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Abstract

BACKGROUND

Strongyloides sterocoralis is a parasitic infection caused by a roundworm that is transmitted through soil contaminated with larvae. It can infrequently cause hepatic abscesses in immunocompromised patients and is rarely reported to form hepatic lesions in immunocompetent hosts.

CASE SUMMARY

We present a case study of a 45-year-old female who presented with right upper quadrant abdominal pain and constitutional symptoms for several weeks. Cross-sectional imaging identified several malignant-appearing liver masses. Further investigation, including serological testing and histopathologic examination, revealed the presence of serum *Strongyloides* antibodies and hepatic granulomas with extensive necrosis. Following treatment with ivermectin for 2 wk, there was complete resolution of the liver lesions and associated symptoms.

CONCLUSION

This case highlights the importance of considering parasitic infections, such as *Strongyloides*, in the differential diagnosis of hepatic masses. Early recognition and appropriate treatment can lead to a favorable outcome and prevent unnecessary invasive procedures. Increased awareness among clinicians is crucial to ensure the timely diagnosis and management of such cases.

Key Words: Hepatic tumor; Liver mass; *Strongyloides sterocoralis*; Parasitology; Case report

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Core Tip: Hepatic pseudotumor is a clinical entity that can mimic malignant tumors of the liver. We report a rare case of hepatic pseudotumor caused by *Strongyloides sterocoralis*. Combined with a review of cases indexed in PubMed, we summarize the infectious causes of hepatic pseudotumor. Recognition of hepatic mass-forming parasitic infections may expedite prompt medical management.

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INTRODUCTION

Inflammatory pseudotumors are mass-like lesions that have been described in various solid organs that mimic the appearance of a neoplasm[1]. Inflammatory pseudotumors represent a heterogeneous group of lesions of both infectious and inflammatory etiology, characterized histologically by the presence of fibroblasts or myofibroblasts and inflammatory cells and the lack of neoplastic cells. When occurring in the liver, hepatic pseudotumor often presents as a solitary, well-defined mass[1]. Due to its radiographic similarity to malignant liver tumors, potential misdiagnosis and unnecessary invasive interventions can occur. Given the variety of clinical presentations, including the absence of symptoms, the incidence of hepatic pseudotumor is not well characterized. It may account for 1% of all resected hepatic tumors[2], highlighting the importance of accurate diagnosis to prevent unneeded surgical resection. The pathogenesis of hepatic pseudotumor is poorly characterized but may be secondary to an exaggerated inflammatory response within the liver[1,3], as they often arise in the setting of infection, autoimmune disease, or recent trauma or surgery[1,3].

Strongyloidiasis is a parasitic infection known to infect the liver and biliary tree[3]. It is caused by the nematode *Strongyloides sterocoralis*, which is endemic in regions of West Africa, the Caribbean, and Southeast Asia – where the prevalence may be as high as 40%[4]. The parasitic larvae penetrate the skin and most commonly distribute to the gastrointestinal and pulmonary systems[5]. Less commonly, disseminated strongyloidiasis occurs when additional organs, including the liver, are involved[5,6]. Disseminated strongyloidiasis with hepatic infection has been primarily described in immunocompromised patients[7] but has rarely been observed in immunocompetent hosts[8].

Herein, we present a rare case of strongyloidiasis with hepatic involvement in an immunocompetent patient leading to the formation of a pseudotumor. By review of this case and the published literature, we summarize the incidence, diagnosis and treatment of *Strongyloides* pseudotumor.

CASE PRESENTATION

Chief complaints

A 45-year-old female was referred from the emergency room to hepatopancreatobiliary clinic for evaluation of liver lesions. She described several weeks of weight loss, decreased appetite, early satiety, fatigue, and right upper quadrant abdominal pain.

History of present illness

The patient was initially evaluated in the emergency room, where she reported a 15 lb weight loss secondary to abdominal pain and anorexia. The patient denied any change in bowel habits, sick contacts, or recent travel. The patient reported that her partner had immigrated from West Africa and that she had traveled there with him approximately 5 years earlier. She had also lived for several years in South Carolina, where *Strongyloides* may be endemic.

History of past illness

The patient had a history of hepatic steatosis, uterine fibroids, and morbid obesity. Upper and lower endoscopy had been performed 3 years earlier and were both found to be within normal limits.

Personal and family history

The patient denied any family history of malignancy.

Physical examination

The vital signs were as follows: Body temperature, 36.5°C; blood pressure, 136/76 mmHg; heart rate, 82 beats per min; and respiratory rate, 12 breaths per min. Abdominal exam was notable for tenderness in the right upper quadrant. There was no hepatosplenomegaly. There were no skin lesions present.

