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REVIEW

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

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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

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

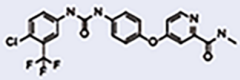
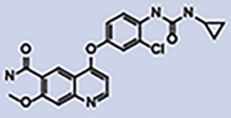
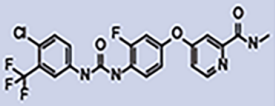
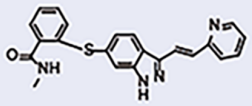
Drugs	Chemical structure	Year of FDA approval	Used dose	Family	Target	Ref.
First line therapies						
Sorafenib		2007	400 mg twice daily	Tyrosine kinase receptor family inhibitor	VEGFR 1, VEGFR 2, PDGFR-β, RET, c-Kitand FMS-like tyrosine kinase-3, Ras/MAPK pathway.	[84,87]
Lenvatinib		2018	12 mg(Child-Pugh 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

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

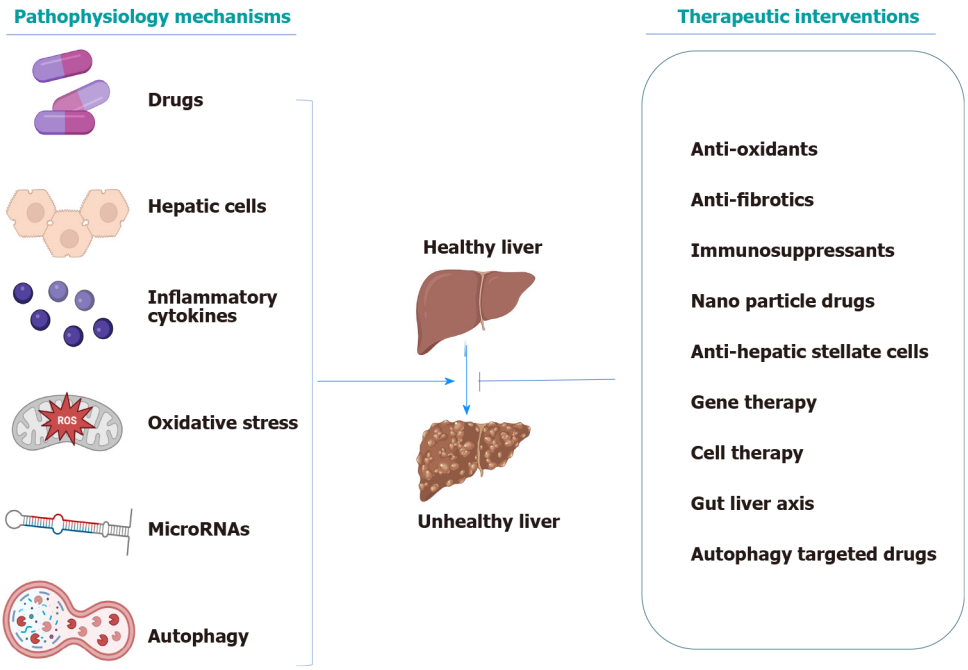

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Figure 1 Graphical abstract. Illustration diagram to explore the pathophysiological mechanisms and possible therapeutic intervenension of acute and chronic liver diseases.

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

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

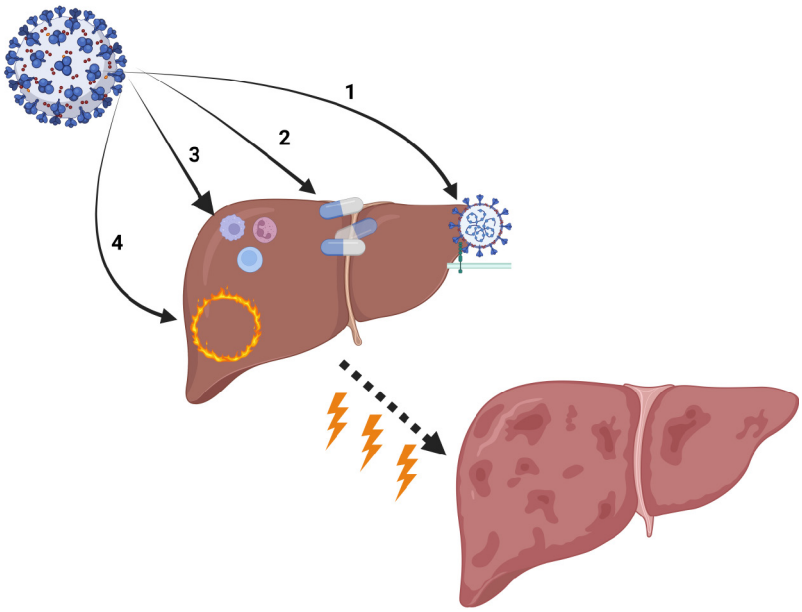

Figure 1 Different mechanisms of liver impairment due to severe acute respiratory syndrome coronavirus 2 infection. 1: Direct effect of the virus on the liver cells; 2: Drug-induced injury; 3: General response of the immune system; 4: Systemic inflammation and tissue hypoxia. The figure was created with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

