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EDITORIAL

Erdafitinib and checkpoint inhibitors for first-line and second-line immunotherapy of hepatic, gastrointestinal, and urinary bladder carcinomas: Recent concept

Mohamed Wishahi

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Abstract

Cancer immunotherapy is administered for first-line, second-line, neoadjuvant, or adjuvant treatment of advanced, metastatic, and recurrent cancer in the liver, gastrointestinal tract, and genitourinary tract, and other solid tumors. Erdafitinib is a fibroblast growth factor receptor (FGFR) inhibitor, and it is an adenosine triphosphate competitive inhibitor of FGFR1, FGFR2, FGFR3, and FGFR4. Immune checkpoint inhibitors are monoclonal antibodies that block programmed cell death protein 1 (PD-1) and its ligand that exert intrinsic antitumor mechanisms. The promising results of first-line treatment of advanced and metastatic urothelial carcinoma with PD-1 blockades with single or combined agents, indicate a new concept in the treatment of advanced, metastatic, and recurrent hepatic and gastrointestinal carcinomas. Cancer immunotherapy as first-line treatment will improve overall survival and provide better quality of life. Debate is arising as to whether to apply the cancer immunotherapy as first-line treatment in invasive carcinomas, or as second-line treatment in recurrent or metastatic carcinoma following the standard chemotherapy. The literature in the field is not definite, and so far, there has been no consensus on the best approach in this situation. At present, as it is described in this editorial, the decision is applied on a case-by-case basis.

Key Words: Programmed cell death protein-ligand 1; Erdafitinib; Liver cancer; Fibroblast growth factor receptor inhibitors; Checkpoint inhibitors; Bladder cancer; Metastases

Core Tip: The promising results of first-line treatment of advanced and metastatic urothelial carcinoma with programmed cell death protein 1 blockades with single or combined agents, indicate a new concept in the treatment of advanced, metastatic, and recurrent hepatic and gastrointestinal carcinomas. Cancer immunotherapy as a first-line treatment will improve overall survival and quality of life. At present, cancer immunotherapy as first-line treatment in invasive carcinomas or as second-line treatment in recurrent or metastatic carcinoma following the standard chemotherapy is applied on a case-by-case basis.

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INTRODUCTION

Recently, critical studies were published on cancer immunotherapy, and these publications addressed recurrent hepatocellular carcinoma (HCC)[1], esophageal squamous cell carcinoma[2], small bowel adenocarcinoma[3], cholangiocarcinomas[4], urothelial carcinomas[4-6], gastric carcinoma[7], colorectal cancer[8], and other solid tumors[5]. Cancer immunotherapy is administered for first-line, second-line, neoadjuvant, or adjuvant treatment of advanced, recurrent, or metastatic carcinoma in the liver, oesophagus, small bowel, colon, and urinary bladder, and other solid tumors[1-6]. This article will address the recently approved two immunotherapeutic drugs for the treatment of advanced, metastatic, and recurrent solid tumors.

ERDAFITINIB

Erdafitinib is a fibroblast growth factor receptor (FGFR) inhibitor, and it is an adenosine triphosphate (ATP) competitive inhibitor of FGFR1, FGFR2, FGFR3, and FGFR4. The United States Food and Drug Administration (FDA) has approved erdafitinib for the treatment of advanced and metastatic urothelial carcinoma in patients ineligible for standard chemotherapy, or refractory to platinum-containing chemotherapy. Erdafitinib has satisfactory clinical activity for metastatic urothelial carcinoma and other solid tumors. Erdafitinib toxicity is acceptable and it has been approved for initial treatment of advanced and metastatic urothelial carcinoma[4-6]. Erdafitinib administration resulted in prolonged progression-free survival. Approved FGFR inhibitors include erdafitinib, pemigatinib, and futibatinib[9]. Erdafitinib is an ATP competitive inhibitor of FGFR1-4. It inhibits FGFR kinase autophosphorylation, thus decreasing the downstream signaling. Normally, FGFR1-4 are bound by fibroblast growth factors to initiate the regulatory effects, which play a crucial role in angiogenesis and damage repair processes[7,8]. When erdafitinib was administered in recurrent HCC therapy, it resulted in increased overall survival (OS)[10]. Erdafitinib has been used in anticancer therapy for cholangiocarcinomas and urothelial carcinomas, and it is also recommended for the treatment of esophageal squamous cell carcinoma and small bowel adenocarcinoma[1-3].

IMMUNE CHECKPOINT INHIBITORS

Immune checkpoint inhibitors (ICIs) are monoclonal antibodies that block programmed cell death protein 1 (PD-1) and its ligand that exert intrinsic antitumor mechanisms[11]. These ICIs are at present the therapeutic option for different cancers and are becoming the standard anticancer therapy for several types of solid malignancies[3,7,8,12]. Recent advances in treatment with ICIs includes treatment of naive patients with locally advanced, or metastatic urothelial carcinoma of the bladder, especially for patients who are ineligible for standard chemotherapy, or refractory to platinum-containing chemotherapy.

Pembrolizumab and nivolumab are both monoclonal antibodies that target the PD-1 receptor on T cells and have been approved for the treatment of advanced HCC. Pembrolizumab has demonstrated consistent efficacy compared with nivolumab. Patients with advanced HCC treated with pembrolizumab had improved OS compared to those treated with placebo. The median OS was 14.6 months in the pembrolizumab group compared to 13.0 months in the placebo group [13].

Nivolumab is one of the ICIs that has shown efficacy in urothelial carcinoma treatment. Nivolumab was initially approved by the FDA for the treatment of metastatic melanoma, metastatic non-small cell lung cancer, advanced renal cell carcinoma, and locally advanced or metastatic urothelial carcinoma. Nivolumab was also approved by the FDA for use in the adjuvant therapy of patients with urothelial tumors who had been treated with radical surgery but are at considerable risk of recurrence after surgery[6].

Nivolumab is a human anti-PD-1 IgG4 monoclonal antibody, and it enhances the native immune defenses. ICIs can restore T-cell activity, which is the sole element for fighting against cancer cells. T cells have an important role in mediating the effects of various immune-related cytokines that assist CD8+ T cells in the elimination of cancer cells[11,12].

CONCLUSION

The promising results of first-line treatment of urothelial carcinoma with cancer immunotherapy indicate a new concept in the treatment of advanced, metastatic, and recurrent cancer in the hepatic, gastrointestinal tract, and genitourinary tract. Cancer immunotherapy as first-line treatment will improve overall survival and provide better quality of life. This will pave the way to consider first-line treatment of gastrointestinal and hepatic cancer with immunotherapy rather than to be applied in metastatic and recurrent disease.

Should we follow the patients with cancer to develop metastasis or recurrence and treat them with cancer immunotherapy, or, start the treatment as a first-line treatment?

The literature in the field is not definite. There is evidence that first-line immunotherapy has a promising result, but it has its side effects and toxicity. Besides, the costs of cancer immunotherapy are much higher compared to those of the standard chemotherapy.

FOOTNOTES

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EDITORIAL

Progress of mitochondrial and endoplasmic reticulum-associated signaling and its regulation of chronic liver disease by Chinese medicine

Yang Zheng, Yi-Hui Zheng, Jia-Hui Wang, Tie-Jian Zhao, Lei Wang, Tian-Jian Liang

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Abstract

The endoplasmic reticulum (ER) is connected to mitochondria through mitochondria-associated ER membranes (MAMs). MAMs provide a framework for crosstalk between the ER and mitochondria, playing a crucial role in regulating cellular calcium balance, lipid metabolism, and cell death. Dysregulation of MAMs is involved in the development of chronic liver disease (CLD). In CLD, changes in MAMs structure and function occur due to factors such as cellular stress, inflammation, and oxidative stress, leading to abnormal interactions between mitochondria and the ER, resulting in liver cell injury, fibrosis, and impaired liver function. Traditional Chinese medicine has shown some research progress in regulating MAMs signaling and treating CLD. This paper reviews the literature on the association between mitochondria and the ER, as well as the intervention of traditional Chinese medicine in regulating CLD.