Laboratory examinations

Laboratory examination was notable for marked eosinophilia [0.9 × 10^3/mL (range 0.0-0.40)] and an elevated sedimentation rate (Westergren; 94 mm/h). Tumor markers were normal (alpha-fetoprotein < 1.8 ng/mL; carbohydrate antigen 19-9 16 U/mL; carcinoembryonic antigen 2.1 ng/mL). Liver function tests were normal, and hepatitis serologies were consistent with previous hepatitis B immunization. Human immunodeficiency virus (HIV) antibody (Ab)/p24 antigen screen, rapid plasma reagin, coccidioides Ab panel, cryptococcus antigen, and quantiFERON-TB Gold Plus were negative. Autoimmune markers were negative (anti-myeloperoxidase Ab < 0.2 units; anti-proteinase 3 Ab < 0.2 units; cytoplasmic antineutrophil cytoplasmic Ab (ANCA) < 1:2 titer; perinuclear ANCA (p-ANCA) < 1:20 titer; atypical p-ANCA < 1:20 titer; angiotensin converting enzyme, serum 42 U/L; anti-cyclic citrullinated peptide Ab, immunoglobulin G (IgG)/IgA 2 U; rheumatoid factor < 10.0 IU/mL; antinuclear Ab direct-negative).

Imaging examinations

An abdominal ultrasound performed in the emergency room noted a focal hypoechoic area within the right hepatic lobe centrally measuring 2.5 cm × 1.6 cm × 1.3 cm. Subsequently, a gadolium-enhanced magnetic resonance image demonstrated a non-cirrhotic liver with a peripherally T2 hyperintense and centrally T2 hypointense 3.7 cm × 3.2 cm × 3.7 cm lesion (Figure 1). Signal characteristics raised suspicion for a malignancy, but the rapid interval growth (from ultrasound-estimated size) suggested possible infection. A computerized tomography scan through the abdomen (Figure 2) demonstrated a low-density mass within the right hepatic lobe measuring up to 4.5 cm × 4.9 cm in the largest dimensions. There was peripheral enhancement with centripetal filling atypical for a hemangioma.

FINAL DIAGNOSIS

The final diagnosis was hepatic pseudotumor caused by *S. sterocoralis*.

TREATMENT

The treatment provided to the patient was ivermectin 24 mg by mouth daily for 2 wk.

OUTCOME AND FOLLOW-UP

Liver lesions and systemic symptoms both resolved shortly after treatment (Figure 3).

DISCUSSION

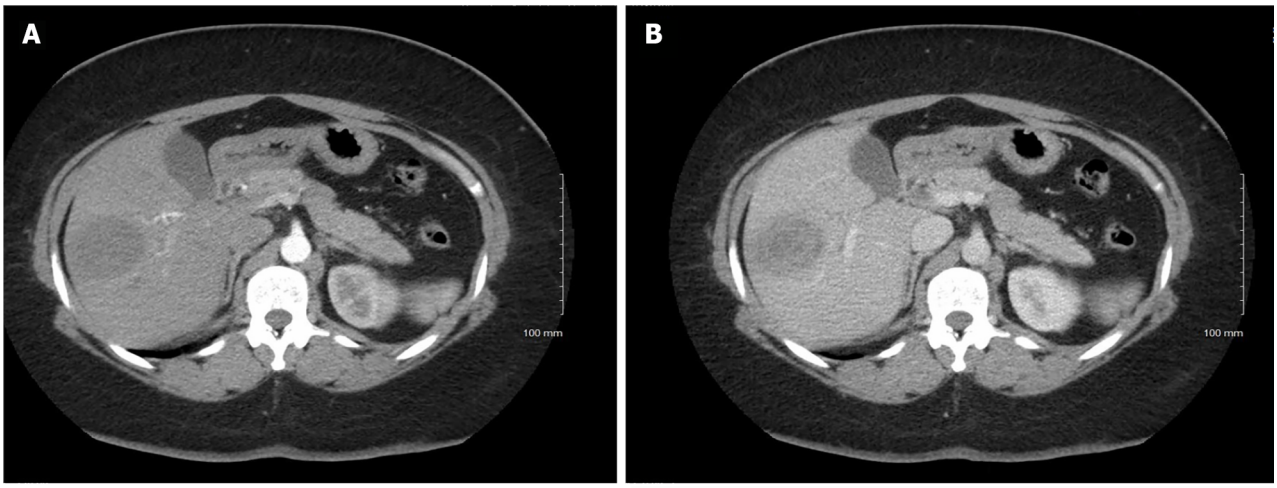
Inflammatory pseudotumor can be idiopathic, infectious, or inflammatory in nature; yet, there are several clinical features of hepatic pseudotumors that suggest an infectious etiology. We present a case of infectious hepatic pseudotumor caused by *S. sterocoralis*. The systemic symptoms of anorexia and malaise, coupled with marked eosinophilia, positive *Strongyloides* serum antibodies, and the presence of granulomas with necrosis on pathologic evaluation, along with clinical improvement with antihelminth therapy, support this rare diagnosis and highlight the importance of considering such infectious etiologies in the management of hepatic masses.