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

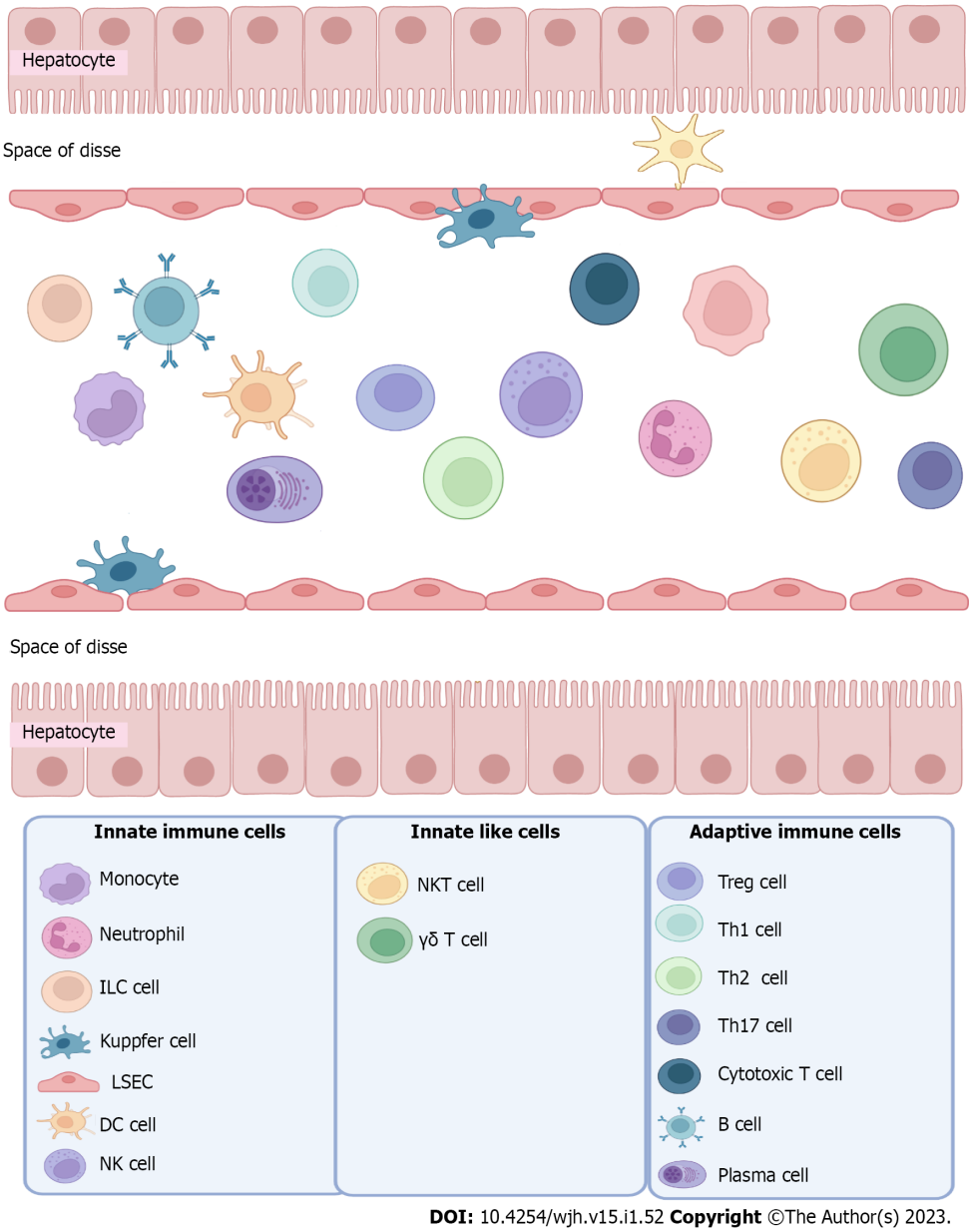

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Figure 1 Cell composition of the healthy liver. ILC: Innate lymphoid cells; DC: Dendritic cells; LSEC: Liver sinusoidal endothelial cells; NK: Natural killer; NKT: Natural killer T.

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].

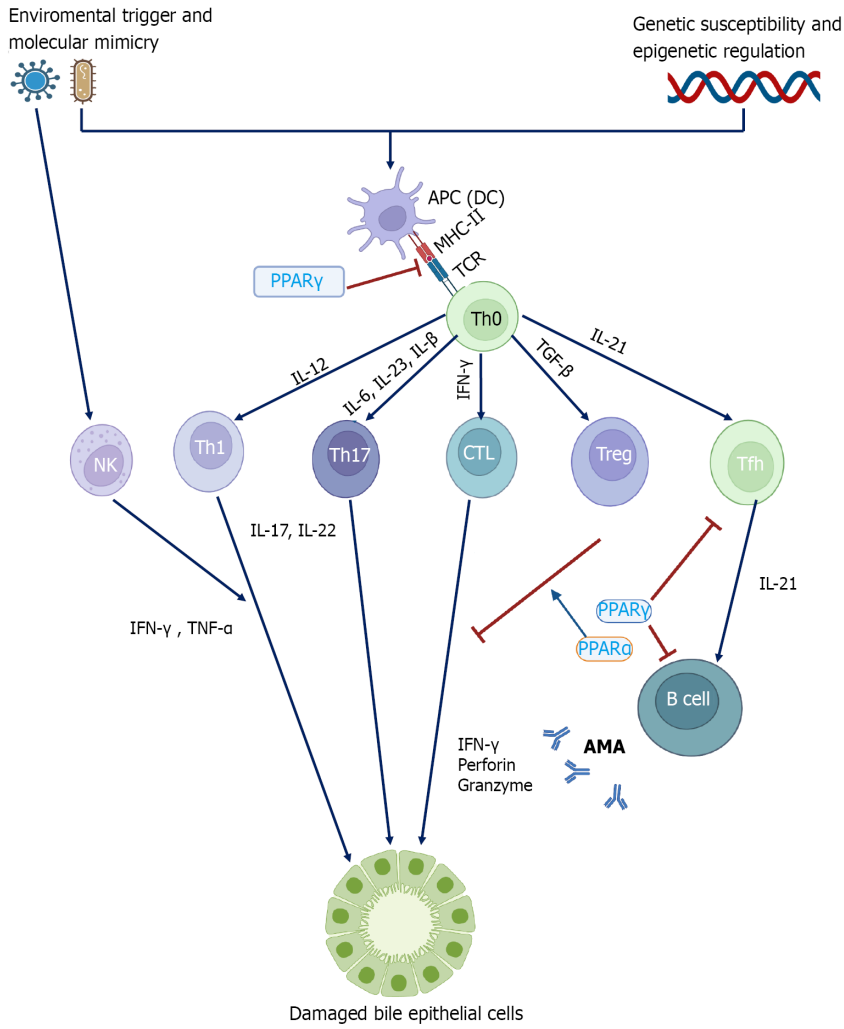

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Figure 2 Model of pathogenic mechanisms in primary biliary cholangitis. APC: Antigen presenting cell; DC: Dendritic cell; NK: Natural killer; IL: Interleukin; TNF: Tumor necrosis factor; TGF-β: Transforming growth factor β; IFN-γ: Interferon-gamma;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