Key Words: Mitochondria; Endoplasmic reticulum; Mitochondria-associated ER membranes; Traditional Chinese medicine; Chronic liver disease

Core Tip: Endoplasmic reticulum mitochondria-associated membranes (MAMs) play a very important role in the pathogenesis of chronic liver disease. MAMs play an important regulatory role in lipid accumulation, inflammatory response and apoptosis of cells, *etc*. Influencing the regulatory function of MAMs by targeting their structure can play a role in ameliorating chronic liver disease, which provides new perspectives and research directions for the development of new therapeutic approaches for chronic liver disease.

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INTRODUCTION

Chronic liver disease (CLD) is a global health threat and a leading cause of human mortality, encompassing non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease, viral hepatitis, liver fibrosis, and hepatocellular carcinoma (HCC)[1]. While early-stage CLD can be reversed, advanced liver cirrhosis and HCC severely impact patients' quality of life and even pose life-threatening risks. Mitochondria, known as the powerhouse of cells, are responsible for generating cellular energy through oxidative phosphorylation. Additionally, mitochondria play a role in calcium homeostasis, reactive oxygen species production, and cell apoptosis. Disruption of mitochondrial function is associated with the progression of CLD, including liver fibrosis, lipid degeneration, and hepatocyte injury. On the other hand, the endoplasmic reticulum (ER) is an interconnected membrane network involved in protein synthesis, folding, and transport. It also plays a crucial role in lipid metabolism, calcium signaling, and cellular stress response. Disturbance of ER homeostasis leads to the accumulation of misfolded proteins, triggering a cellular stress response known as ER stress. ER stress has emerged as a key factor in liver injury, inflammation, and the development of CLD. There is crosstalk between the ER and mitochondrial signaling pathways, with mitochondria-associated ER membranes (MAMs) connecting the ER and mitochondria in terms of structure and function. MAMs play important roles in cellular signaling and function, including calcium signaling, mitochondrial dynamics, energy metabolism, inflammation response, lipid transport, and cell apoptosis. Thus, they have a significant impact on the interplay and regulation of CLD[2]. Given the interdependence and coordinated functions of mitochondria and the ER, understanding the potential mechanisms of mitochondria-ER signaling in CLD has become an area of in-depth research. Targeted therapeutic approaches may offer potential strategies to improve liver damage and prevent disease progression.

MITOCHONDRIA AND CLDS

Mitochondria, also known as the "powerhouses" of cells, play a crucial role in various metabolic processes[3]. They serve as the site for cellular oxidative phosphorylation and adenosine triphosphate (ATP) synthesis, and are involved in a wide range of important biological functions, including energy conversion, the citric acid cycle, regulation of cellular calcium concentration, as well as being the primary source of reactive oxygen species (ROS) and a regulatory center for cell apoptosis[4].

Mitochondrial dysfunction and NAFLD

Research suggests that NAFLD is characterized by excessive accumulation of fat in liver cells, leading to abnormal fatty acid oxidation, significant increase in mitochondrial reactive ROS, and alterations in mitochondrial membrane lipids and proteins[5,6]. Zeng *et al*[7] found that fatty acid translocase (FAT/CD36) on the mitochondrial membrane is heavily glycosylated in NAFLD, reducing its ability to transport long-chain fatty acids into the mitochondria and inhibiting fatty acid oxidation. Additionally, the lipid composition of the mitochondrial membrane changes with the continuous accumulation of lipids[7]. The increased electron transport chain (ETC) activity leads to excessive ROS production, which directly attacks biomolecules, reduces intracellular antioxidant enzyme levels, and causes oxidative damage to cells. This results in metabolic changes and ultimately leads to ETC dysfunction. The combination of increased rates of fatty acid beta-oxidation and elevated ROS levels leads to damage to the mitochondrial respiratory chain, insulin resistance, and inflammation[8], See Figure 1 for details.

Mitochondrial dysfunction and alcoholic liver disease

In AFLD, various changes occur in liver mitochondria, such as increased levels of reactive ROS, decreased mitochondrial membrane potential, abnormal fatty acid beta-oxidation, and mitotic abnormalities[9]. Alcohol metabolism in the liver interferes with the oxidation of fatty acids, leading to the accumulation of triglycerides (TG). Simultaneously, alcohol metabolism generates a large amount of ROS, which causes oxidative damage to cells, resulting in metabolic changes, liver cell injury, and inflammation leading to the development of alcoholic liver disease (ALD)[10]. Furthermore, alcohol-induced mitochondrial damage exacerbates liver injury and steatosis. Mitochondria undergo structural remodeling to

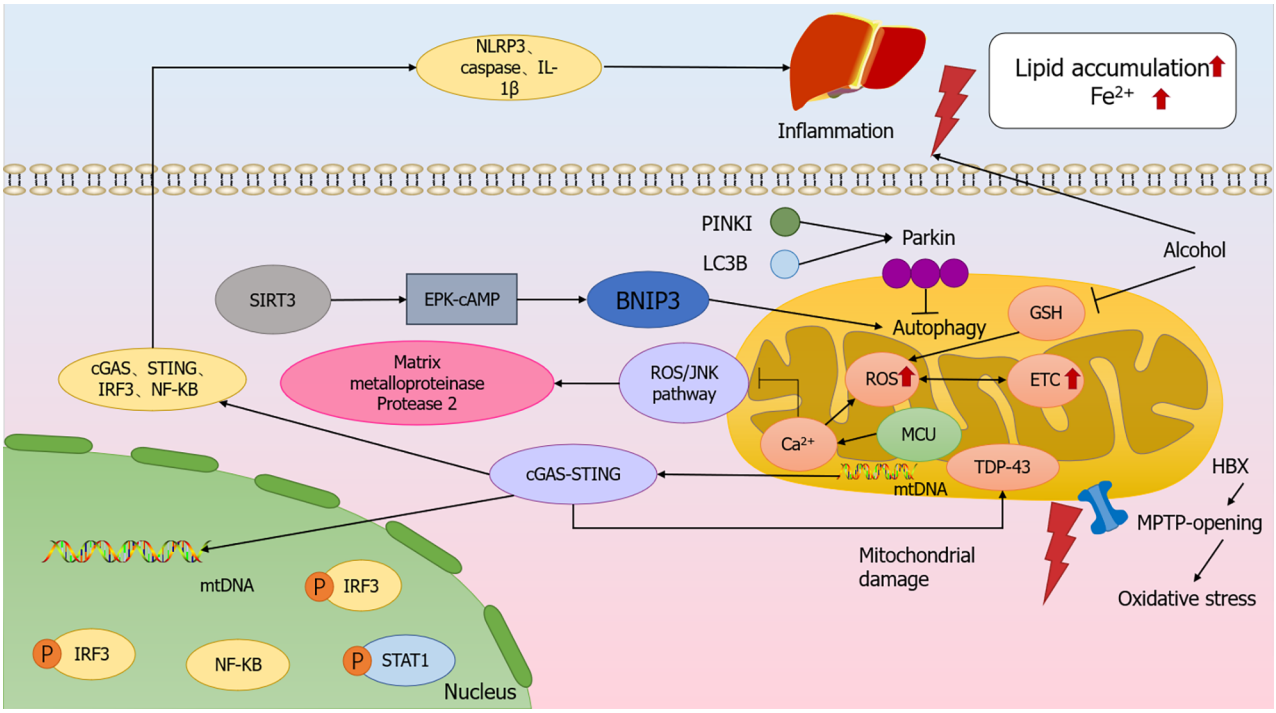

Figure 1 Molecular mechanism of mitochondrial dysfunction modulating the relationship between chronic liver disease. Increasing electron transport chain (ETC) leads to excess reactive oxygen species (ROS), causing corresponding metabolic changes and ultimately ETC dysfunction. Alcohol metabolism, which decreases GSH levels in mitochondria, increases ROS production and causes iron death. Interfering with autophagy by silencing Parkin led to enhanced apoptotic signaling, while SIRT3 increased BNIP3 expression in hepatocytes *via* the ERK-cAMP pathway, promoting BNIP3-mediated mitochondrial autophagy. Mitochondrial injury can lead to mtDNA leakage, which triggers an inflammatory response through the cGAS-String pathway. cGAS-STRING signaling pathway key molecules, cGAS, STING, and IRF3 expression levels, as well as the downstream NF-κB nuclear translocation, were significantly increased, and at the same time, it caused the accumulation of TDP-43 in the mitochondria of hepatocytes, which induced mitochondrial damage. Inflammatory response significantly increased the expression levels of NLRP3, caspasel and IL-1β in the liver, resulting in iron accumulation and lipid accumulation. ETC: Electron transport chain; ROS: Reactive oxygen species.

enhance alcohol metabolism. Studies have shown that alcohol can damage mtDNA, impair cellular energy metabolism, and increase ROS production. Additionally, alcohol induces excessive accumulation of iron in the liver. Alcohol metabolism also reduces the levels of the antioxidant GSH in mitochondria, leading to increased ROS production, lipid peroxidation in liver cells, and iron-induced cell death[11], See Figure 1 for details.