To date, the medical literature includes only two published reports of *Strongyloides* hepatic pseudotumor[7,8], which were diagnosed in patients with immunocompromise secondary to HIV or steroid use, respectively. While expected to be rare in the United States, the true prevalence of *Strongyloides* is not known. In the southeastern United States, particularly Appalachia, *Strongyloides* seroprevalence may approach 2%, and may be as high as 10% in South Carolina[9] - where the patient had previously resided. More commonly, *Strongyloides* is endemic to the soil of tropical and subtropical countries, where the nematode larvae migrate through the skin into human hosts and mature to the adult stage[10-12]. *Strongyloides* can perpetually reinfect a patient as the rhabditiform larvae develop into infective filariform larvae and pass into the feces. Strongyloidiasis may remain asymptomatic for many years in immunocompetent patients[10], whereas hyperinfection syndrome and disseminated strongyloidiasis are more common among immunosuppressed hosts[11,12]. In healthy individuals, nematodes have been shown to stay in the body for over 50 years without causing symptoms. It is plausible that this particular case represents a delayed presentation of a clinical exposure years prior with a long period of asymptomatic infection.



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Figure 1 Representative magnetic resonance imaging of hepatic pseudotumor. Gadolium-enhanced magnetic resonance imaging demonstrated a peripherally T2 hyperintense and centrally T2 hypointense lesion.

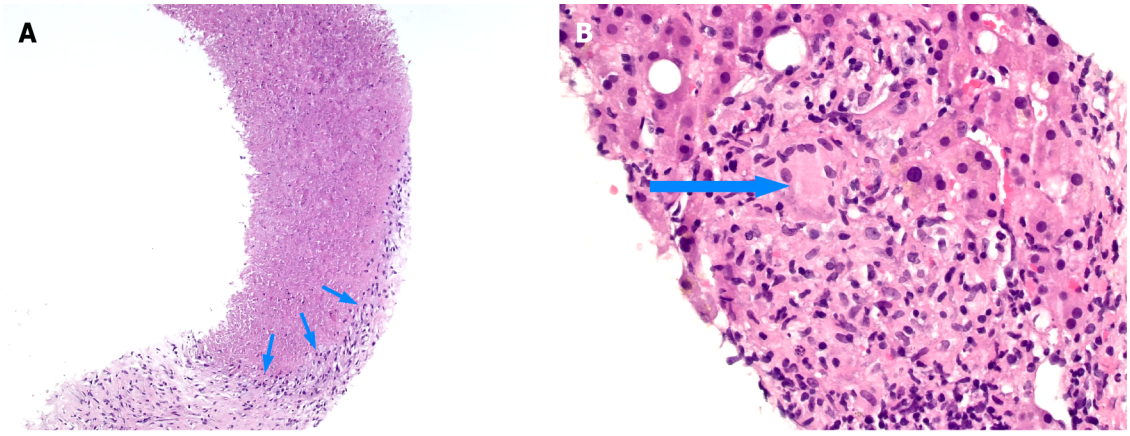


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Figure 2 Representative computerized tomography imaging of hepatic pseudotumor. A-B: Computerized tomography scan through the abdomen with arterial (A) and venous (B) phases demonstrated a low-density mass within the right hepatic lobe measuring up to 4.5 cm × 4.9 cm in largest dimensions. There was peripheral enhancement with centripetal filling; however, the enhancement was contiguous without significant nodularity and thus atypical for a hemangioma.