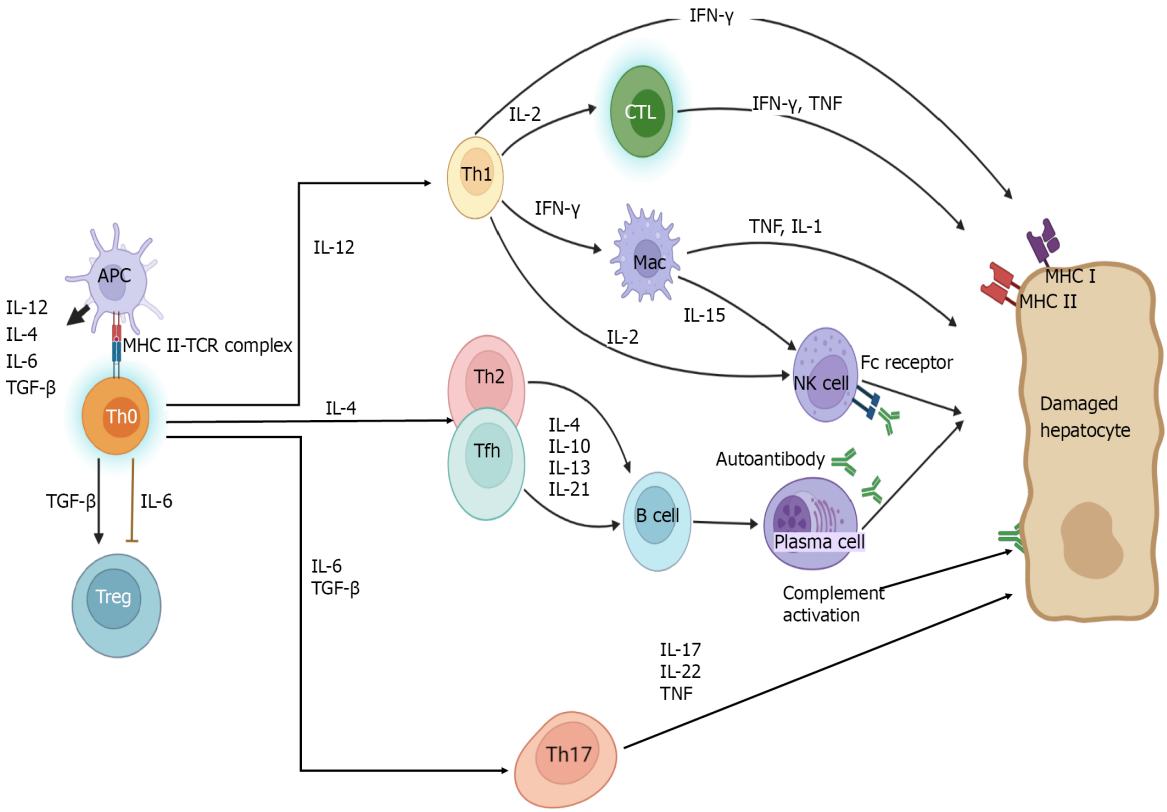

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Figure 3 Pathogenic pathways of autoimmune hepatitis. APC: Antigen-presenting cell; CTL: Cytotoxic T lymphocyte; Mac: Macrophage; IL: Interleukin; TNF: Tumor necrosis factor; MHC: Major histocompatibility complex; TGF-β: Transforming growth factor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

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).

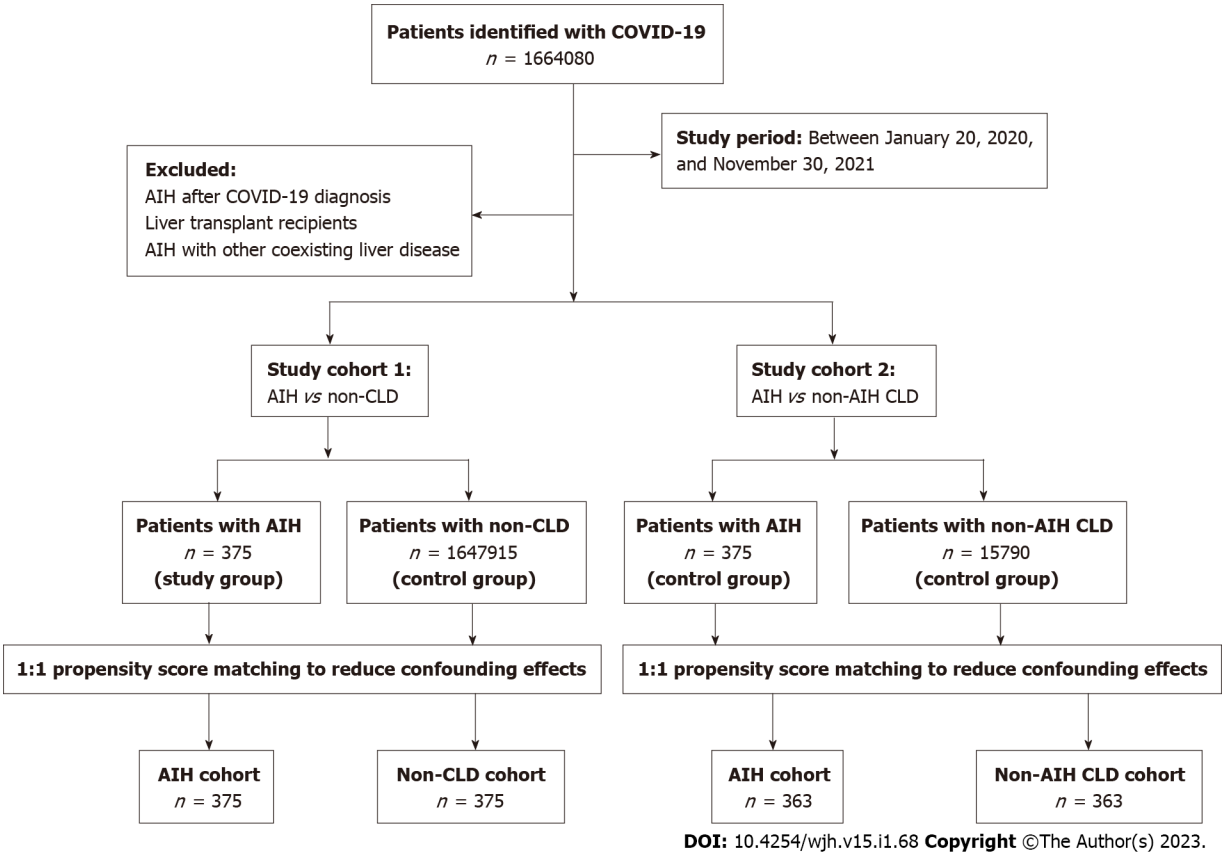

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Figure 1 Flow chart showing patient selection for study cohorts. COVID-19: Coronavirus disease-2019; AIH: Autoimmune hepatitis; CLD: Chronic liver disease.

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).