Mitochondrial dysfunction and viral hepatitis

The incidence and contagiousness of viral hepatitis are high, with hepatitis B (HBV) and hepatitis C (HCV) being common forms of chronic hepatitis. Han *et al*[12] demonstrated through their experiments that morphological and structural changes in mitochondria can typically be detected in patients with hepatitis B[13]. HBV can induce perinuclear aggregation of mitochondria and trigger their translocation through phosphorylation of the core protein at Ser616, leading to mitochondrial division[14]. Hsu *et al*[15] found that the absence of BNIP3 promotes excessive accumulation of reactive ROS, inflammatory reactions, and the formation of fatty liver in liver tissue. The characteristic of HCV-related CLD is an increase in ROS, which causes metabolic disorders, resulting in insulin resistance, hepatic steatosis, and iron accumulation in the liver. Ezaki *et al*[16] discovered that the HCV core protein NS5A can induce mitochondrial division, inhibit the translocation of Parkin to mitochondria, reduce autophagic activity, and maintain HCV-induced mitochondrial damage, See Figure 1 for details.

Mitochondrial dysfunction and liver fibrosis

Yong *et al*[17] discovered that pathological factors can cause mitochondrial damage, leading to the release of mtDNA and triggering an inflammatory response through the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway. In their experiments, abnormal mitochondrial morphology was observed, and RT-PCR revealed an increase in mtDNA content in the cytoplasm, making mtDNA highly susceptible to ROS attack[18]. Mutations in mtDNA can easily affect genes encoding important functional regions, leading to the occurrence of mitochondrial dysfunction-related diseases and inducing activation of the hepatic inflammatory signaling pathway[19]. In the liver, the expression levels of NLRP3, caspase, and IL-1β are significantly increased, along with the expression levels of key molecules in the cGAS-STING signaling pathway, including cGAS, STING, IRF3, and downstream NF-κB nuclear translocation. This also leads to the accumulation of TDP-43 in liver cells, inducing mitochondrial damage. The release of mtDNA from mitochondrial damage can activate the cGAS-STING signaling pathway, collectively triggering an inflammatory response and promoting the formation of liver fibrosis. The progression of liver fibrosis is believed to be associated with iron

overload, which promotes hepatocyte iron death through excessive induction of lipid peroxidation[20]. Inhibition of hepatic stellate cell (HSC) activation and alleviation of liver fibrosis can be achieved by regulating iron death[21], See Figure 1 for details.

Mitochondrial dysfunction and HCC

Mitochondrial dysfunction-mediated accumulation of reactive ROS and damage to mitochondrial DNA may contribute to the development of liver cancer. Research has shown that mitochondrial fission promotes the survival of liver cancer cells by enhancing autophagy and suppressing mitochondria-dependent cell apoptosis, and inhibitors of mitochondrial fission significantly inhibit tumor growth[18]. The characteristic feature of mitochondria in cancer cells is the excessive production of ROS, which promotes cancer development by inducing genomic instability, modifying gene expression, and participating in signaling pathways. The relative increase in ROS is due to a slowdown in electron transfer in the respiratory chain, leading to an absolute increase in ROS production, as well as a decrease in antioxidant enzyme activity. The decrease in cellular antioxidant enzyme levels leads to oxidative damage in cells and signaling transduction, resulting in corresponding metabolic changes[8]. Mechanistic analysis has shown that mitochondrial fission leads to increased ROS production, mediates the activation of AKT, and synergistically interacts with the TP53 and NF-κB pathways to promote tumor progression[22], See Figure 1 for details.

ENDOPLASMIC RETICULUM AND CLD

The ER is a vital organelle involved in calcium storage, lipid metabolism, and synthesis of steroid hormones[23]. External stimuli that disrupt protein folding or lipid homeostasis can trigger ER stress (ERS), activating the unfolded protein response (UPR) signaling pathway in cells. Research has shown that ERS is closely associated with the development of various diseases, including inflammation, dysregulation of glucose and lipid metabolism, liver fibrosis, and cancer.

Endoplasmic reticulum stress and NAFLD

ERS can contribute to the progression of NAFLD from simple steatosis to NASH, which is characterized by the development of inflammation and varying degrees of fibrosis[24]. The "second-strike theory" suggests that the liver undergoes oxidative stress, resulting in increased cellular activity and a massive release of inflammatory cytokines, causing hepatocellular inflammation and the formation of NAFLD[25]. In NAFLD, cytotoxic lipids modify the ER structure and directly activate the UPR, leading to dysregulation of protein homeostasis. Although hepatocytes can tolerate TG storage, saturated fatty acids and lysophosphatidylcholine cause lipotoxicity[26,27]. Once taken up by hepatocytes, these lipids are stored in cytoplasmic droplets, and excess saturated fatty acids trigger hepatocyte ERS through various mechanisms[28]. ERS and IR trigger NAFLD by acting on hepatocytes and causing massive intracellular lipid deposition, See Figure 2 for details.

Endoplasmic reticulum stress and ALD

Homocysteine is known to enhance the production of NF-κB, IL-1b, IL-6, and IL-8, induce endoplasmic ERS, and promote various cellular damage processes[29]. Homocysteine can activate pathways such as GRP78, CHOP, IRE1α, sterol regulatory element-binding protein, and JNK[30]. Long-term alcohol administration in animals leads to increased expression of cytochrome P450, which in turn enhances the expression of superoxide dismutase and activates nuclear factor erythroid 2-related factor 2, a key factor in the ESR response. Sun *et al*[31] found that changes in gene expression in ALD are closely related to its pathogenesis. Gene ontology enrichment analysis revealed two main functional groups: angiogenesis and stress response. Abnormal angiogenesis is also observed in the progression of ALD and exacerbates alcohol-induced liver injury. Studies have shown that excessive ethanol intake can cause chronic inflammation in liver parenchyma, leading to liver fibrosis and pathological angiogenesis, See Figure 2 for details.

Endoplasmic reticulum stress and viral hepatitis

ERS plays a crucial role in the progression of viral hepatitis, particularly in cases of HBV infection. The HBV inflammatory surface antigen can activate the UPR, leading to hepatocyte apoptosis and precancerous phenotypic changes. Notably, elevated levels of GRP78, an ER chaperone protein, have been observed in HBV patients, suggesting its association with HBV infection[32]. Additionally, research indicates that HCV-related liver cancer patients exhibit upregulation of vascular endothelial growth factor (VEGF) in tissue serum, leading to abnormal angiogenesis acceleration [33]. Accumulation of misfolded proteins triggers ER stress and the expression of GRP78[34]. GRP94, which binds to Ca^{2+}, facilitates translocation and folding of newly synthesized polypeptides, participates in oligomer assembly and degradation, and inhibits the formation of misfolded proteins. Furthermore, GRP94 acts as an antigen-presenting cell, activating the UPR and initiating cellular self-regulation[35]. XBP1 promotes the expression of molecular chaperones such as GRP78 and GRP94, enhancing the cell's ability to handle unfolded proteins and activating ER-associated degradation (ERAD) and other ER degradation pathway-related genes, See Figure 2 for details.