Other etiologies of infectious hepatic pseudotumor include *Mycobacterium tuberculosis*, *Brucella*, *Bartonella*, *syphilis*, *Candida,* and *Actinomyces*. *Tuberculosis* can cause a localized hepatic lesion, either as a tuberculoma with confluent granulomas and few organisms in immune competent individuals, or a tuberculous abscess, which is often suppurative and contains numerous organisms in immunocompromised hosts[13]. A *Brucelloma* is typically seen in the context of reactivation of remote infection, usually originating from animals or animal products from high-risk regions, typically surrounding the Mediterranean[14].The clinical presentation of hepatic *Brucelloma* overlaps with many of the symptoms of brucellosis, typically chronic, undulating fevers, malaise, and anorexia. There may be right upper quadrant pain. Diagnosis of hepatic *Brucelloma* is validated by serological positivity and positive cultures on blood or tissue samples. Hepatic cat scratch disease, caused by *B. henselae*, may present with abdominal lymphadenopathy and skin papules. It can form irregular stellate hepatic abscesses, histologically characterized by palisading histiocytes, lymphocytes, and a rim of fibrosis[13]. Hepatic syphilis can occur in both immunocompetent and immunocompromised patients[15-17]. Immunohistochemical stain for *Treponema pallidum* highlights the organisms in these pseudotumors; additionally, there is often periductal edema and neutrophilic pericholangitis in portal tracts outside the mass lesion. Hepatic *candidiasis* is most often observed in immunocompromised patients[18] and on histologic examination, may show granulomas or abscesses containing pseudohyphae and/or yeast forms[13]. Lastly, *Actinomycosis* can cause hepatic fibrotic masses containing characteristic sulfur granules and colonies of filamentous organisms[19,20]. In summary, patient history and exposures are important considerations when considering these infectious causes of hepatic pseudotumor in order to guide further testing for diagnostic confirmation.

While most hepatic pseudotumors are presumed to be a response to infection, the causative agent is not always identified. Additionally, there are several malignancies and inflammatory diseases that must be considered. Beyond primary (*i.e.* cholangiocarcinoma and hepatocellular carcinoma) and metastatic liver tumors, there are several neoplasms that particularly resemble hepatic pseudotumor. These include inflammatory myofibroblastic tumor (IMT), follicular



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Figure 3 Histologic examination of the liver lesion. A: The biopsy shows loose fibrous tissue (bottom) with large areas of necrosis rimmed by histiocytes (arrows), hematoxylin and eosin stain (100 ×); B: The adjacent viable hepatic parenchyma contains few non-necrotic granulomas. The arrow denotes a multinucleated giant cell within the granuloma, hematoxylin and eosin stain (400 ×).

dendritic cell tumor, and less commonly, Hodgkin lymphoma. When in question, further testing on tissue samples can help distinguish these diagnoses (IMT with *ALK* or *ROS1* expression; follicular dendritic cell tumor with *in situ* hybridization for Epstein-Barr virus; Hodgkin lymphoma with immunohistochemical analysis). Non-infectious inflammatory hepatic pseudotumor can be observed in IgG4-related disease. While most commonly manifested as a form of sclerosing cholangitis and thus a mimic of cholangiocarcinoma, IgG4-related hepatic pseudotumors can form adjacent to involved ducts[21,22]. Histologically, IgG4-related hepatic pseudotumors are characterized by numerous IgG4-positive plasma cells on immunohistochemical staining.

Several limitations warrant emphasis. For this case, the *Strongyloides* IgG Ab titer was not available, and was only reported by the laboratory as a binary (*i.e.* positive/negative); thus, we cannot comment on the degree of elevation that may be observed with this subset of hepatic disease. Second, for completeness of the presentation of the history and evaluation of this case, we hoped to include a representative image of the *Strongyloides* larvae from the stool examination, but this could not be provided by the commercial laboratory involved.

CONCLUSION

Hepatic masses are a heterogenous pathology of malignant, inflammatory and infectious etiologies. This rare case study of hepatic pseudotumor caused by strongyloidiasis infection highlights the broad diversity of diagnoses that need be considered, even in geographic regions and patient cohorts not typical of opportunistic infections. Moreover, the clinical presentation – namely, eosinophilia, positive serum antibodies and necrotic granulomas – review the typical features of *Strongyloides*-induced pseudotumor. Further research is warranted to elucidate the underlying biology of hepatic pseudotumor formation and improve diagnostic accuracy. Recognizing the pivotal role of infections in the differential diagnosis of hepatic pseudotumor is paramount for safeguarding patients from undergoing unnecessary invasive procedures. This approach not only facilitates accurate diagnosis and timely initiation of appropriate treatments but also prevents unwarranted interventions, underscoring the significance of a comprehensive consideration of infections in the diagnostic algorithm of hepatic pseudotumor cases.

FOOTNOTES

Author contributions: Grandhi MS, Alexander HR, and Ecker BL contributed to the conceptualization and supervision of the research; Gialanella JP, Alexander RH, Korpela K, and Ecker BL contributed to data collection and data analysis; Gialanella JP and Ecker BL contributed to the manuscript writing; All authors have read and approved the final manuscript.

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