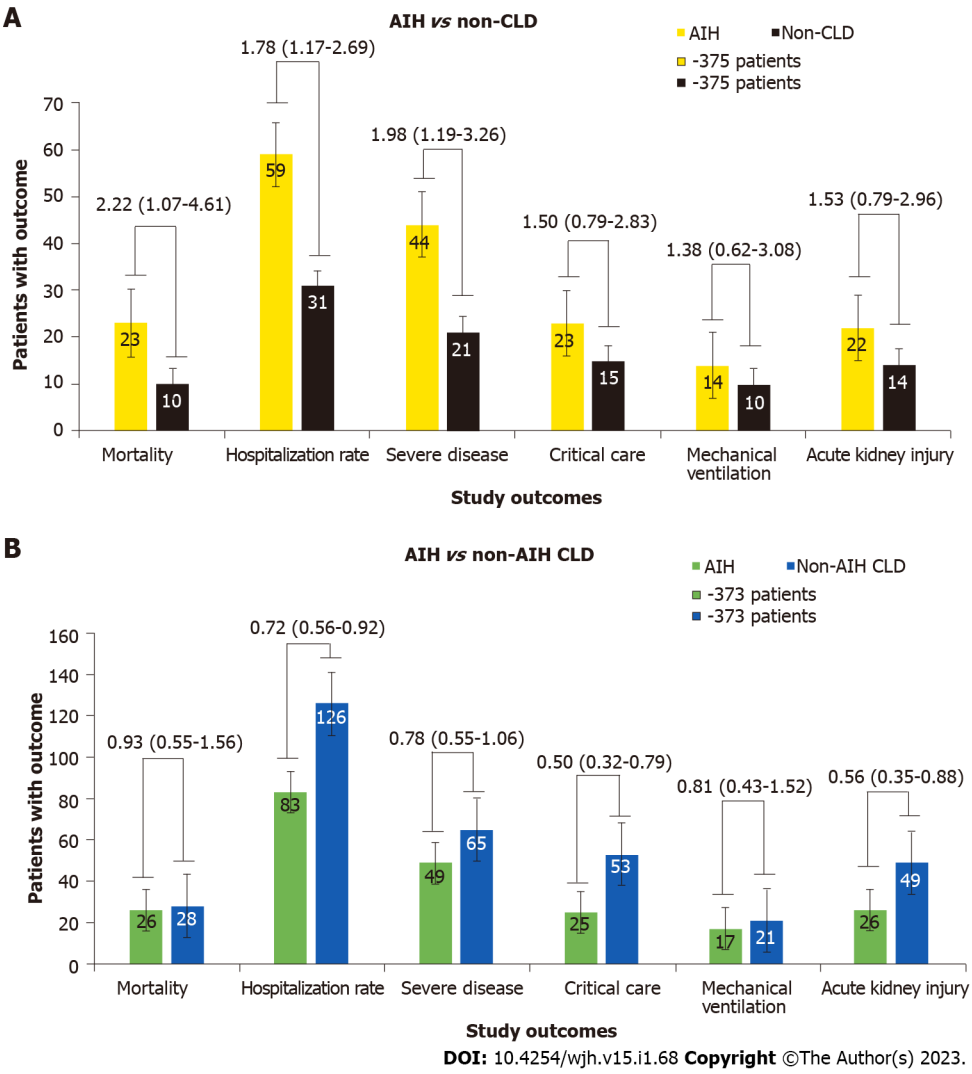

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Figure 2 Risk of all-cause mortality, rates of hospitalization, severe diseases, need for critical care, need for mechanical ventilation, and acute kidney injury, at 30 d from coronavirus disease 2019 diagnosis. A: Between patients with autoimmune 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

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.

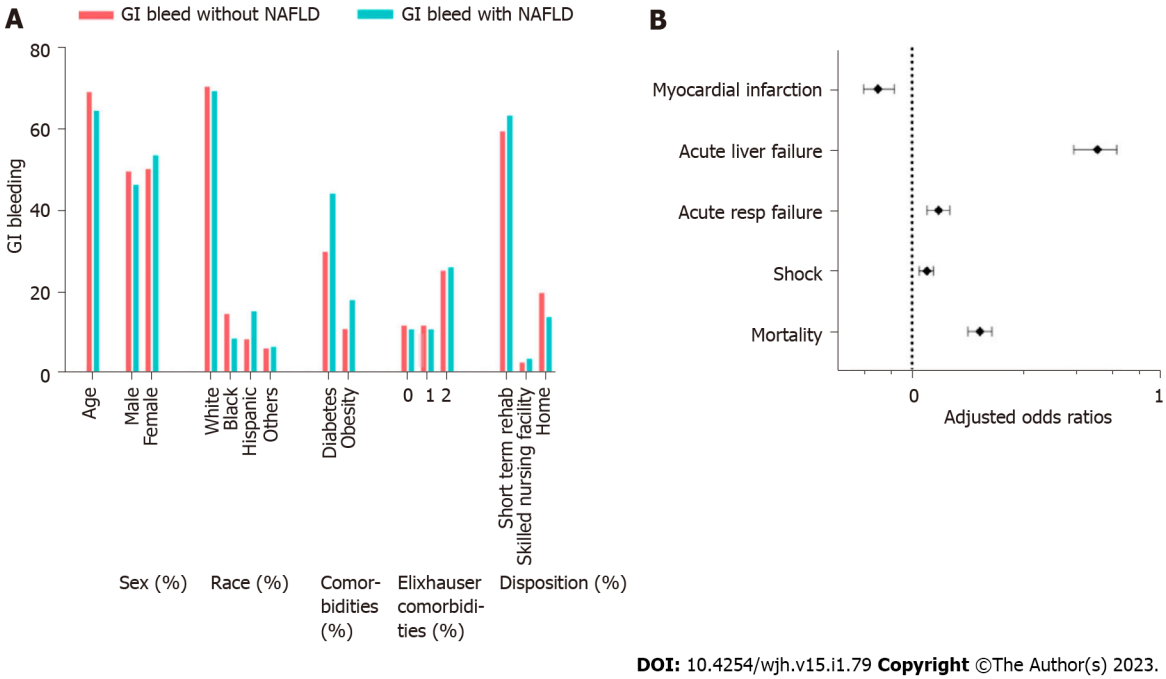

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Figure 1 Gastrointestinal bleeds and patient characteristics. A: Gastrointestinal bleeds and patient characteristics; B: Outcomes. GI: Gastrointestinal; NAFLD: Non-alcoholic fatty liver disease.

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.

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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

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

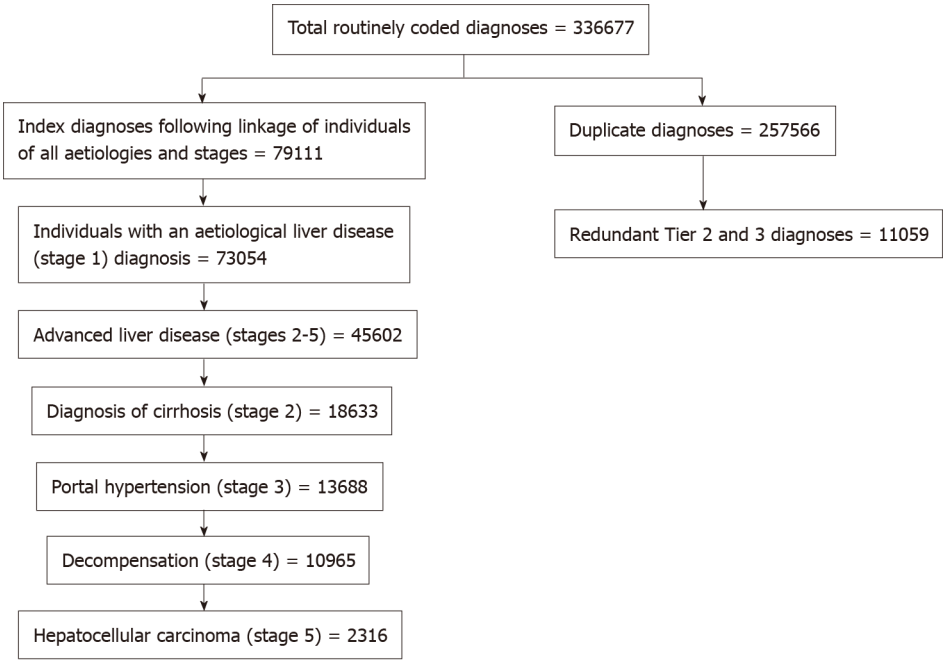

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Figure 1 Modified CONSORT flow chart of liver disease diagnoses Wales 1999-2019. There were 336677 liver disease diagnoses recorded by routine inpatient, day case and death certificate coding. 257566 were duplicate diagnoses. 11059 aetiological diagnoses 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