Endoplasmic reticulum stress and liver fibrosis

ERS is a cellular response to various stressors that disrupt protein folding in the endoplasmic ER. Three transmembrane proteins on the ER membrane, namely ATF6, PERK, and IRE1α, play crucial roles in releasing unfolded/misfolded proteins and initiating the UPR. During ERS, BiP dissociates from these transmembrane proteins and binds to unfolded/

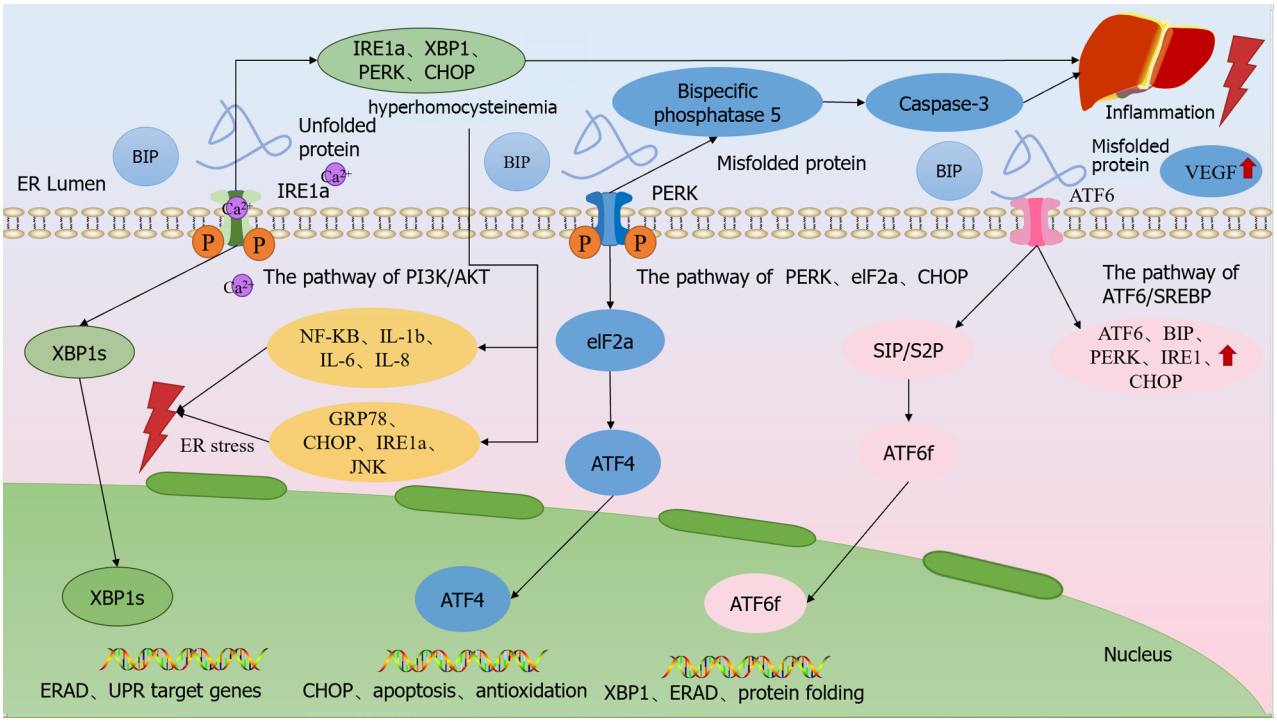

Figure 2 Endoplasmic reticulum dysfunction modulates chronic liver disease. Upon occurrence of endoplasmic reticulum stress (ERS), BiP dissociates from the three ER transmembrane proteins and binds to unfolded proteins with high affinity, and the dissociated transmembrane proteins shift to an active state and activate downstream signaling. Upon dissociation from BiP upon ERS, PERK endoplasmic reticulum stress increases the expression of bispecific phosphatase 5 in hepatocytes through the PERK/eIF2α/CHOP pathway as a means of raising the intracellular activated caspase-3 levels, which ultimately induces hepatocyte death. ATF6 is cleaved upon dissociation from BiP during ERS onset, and cleaved ATF6α activates the transcription of XBP1u.IRE1α is activated and translocates to the cell membrane *via* the PI3K/AKT pathway, leading to extracellular Ca^{2+} inward flow, which disrupts the intracellular calcium homeostasis and triggers ERS, so that IRE1α, XBP1, PERK and CHOP upregulation, which ultimately leads to hepatic stellate cell activation and proliferation and promotes liver fibrosis. ER: Endoplasmic reticulum; VEGF: Vascular endothelial growth factor.

misfolded proteins, activating downstream signaling pathways[36]. In liver cells, PERK-mediated ERS induces the upregulation of dual-specificity phosphatase 5, which reduces the phosphorylation levels of extracellular signal-regulated kinases and inhibits their activity. Additionally, PERK-mediated ERS increases the activation of caspase-3, leading to hepatocyte death and liver fibrosis[37]. Overexpression of IRE1α in HSCs downregulates IRE1α, XBP1, PERK, and CHOP, preventing HSC activation and reducing liver injury and fibrosis. In PDGF-induced HSCs, the activation of the PI3K/AKT pathway disrupts calcium homeostasis, leading to the accumulation of misfolded proteins and the induction of ERS, ultimately promoting HSC activation, proliferation, and liver fibrosis[38], See Figure 2 for details.

Endoplasmic reticulum stress and HCC

ATF6 is a type II ER transmembrane protein that dissociates from BiP and undergoes cleavage when ER stress occurs[39]. The cleaved form of ATF6α not only activates the transcription of XBP1u, but also induces genes related to protein folding and ERAD[40]. The rapid proliferation of tumor cells is accompanied by a sharp increase in protein synthesis, inevitably leading to UPR activation, while miRNA imbalance exists in both hepatitis and HCC. Some studies have also shown that the endoribonuclease activity of IRE1 is not only responsible for cleaving XBP1, but also for cleaving and regulating miRNAs[41]. Therefore, ER stress leads to miRNA imbalance in inflammation and cancer, promoting tumor development and progression. Tumor cells can transmit ER stress and UPR signals to neighboring macrophages, upregulating UPR target genes and pro-inflammatory factors, thereby promoting the pro-inflammatory effect of tumors and regulating the tumor microenvironment[42,43]. VEGF, as an important regulator of angiogenesis, inhibits tumor growth by restricting blood vessel formation and reducing tumor blood supply when its levels decrease[44], See Figure 2 for details.

MITOCHONDRIAL AND ENDOPLASMIC RETICULUM ASSOCIATION SIGNALING AND CLD

There is crosstalk between ER and mitochondrial signaling pathways, and the opening of endoplasmic reticulum Insp3/Ca ion channels affects mitochondrial Ca^{2+} homeostasis, and disruption of mitochondrial Ca^{2+} homeostasis leads to alterations in mitochondrial membrane potential, permeability transition pore, and other alterations in mitochondria, which are one of the most important organelles in hepatocytes, providing the majority of the cell's energy[45]. The ER is connected to the mitochondria by MAMs connected to the mitochondria, a dynamic structure that is highly sensitive to the cellular physiological environment and is mainly involved in the regulation of ERS, oxidative stress, apoptosis, cellular autophagy, changes in mitochondrial dynamics and inflammation[46].

MAMs and NAFLD

The molecular mediators of mitochondrial fusion are primarily Mfn1/2 (mitofusin1/2). Both Mfn1 and Mfn2 are located on the outer mitochondrial membrane, with Mfn2 also being localized on MAMs. Together, they regulate the structure and function of MAMs, inhibit the proximity of ER and mitochondria, and promote mitochondrial fusion[47]. Reduced expression of Mfn2 has been observed in liver biopsies of NASH patients, and specific ablation of liver Mfn2 *in vivo* leads to inflammation, TG accumulation, fibrosis, and liver cancer. This suggests that Mfn2 binds to phosphatidylserine (PS) and can selectively extract PS into membrane domains, facilitating its transfer to mitochondria and the synthesis of mitochondrial phosphatidylethanolamine (PE). Therefore, the deficiency of Mfn2 in the liver reduces PS transfer and phospholipid synthesis, leading to ER stress and the development of NASH. Specifically, PS is mainly synthesized in the ER and enters mitochondria through transient membrane contacts between MAMs and the outer mitochondrial membrane. In mitochondria, PS is converted to PE, which then enters the endoplasmic reticulum to be converted to phosphatidylcholine (PC). Thus, MAMs play a crucial role in phospholipid synthesis and transport[48], See Figure 3 for details.

MAMs and ALD

Under the influence of alcohol, PERK, as a connecting protein of MAMs, mediates liver cell death through the regulation of MAMs. When PERK in MAMs is knocked out, cells exhibit abnormal ER morphology, as well as MAMs disruption and calcium imbalance[49]. After passing through the gastrointestinal tract, alcohol is absorbed into the bloodstream and enters the liver for metabolism. Within liver cells, enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase convert alcohol into acetic acid, which enters the tricarboxylic acid cycle (TCA), leading to a decrease in the NAD^+/NADH ratio and further mitochondrial dysfunction. Damage to the mitochondria impairs the TCA, leading to disturbances in fatty acid metabolism and excessive fat deposition in liver cells, resulting in fatty liver. Additionally, alcohol can also cause damage to ER morphology and function in liver cells through various pathways. In alcohol-induced liver tissue with severe impairment of both mitochondrial and ER function and morphology, MAMs, as a functional platform where these two organelles interact, may be more sensitive to alcohol exposure[50], See Figure 3 for details.

MAMs and viral hepatitis

Our findings demonstrate that STING can bind to mitochondrial antiviral signaling protein at MAMs, thereby enhancing the interferon response to viral infections. Additionally, HCV proteins were found to localize to MAMs, potentially leading to increased mitochondrial ROS levels through Ca^{2+} manipulation. Furthermore, certain molecules present on the structure of MAMs, such as Ero1 and p66Shc, promote ROS production. Ero1, an important molecule involved in redox homeostasis, consists of two isoforms, Ero1-α and Ero1-β. The majority of Ero1-α is localized on MAMs, and its upregulation can result in increased ROS production. Notably, the dephosphorylation of the Ser36 site enables its transfer to MAMs, mediating ROS generation. This process facilitates the flow of Ca^{2+} from the endoplasmic ER to mitochondria through MAMs. Consequently, the accumulation of mitochondrial Ca^{2+} leads to mitochondrial depolarization and abnormalities in oxidative phosphorylation. As a result, the mitochondrial electron transport chain uncouples with respiratory complexes I and III, further augmenting ROS production. This process can be described by $ERCa^{2+}$, which serves as the basis for mitochondrial ROS production. Importantly, the inhibition of $ERCa^{2+}$ channels can effectively block this process[51,52], See Figure 3 for details.

MAMs and liver fibrosis

TMAMs can have an effect on mitochondrial function, including fusion/fission, mitophagy, and energy metabolism. MAMs play a dual role in maintaining cellular homeostasis by promoting mitochondrial division and mitophagy while also potentially causing pathological damage through excessive mitochondrial fission, calcium overload, or oxidative stress. Evidence suggests that MAMs are involved in PINK1/Parkin-mediated mitophagy, with core proteins associated with MAMs regulating their integrity and functionality[49]. *In vivo* studies have demonstrated that liver-specific ablation of Mfn2, a protein involved in MAMs, leads to inflammation, triglyceride accumulation, fibrosis, and HCC. This suggests that Mfn2 binds to PS and selectively extracts it to membrane domains, facilitating PS transfer to mitochondria and mitochondrial phosphatidylethanolamine synthesis[53], See Figure 3 for details.

MAMs and HCC

Research has shown that enhancing the physical contact between the endoplasmic ER and mitochondria leads to mitochondrial calcium overload and cell apoptosis. Conversely, disrupting the ER-mitochondria contact points stimulates mitochondrial oxidative respiration and ATP production[54]. Increased mitochondrial fission has been observed in liver cancer tissues, and it has been found that enhanced mitochondrial fission promotes the growth of liver cancer cells. Overexpression of Drp1 in liver cancer cells, followed by overexpression of Rab32, leads to a decrease in MAMs structure formation, a significant increase in mitochondrial calcium concentration, and subsequently promotes liver cancer cell apoptosis while inhibiting cell proliferation. Numerous studies have found that MAMs play important roles in both calcium signal transduction and the transfer of calcium from the ER to mitochondria[55]. Excessive uptake of mitochondrial calcium can result in mitochondrial calcium overload, opening of the permeability transition pore, mitochondrial swelling, rupture of the outer mitochondrial membrane, and subsequently, release of cytochrome c and cell apoptosis[54], See Figure 3 for details.

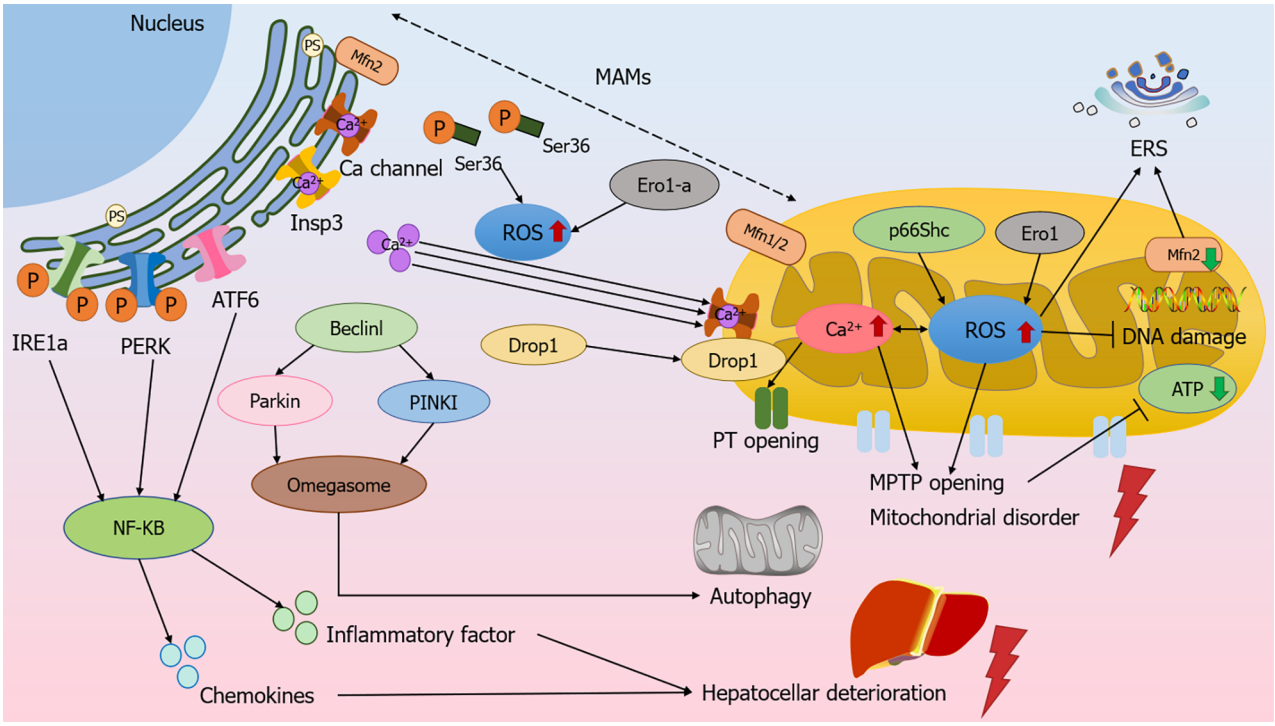

Figure 3 Mitochondrial and endoplasmic reticulum-associated signaling regulates the molecular mechanism of chronic liver disease. Reactive oxygen species (ROS) in the endoplasmic reticulum (ER) initiates Ca^{2+} efflux from Insp3 and Ca ion channels, and the Ser36 site is dephosphorylated and can be transferred to mitochondria-associated ER membranes (MAMs) to mediate ROS production. ROS promotes Ca^{2+} in the ER to flow to the mitochondria through the MAMs and increases mitochondrial ROS production, and conversely, the increase in ROS affects Ca^{2+} and initiates the opening of permeability transition pores, and the swelling of the mitochondria causes the rupture of the outer membrane, which can lead to oxidative damage to DNA and cause adenosine triphosphate depletion. Beclinl and PINK1/Parkin mediate mitochondrial autophagy, and together with PINKI, promote an increase in MAMs and omegasome formation. Excessive accumulation of ROS leads to endoplasmic reticulum stress, activation of the ATF6, IRE1, and PERK, the three unfolded protein response pathways, and the up-regulation of NF-κB activity, promoting the secretion of hepatocyte inflammatory factors and chemokines, leading to functional deterioration of hepatocytes. MAMs: Mitochondria-associated ER membranes; ERS: Endoplasmic reticulum stress; ATP: Adenosine triphosphate.