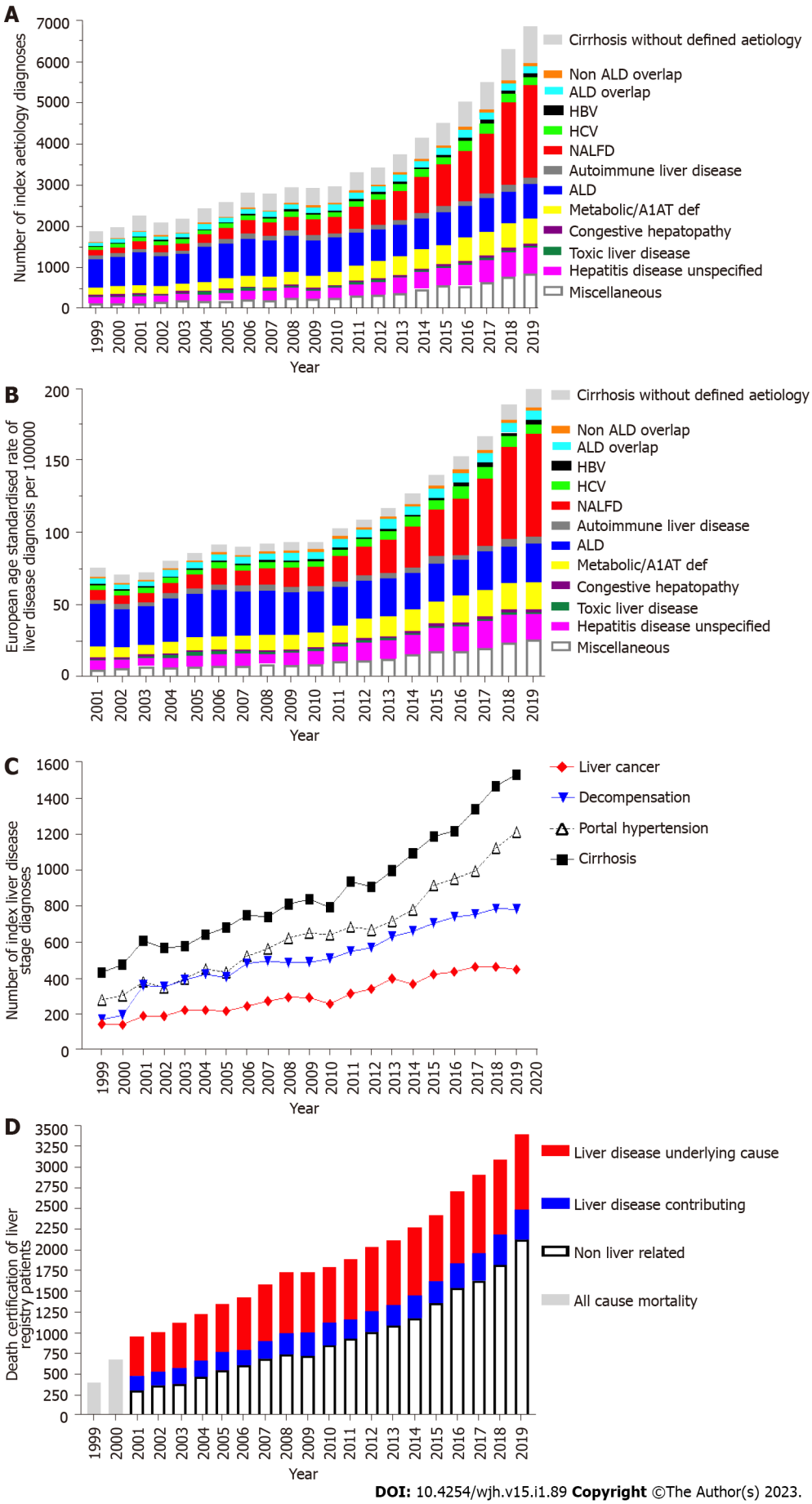

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Figure 2 Incidence of liver disease in Wales 1999-2019. A: The incidence of liver disease by aetiology recorded by routine coding following inpatient and

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.