TRADITIONAL CHINESE MEDICINE TARGETS MITOCHONDRIAL ENDOPLASMIC RETICULUM MODULATION TO INTERVENE IN CLD

The research on traditional Chinese medicine (TCM) in regulating MAMs signaling and treating CLDs has shown significant progress. Certain TCM components have been proven to have a reparative effect on the structure and function of MAMs, thereby improving the progression of CLDs. Additionally, some active ingredients in TCM exhibit antioxidant, anti-inflammatory, and insulin-sensitivity regulating properties, some of which may be related to their modulation of MAMs signaling. The modulation of MAMs signaling by TCM components is an important research direction for the treatment of CLDs. Current studies have demonstrated that certain TCM components can regulate MAMs signaling and improve liver pathological responses, providing new insights and approaches for the treatment of CLDs.

Silymarin

Silymarin is a novel flavonoid compound extracted from the seeds of Silybum marianum[56]. This drug has been shown to protect the liver, promote liver cell regeneration, enhance liver metabolism, and reduce serum transaminase levels. It is suitable for adjunctive treatment of chronic hepatitis, cirrhosis, and other liver diseases. Animal experiments have shown that silymarin can increase the expression of superoxide dismutase (SOD) in lymphocytes of mice with CLD, thereby reducing the production of reactive ROS and protecting liver cell membranes[57]. Silymarin can also stabilize endoplasmic ER function and exert anti-inflammatory and hepatoprotective effects by reducing the levels of TNF-α, IL-6, IL-8, and other inflammatory cytokines[58], See Figure 4 for details.

Tartaric acid

Tartaric acid can inhibit lipid peroxidation and clear ROS, effectively preventing the occurrence and development of liver fibrosis induced by carbon tetrachloride (CCl_4). Multiple studies have found that rhein can inhibit the chemotaxis and phagocytosis of neutrophils, as well as the activation of macrophage lipid inflammatory mediators, and suppress the activity of TNF-α and IL-1β[59,60]. Rhein has the ability to clear ROS and inhibit lipid peroxidation[61]. Research has shown that in animal models of CCl_4 and ethanol-induced liver fibrosis, rhein can significantly reduce malondialdehyde levels and increase SOD levels, thereby reducing the excessive generation of ROS in mitochondria, See Figure 4 for details.

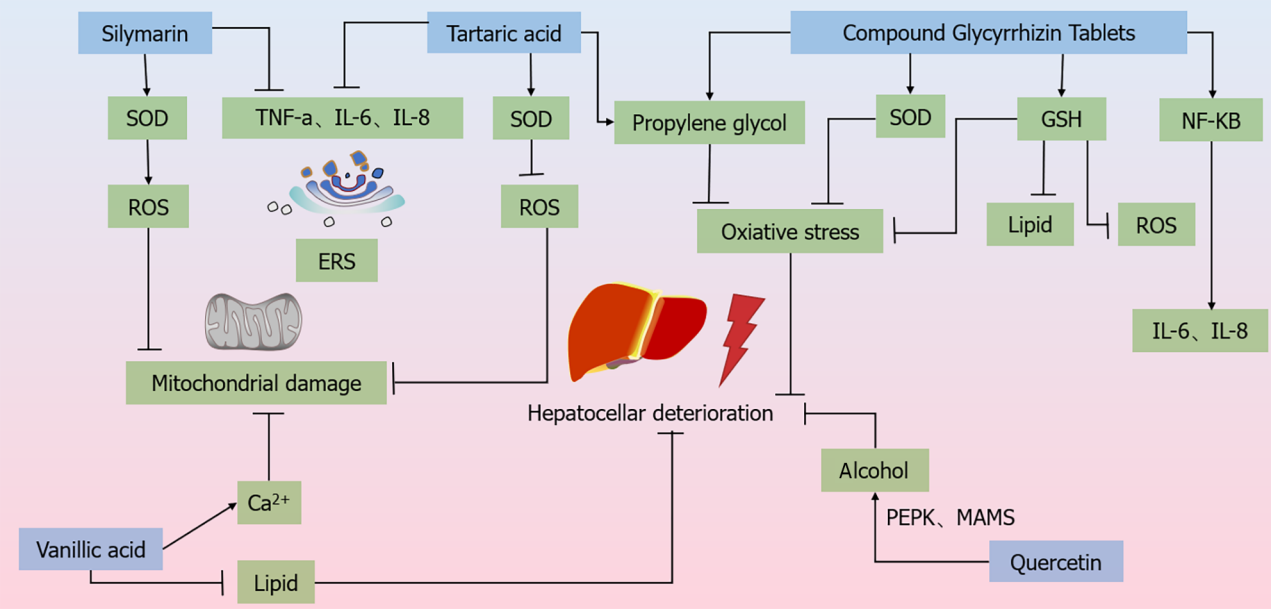

Figure 4 Molecular mechanism of Chinese medicines regulating the treatment of chronic liver disease. Silymarin increases the expression of superoxide dismutase (SOD) to reduce the levels of reactive oxygen species (ROS) and TNF-α, IL-6, IL-8, *etc.* to stabilize the endoplasmic reticulum (ER). Rhein is capable of anti-lipid peroxidation and scavenging of ROS, and inhibits the activities of TNF-a and IL-1β to stabilize the ER. vA significantly reduces malondialdehyde and elevates the levels of SOD to reduce ROS in mitochondria. vA has the ability to reduce lipid accumulation in hepatocytes and protect ER and Ca^{2+} homeostasis. quercetin inhibits alcohol and Ca^{2+} homeostasis through the regulation of PERK-MAMs pathway. VA has the ability to reduce intracellular lipid accumulation in hepatocytes and effectively protect ER and Ca^{2+} homeostasis. quercetin inhibits alcohol-induced iron death in hepatocytes and alleviates alcoholic liver injury through the regulation of the PERK-MAMs pathway. Composite glycyrrhizin tablets inhibit NF-κB pro-inflammatory signaling and IL-6 and IL-1β activities by inhibiting NF-κB pro-inflammatory signaling and IL-6 and IL-1β activities. transduction and IL-6 and IL-8 expression to inhibit hepatic injury, and also by promoting SOD and GSH expression as well as inhibiting malondialdehyde synthesis, induced hepatocellular injury. GSH passages inhibit ROS generation and attenuate hepatic injury caused by oxidative stress. ROS: Reactive oxygen species; ERS: Endoplasmic reticulum stress; SOD: Superoxide dismutase.

Quercetin

Quercetin is a flavonoid compound. Recent studies have found that subcellular mechanisms related to calcium imbalance, endoplasmic ERS, and mitochondrial damage are closely related to the progression of CLD and the activation of NLRP3 inflammasomes. During metabolic-related fatty liver disease, the expression of NLRP3 inflammasomes increases, and the homeostasis of MAMs is disrupted. Quercetin intervention has been shown to effectively protect against liver damage and reverse changes in MAMs. The results suggest that quercetin may exert its protective effects through the MAMs-NLRP3 pathway, providing new insights for nutritional interventions in the treatment of metabolic-related fatty liver disease. Quercetin inhibits alcohol-induced hepatocyte ferroptosis by regulating the PERK-MAMs pathway, thereby alleviating alcohol-induced liver injury[62], See Figure 4 for details.

Vanillic acid

One such TCM component is vanillic acid (VA), an edible plant compound, which has been studied for its beneficial effects on calcium (Ca^{2+}) complications induced by hyperinsulinemia (HI), intracellular homeostasis, MAM integrity, and liver metabolism under *in vivo* and *in vitro* conditions. VA has been found to possess various pharmacological effects, such as antioxidant, antidiabetic, and anti-inflammatory properties[63]. The liver plays a crucial role in regulating lipid metabolism balance in the body, and excessive lipid accumulation in liver cells contributes to the occurrence and development of NAFLD. During the HI phase in HepG2 cells, distorted MAMs lead to insulin resistance (IR) and excessive lipid synthesis, accompanied by inflammation and oxidative stress. VA effectively protects ER and Ca^{2+} homeostasis during the HI phase[64], See Figure 4 for details.