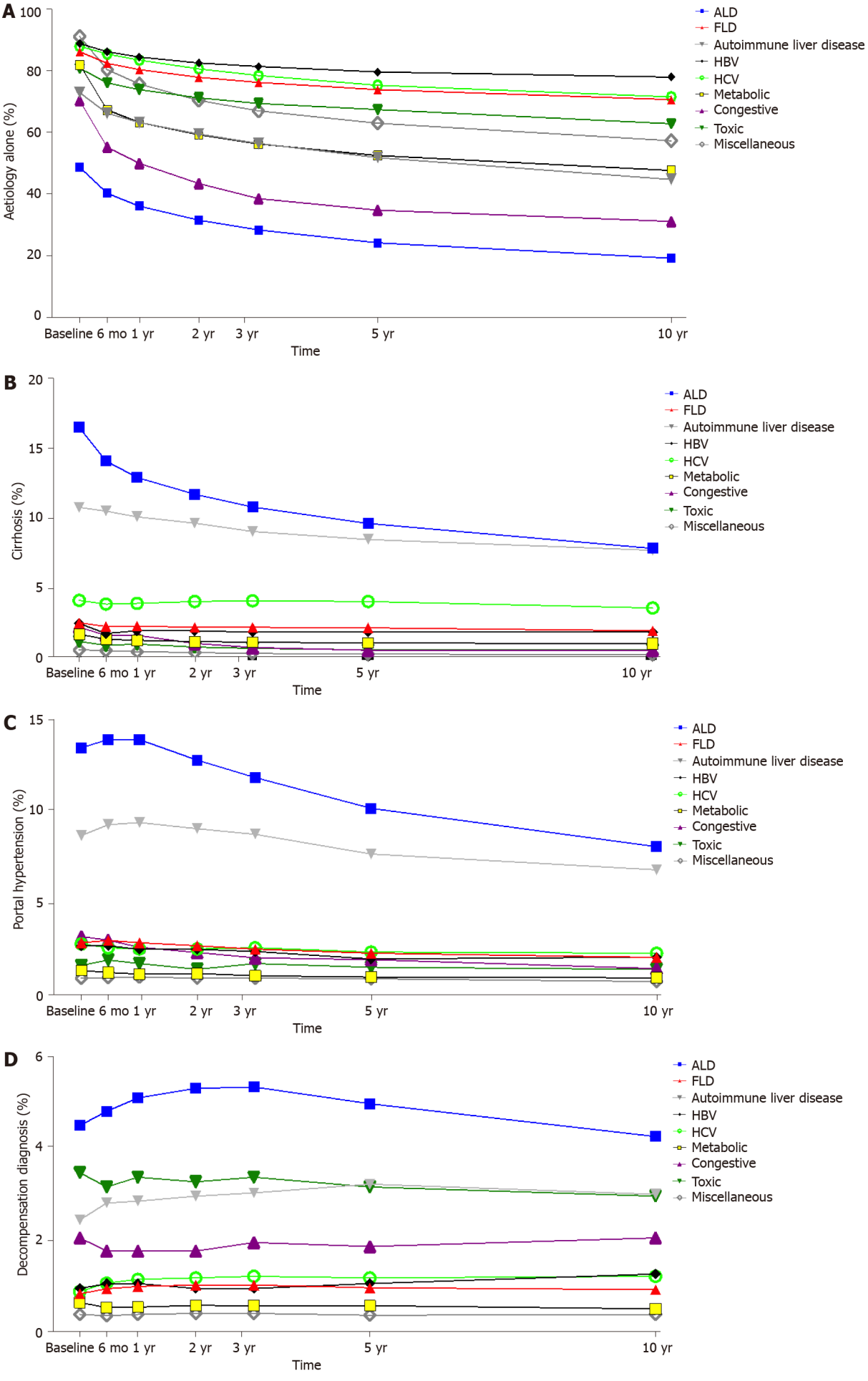
The Wales Liver Registry methodology builds on the Hepahealth project grouping of ICD-10 code which was predominantly applied to mortality data[15]. To increase the relevance to longitudinally collected routine hospital coding data these groupings have been modified with the addition of a hierarchical 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
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Portal hypertension (%)
15
10
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0
Baseline 6 mo 1 yr 2 yr 3 yr 5 yr 10 yr
Time
ALD
FLD
Autoimmune liver disease
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Decompensation diagnosis (%)
6
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Baseline 6 mo 1 yr 2 yr 3 yr 5 yr 10 yr
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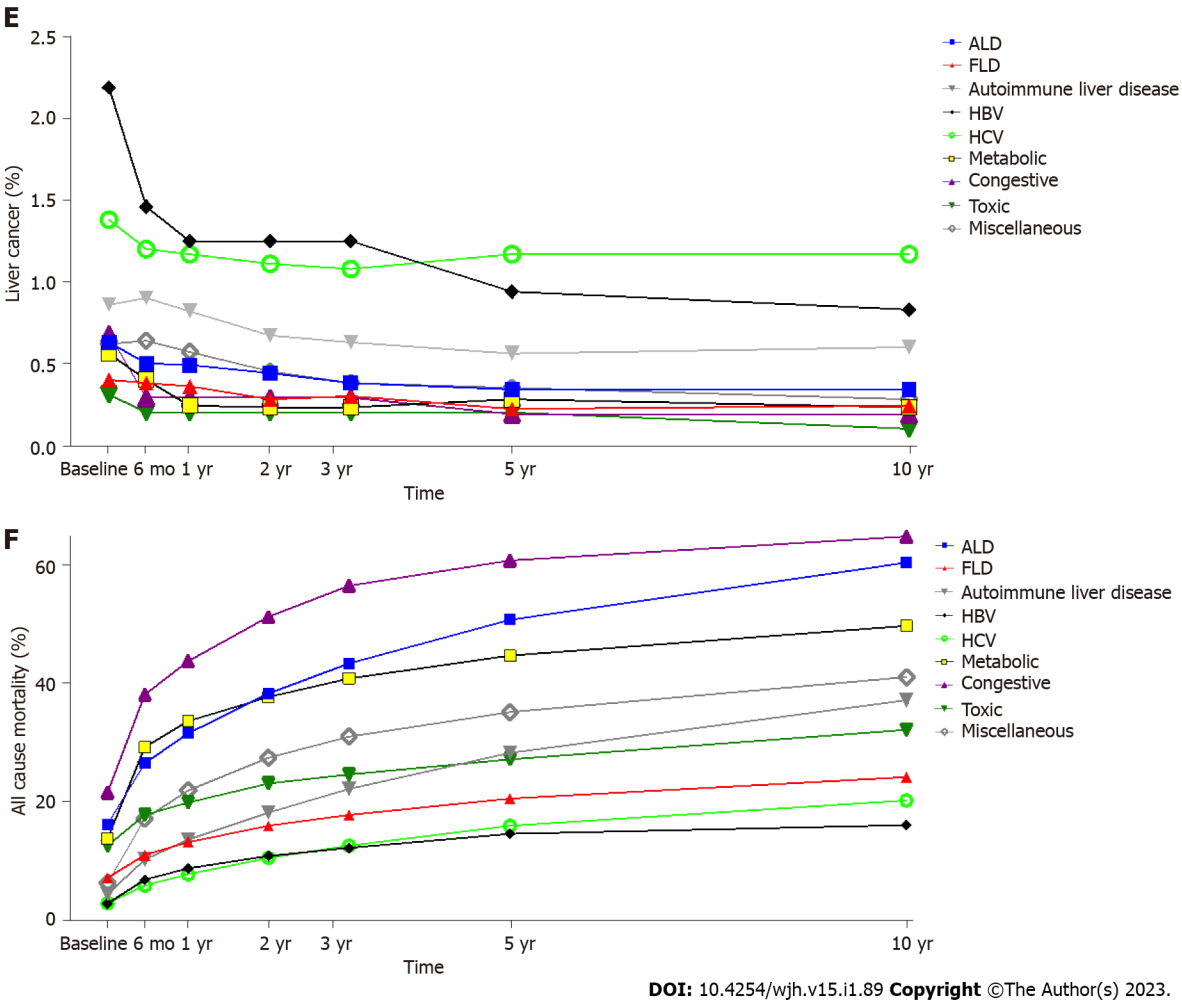

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Figure 3 Progression of liver diseases in Wales. The proportion of individuals at their most advanced stage of liver disease at baseline, 6 mo, 1, 2, 3, 5 and 10 years in Wales by aetiology. A: Aetiology (stage 1) diagnosis alone (not progressed); 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

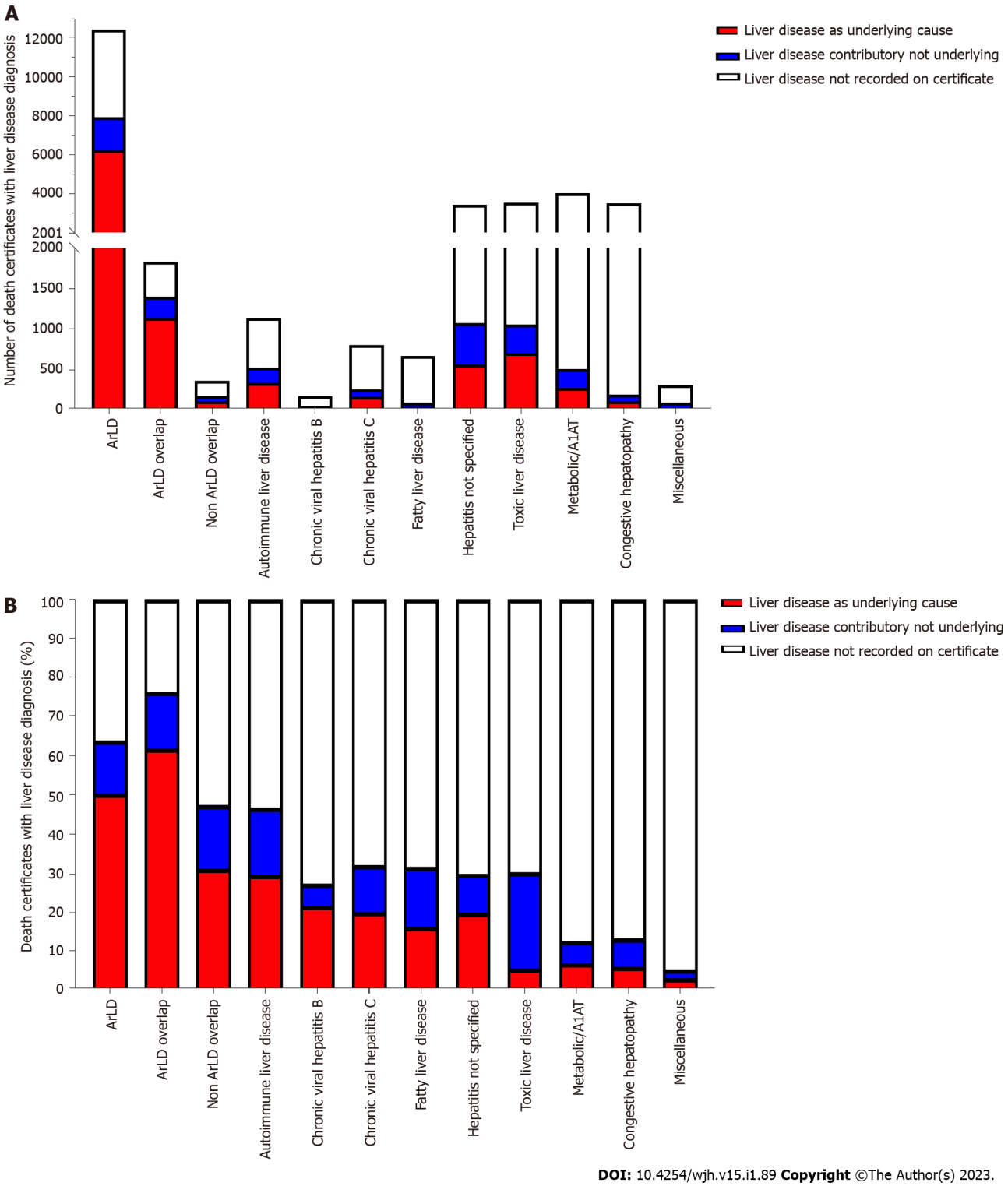

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Figure 4 Liver disease diagnoses on death certificates reported for individuals in the Wales Liver Disease Registry 1999-2019. A: The number of liver disease diagnoses that were reported as the underlying cause, reported on the death certification 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

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.

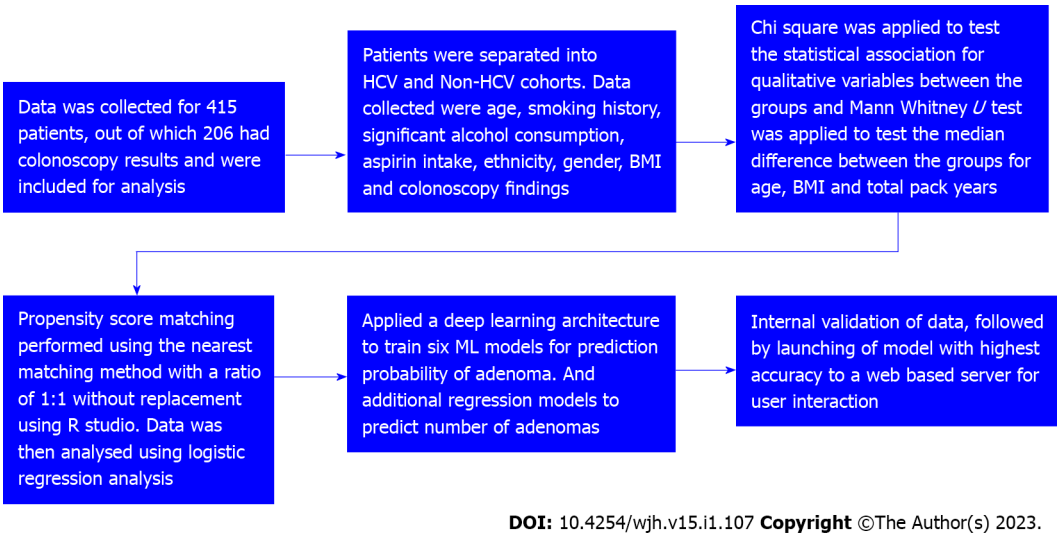

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Figure 1 Strobe diagram. HCV: Hepatitis C virus; ML: Machine learning.

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

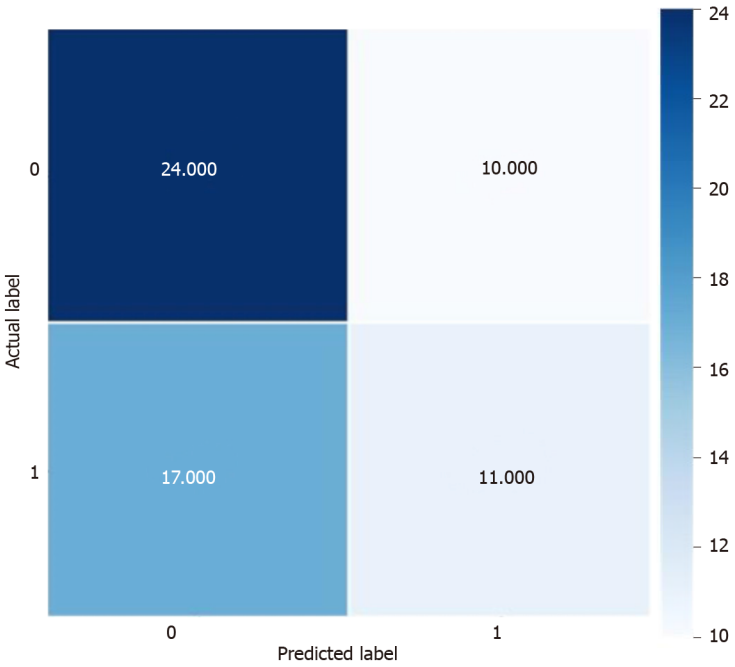

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Figure 2 Distribution of actual label *vs* predicted label during internal testing.

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.

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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

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.

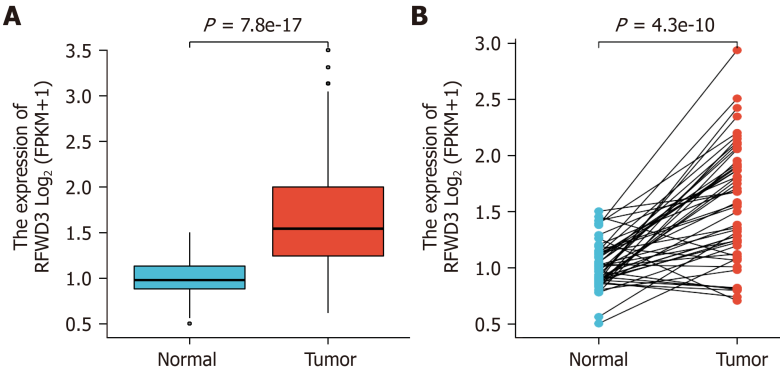

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Figure 1 Differential expression levels of ring finger and WD repeat domain 3 in hepatocellular carcinoma and normal tissues. A: Non-paired hepatocellular carcinoma (HCC) and normal samples; B: Paired HCC and normal samples. Data source: mRNA-Seq data 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.