Compound glycyrrhizin tablets

Compound glycyrrhizin tablets have a positive effect on the prevention and treatment of liver diseases, with glycyrrhizin, glycine, and cysteine hydrochloride as its main components[65]. Animal experiments have shown that compound glycyrrhizin tablets can protect the liver by inhibiting the transduction of the NF-κB pro-inflammatory signaling pathway and the expression of downstream inflammatory molecules[66]. Studies have found that glycyrrhizin not only inhibits the expression of IL-6 and IL-8, significantly increasing the activity of liver cells, but also promotes the expression of SOD and glutathione, and inhibits malondialdehyde synthesis, thereby alleviating oxidative stress-induced liver cell damage. Glycine can inhibit the generation of reactive ROS, reduce ROS and lipid peroxidation, and alleviate liver damage caused by oxidative stress reactions[67], See Figure 4 for details.

CONCLUSION

The signaling crosstalk between mitochondria and ER, mainly mediated by ROS and calcium loading, influences each other's endoplasmic reticulum and mitochondrial function mechanisms to regulate CLD. The mechanisms of mitochondrial and ER dysfunction are complex and diverse, and they are interrelated and interact with each other. With the deepening of the basic theoretical research on the structure and function of mitochondria and ER, it is possible to combine the application of genomics, proteomics, metabolomics, and other modern technologies to explore in depth the signaling links between mitochondrial and ER function in various CLDs. In this paper, we elucidate the treatment of CLDs by TCM from traditional Chinese medicine to provide new ideas and means for the diagnosis and treatment of CLDs in the future. MAMs are the key factors linking the material and communication signals of mitochondria and ER. However, there are more methods for evaluating MAMs, and the accuracy of their detection varies in different studies, such as subjective judgment errors in techniques such as fluorescence localization. Under different pathologic conditions, the body's compensatory effects on MAMs proteins can vary. Therefore, future studies need to further define the criteria for judging the number of MAMs, such as the promotion of dynamic observation techniques at the cellular level, which will increase the credibility and rigor of the study.

FOOTNOTES

Co-first authors: Yang Zheng and Yi-Hui Zheng.

Author contributions: Zheng Y, Zheng YH, Wang JH, Zhao TJ, Wang L and Liang TJ designed the study; Zheng Y and Zheng YH wrote the first draft; Wang JH, Zhao TJ and Wang L were responsible for the production of the images; Zheng Y and Liang TJ revised the paper; All authors have read and approve the final manuscript.

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EDITORIAL

Subclinical hepatitis E virus genotype 1 infection: The concept of "dynamic human reservoir"

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Abstract

Hepatitis E virus (HEV) is hyperendemic in South Asia and Africa accounting for half of total Global HEV burden. There are eight genotypes of HEV. Among them, the four common ones known to infect humans, genotypes 1 and 2 are prevalent in the developing world and genotypes 3 and 4 are causing challenge in the industrialized world. Asymptomatic HEV viremia in the general population, especially among blood donors, has been reported in the literature worldwide. The clinical implications related to this asymptomatic viremia are unclear and need further exploration. Detection of viremia due to HEV genotype 1 infection, apparently among healthy blood donors is also reported without much knowledge about its infection rate. Similarly, while HEV genotype 3 is known to be transmitted *via* blood transfusion in humans and has been subjected to screening in many European nations, instances of transmission have also been documented albeit without significant clinical consequences. Epidemiology of HEV genotype 1 in endemic areas often show waxing and waning pattern. Occasional sporadic occurrence of HEV infection interrupted by outbreaks have been frequently seen. In absence of known animal reservoir, where HEV exists in between outbreak is a mystery that needs further exploration. However, occurrence of asymptomatic HEV viremia due to HEV genotype 1 during epidemiologically quiescent period may explain that this phenomenon may act as a dynamic reservoir. Since HEV genotype 1 infection cannot cause chronicity, subclinical transient infection and transmission of virus might be the reason it sustains in interepidemic period. This might be the similar phenomenon with SARS COVID-19 corona virus infection which is circulating worldwide in distinct phases with peaks and plateaus despite vaccination against it. In view of existing evidence, we propose the concept of "Dynamic Human Reservoir." Quiescent subclinical infection of HEV without any

clinical consequences and subsequent transmission may contribute to the existence of the virus in a community. The potential for transmitting HEV infection by asymptomatic HEV infected individuals by fecal shedding of virus has not been reported in literature. This missing link may be a key to Pandora's box in understanding epidemiology of HEV infection in genotype 1 predominant region.

Key Words: Hepatitis E; Viral hepatitis; Genotype 1; Dynamic human reservoir; Subclinical infection

Core Tip: Epidemiology of hepatitis E virus (HEV) is yet to be known and unraveled. HEV genotype 1 outbreaks tend to reoccur in periodic fashion in certain endemic areas. The virus often disappears even during conducible seasons and living conditions in between these outbreaks. There are no known animal reservoirs for human HEV genotype 1. Occurrence of asymptomatic viremia and transmission during epidemic quiescence in endemic areas may show humans acting as transient reservoir keeping the virus viable in the community. We propose this phenomenon as "Dynamic Human Reservoir" and emphasize the need for further research and data on this area for better understanding of HEV epidemiology.

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INTRODUCTION

Hepatitis E virus (HEV) infection is a global health concern that leads to 20 million infections, 3.3 million symptomatic cases, and 44000 deaths annually[1]. There is a dichotomy in the distribution of its genotypes and subsequent clinical manifestation worldwide. Previously considered a disease of developing and underdeveloped countries where poor sanitation and water hygiene prevail, is now being progressively recognized as an equally important public health problem in the industrialized world[2]. HEV genotype 1 and 2 is transmitted through contaminated drinking water, limited to humans only, and is prevalent in developing countries of Asia and Africa. It is known to manifest as a spectrum starting from asymptomatic infection, uncomplicated acute hepatitis, acute liver failure, especially among pregnant women, acute on chronic liver failure, and other extrahepatic manifestations. Genotypes 3 and 4 are initially zoonotic diseases that have crossed the species barrier and infected humans. It is endemic in industrialized nations of Asia and Europe, transmitted by undercooked meat products[3,4]. Its manifestations are less dramatic, with only a milder form of illness, but can potentially lead to chronic infection in immunocompromised hosts, culminating in the form of chronic liver disease[5-7]. Considering this dichotomy and completely distinct epidemiology of genotypes 1 and 2 from genotypes 3 and 4, this manuscript intends to discuss the former.

EPIDEMIOLOGY

The epidemiology of HEV genotypes 1 and 2 is heterogeneous across the region. While young adults are more affected in the Asian population, Egyptians experience more severe disease and high infection rates among children[8]. Similarly, the Global Burden of Disease (GBD) due to HEV is difficult to estimate due to data gaps in understanding its epidemiology. While symptomatic cases of HEV are easy to confirm and report, asymptomatic HEV infection is challenging to detect. Data on what proportion of HEV infection results in symptomatic infections is lacking, even in GBD estimation of HEV, extrapolation from the natural history of HAV infection, which may not truly reflect the behavior of HEV[9]. The actual burden of asymptomatic HEV viremia and its significance in the context of HEV genotype 1 and 2 is yet to be explored and understood.

HEV OUTBREAKS AND PERIOD OF QUIESCENCE

At least forty-four major outbreaks of HEV have been reported in Asia and Africa between 2011-2022[10]. Each of these significant outbreaks has witnessed thousands of symptomatic infections and a fair number of deaths, especially among the pregnant women. Except for recurrent outbreaks seen in Kathmandu Valley (Nepal), other outbreaks are temporally and spatially separated. It is not well understood why these large outbreaks occur interspersed by a period of inactivity or low level of sporadic cases. Having documented at least 4 significant outbreaks since 1973, we have taken Kathmandu Valley as a model for our discussion[11]. HEV once used to account for nearly half of the sporadic acute hepatitis among adults in 1997[12]. After a large outbreak of HEV between 2007-2008 in Kathmandu valley, HEV infection went into

dormancy. It is now rare to see acute HEV hepatitis in Kathmandu Valley other than occasional cases, which are more likely to be imported based on their travel history. After the 2015 mega earthquake, it created a perfect humanitarian setting due to internal displacement of people in Nepal, prediction of outbreak was anticipated[13]. However, HEV defied the prediction and remains a rare entity in Kathmandu Valley, once known as the epicenter of HEV.