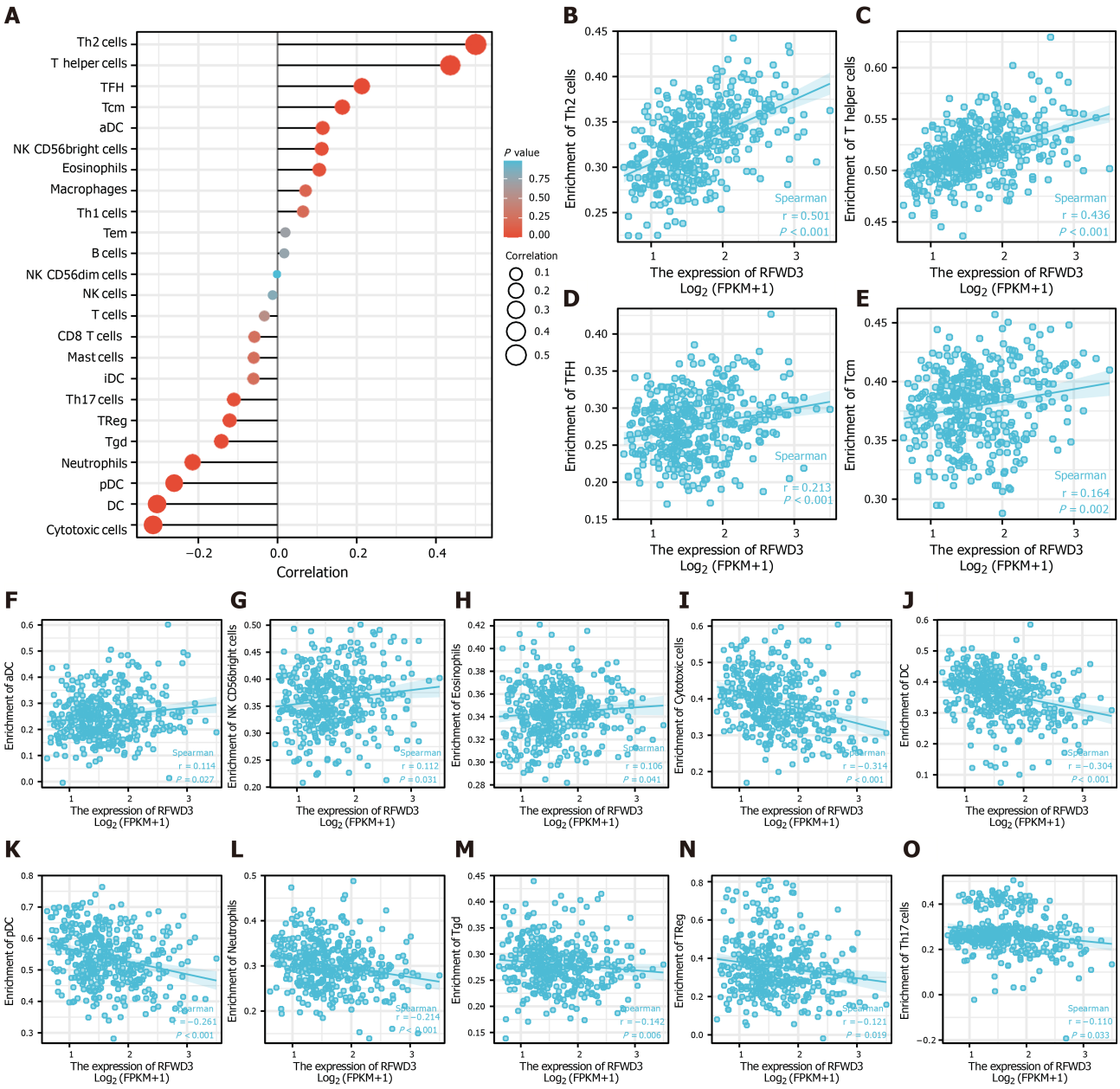

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Figure 2 Correlation analysis of ring finger and WD repeat domain 3 expression and immune cell infiltration in hepatocellular carcinoma.
A: Lollipop plot manifesting the correlation between ring finger and WD repeat domain 3 (RFWD3) expression and 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.

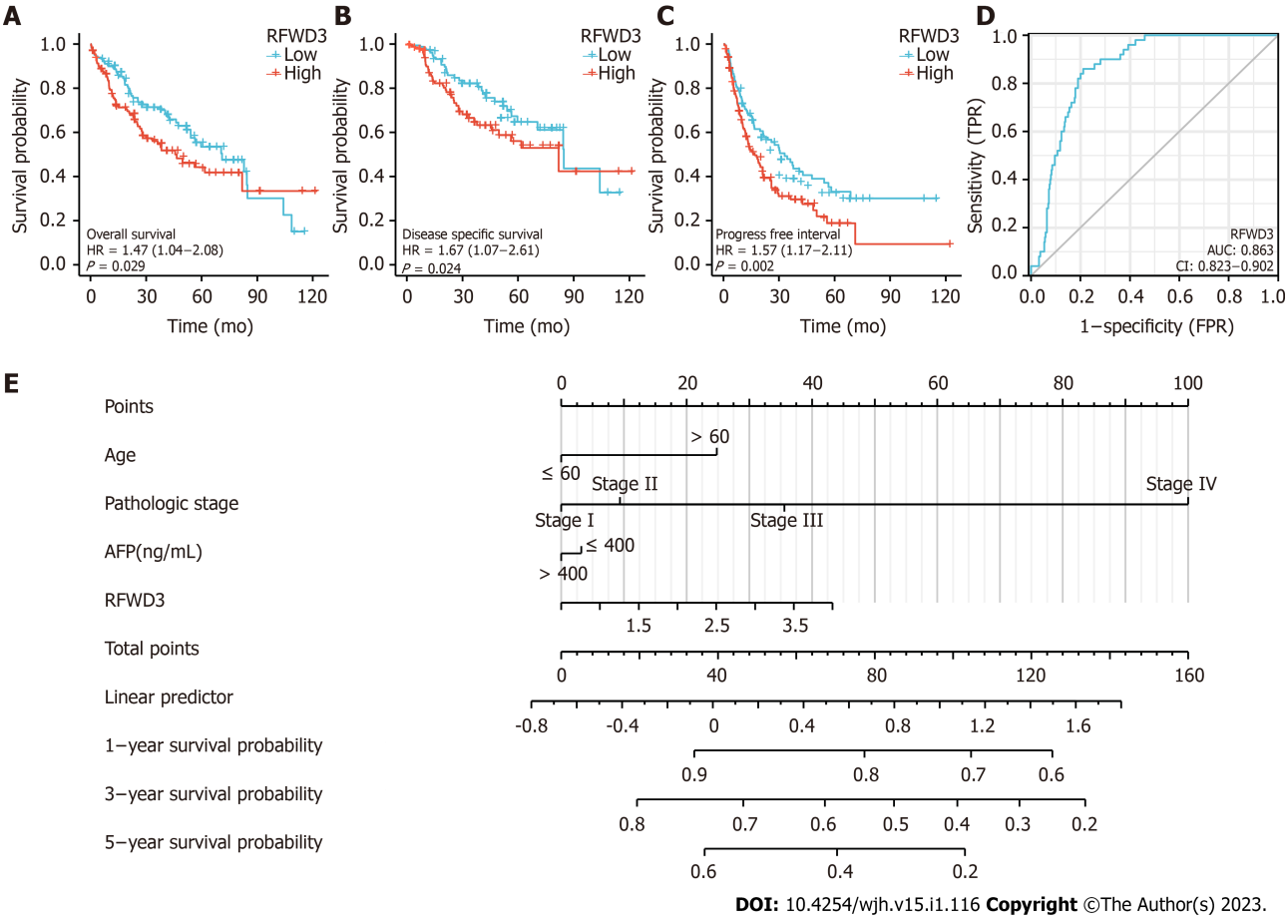

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Figure 3 Survival analysis of ring finger and WD repeat domain 3 in hepatocellular carcinoma and the nomogram for prognosis. A-C: Ring finger and WD repeat domain 3 (RFWD3) expression is related to overall survival, disease-specific survival (B) and 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

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