SUBCLINICAL VIREMIA IN ENDEMIC AREAS

During our investigation among residents of Kathmandu, to our surprise, we detected viremia among asymptomatic healthy blood donors amid quiescence of HEV[14]. Out of 581 blood donors evaluated in 2014, HEV RNA was isolated in eight subjects (1.5%), all belonging to genotype 1a. Rate of anti-HEV IgM and anti-HEV IgG detection were 3.6% and 8.3%, respectively. Serum transaminase levels were normal in all the subjects and majority of these viremic subjects did not have any serological evidence of infection[14]. Similar reports have been published from other genotype 1 predominant regions, and some of the studies have even shown the possibility of transfusion transmitted HEV[15-17]. However, these infections were milder, subclinical, and of unknown significance, hence classifying this as an unimportant route of transmission.

HEV viremia among healthy asymptomatic blood donors has been reported in several studies from India. Arankalle *et al*[15] reported HEV RNA detection among 3 out of 200 healthy blood donors with Anti HEV IgM antibody in only one case. Similarly, Khuroo *et al*[17] found HEV viremia among 0.8%-3.7% of healthy controls and evidence that blood transfusion can transmit HEV infection. Occurrences of subclinical infection during an outbreak have also been well documented[18,19].

IMPLICATIONS OF SUBCLINICAL HEV VIREMIA

These findings indicate that subclinical HEV viremia is frequent in endemic areas both during an outbreak and even in the absence of sporadic cases or an outbreak. One may assume that without sporadic outbreaks of HEV in a community, contamination of the drinking water supply may not occur. However, these findings refute such assumptions. Even during quiescence of HEV infection, subclinical infection, subsequent fecal shedding, and contamination of water sources might keep the virus transmitting in the community. Now, there is evidence in both experimental animal models as well as humans that asymptomatic viremic subjects can shed HEV in feces[18,20]. Unlike in symptomatic subjects, where both viremia and fecal shedding occur for a short duration and until symptomatic and biochemical resolution, the duration of viremia in asymptomatic subjects is unknown. However, it is unlikely that asymptomatic subjects can shed the virus protractedly.

Why some individuals with HEV infection do not develop clinical hepatitis is unknown. One may speculate that for any symptomatic infection, there could be asymptomatic infections, but the proportion is yet to be determined. There is preliminary data to suggest that inoculum size may be an essential factor in determining the severity of HEV infection, and for that matter, low inoculum size may be the reason individuals do not develop disease despite viremia[21]. There is evidence that patients with subclinical infections during outbreak setting have lower viral load than those with clinical acute hepatitis[19]. Lower dose of inoculum at infection leading to low viral load may result in subclinical infection, but this association is yet to be proven. Fecal shedding of HEV from subclinical infection causing low-grade to modest contamination of drinking water sources could be the reason HEV keeps circulating in the community as subclinical viremia even when clinical HEV is nonexistent in the community. These subclinical human infections might function as transient reservoirs or "dynamic human reservoirs" and have a strong implication in understanding the epidemiology of HEV.

Another essential characteristic of subclinical infection is the frequent absence of IgM and IgG antibodies against HEV [14,15,17]. Low viral load of HEV in subclinical infections may be the reason for lower immune response leading to the absence or short-lived IgM and IgG in these cases. This might as well explain the reason for the low prevalence of anti-HEV IgG among blood donors despite subclinical viremia being common and may indicate subclinical infection and circulation may not provide immunity against future infections. Contrary to these observations, Egyptian children have a high seroconversion rate despite acquiring asymptomatic infections. Our observations cannot explain this phenomenon, but despite frequent subclinical infections in children, significant outbreaks have not been observed in Egypt, and adverse maternal outcomes due to HEV infection during pregnancy are less frequent[22]. Antibody response due to early subclinical infection in childhood could have prevented both outbreaks and adverse outcomes in pregnancy in Egypt.

CONCLUSION

Hepatitis E Virus infection is an enigma. Heterogeneity, even within genotype 1 infection in terms of epidemiological and clinical manifestation, has stood as a barrier to proper understanding of this disease. Frequent detection of HEV viremia in asymptomatic healthy subjects during periods of low incidence of clinical acute hepatitis in the community argues towards possible transient human reservoirs. In view of limited available data, more studies to characterize these subclinical infections and to better understand their clinical significance are warranted.

FOOTNOTES

Author contributions: Shrestha A developed the core concept, reviewed literature, drafted the manuscript; KC S contributed to improvising the concept, reviewed literature, drafted manuscript; Basnet S contributed by literature review, improvement and critical review of the manuscript.

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Metabolic dysfunction-associated steatotic liver disease: A silent pandemic

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Abstract

The worldwide epidemiology of non-alcoholic fatty liver disease (NAFLD) is showing an upward trend, parallel to the rising trend of metabolic syndrome, owing to lifestyle changes. The pathogenesis of NAFLD has not been fully understood yet. Therefore, NAFLD has emerged as a public health concern in the field of hepatology and metabolisms worldwide. Recent changes in the nomenclature from NAFLD to metabolic dysfunction-associated steatotic liver disease have brought a positive outlook changes in the understanding of the disease process and doctor-patient communication. Lifestyle changes are the main treatment modality. Recently, clinical trial using drugs that target 'insulin resistance' which is the driving force behind NAFLD, have shown promising results. Further translational research is needed to better understand the underlying pathophysiological mechanism of NAFLD which may open newer avenues of therapeutic targets. The role of gut dysbiosis in etiopathogenesis and use of fecal microbiota modification in the treatment should be studied extensively. Prevention of this silent epidemic by spreading awareness and early intervention should be our priority.

Key Words: Metabolic dysfunction; Fatty liver; Obesity; Insulin resistance

Core Tip: Non-alcoholic fatty liver disease is often considered the hepatic manifestation of metabolic syndrome. The new nomenclature of "metabolic dysfunction associated steatotic liver disease" emphasizes the role of disordered metabolism in the pathogenesis. Weight reduction by lifestyle changes is the mainstay of treatment.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a broad spectrum of liver disorders related to dysmetabolic conditions. It is characterized by macrovesicular steatosis with or without hepatocellular ballooning, lobular inflammation, and hepatic fibrosis[1]. NAFLD is the leading cause of hepatic morbidity and mortality worldwide and is now the most common indication of liver transplantation (LT)[2-5]. Thus, NAFLD is associated with exorbitant healthcare costs[6,7]. It affects nearly one third of the adult population[8-11] and 9%-12% of the pediatric population[12-14]. The latest meta-analysis by Younossi *et al*[15], showed a global rise in NAFLD prevalence at an alarming rate- from 25.26% (21.59-29.33) in 1990-2006 to 38% (33.71-42.49) in 2016-2019 ($P < 0.001$)[15]. The prevalence in Asia is following a trajectory similar to that in the western countries[16-18].

THE DEBATE OVER THE NEW NOMENCLATURE: NAFLD *VS* MASLD

NAFLD is usually defined as the presence of steatosis in >5% hepatocytes, detected by imaging or histopathology after exclusion of secondary causes for hepatic steatosis[19]. Pathologically, it is strongly linked to metabolic syndrome, which is a constellation of obesity, hypertension, hyperlipidemia, type 2 diabetes mellitus (T2DM)[20]. Patients with NAFLD are at higher risk of liver-related complications as well as cardiovascular complications and mortality[20,21]. The most common cause of mortality in patients with NAFLD is cardiovascular complications, followed by extrahepatic malignancies and hepatic complications, highlighting the fact that NAFLD is a multisystemic disease[22,23]. Recently, an international expert group proposed to change the existing nomenclature "Non-Alcoholic Fatty Liver Disease" and adopt the acronym MASLD, or "Metabolic dysfunction-Associated Steatotic Liver Disease", thus emphasizing the role of systemic metabolic dysfunction in the etiopathogenesis[24]. The shift in the nomenclature introduces a "positive" diagnostic criteria and highlights the cardiovascular risk profile of these individuals. The new nomenclature thus aims for a better understanding of the disease and patient-physician communication.

The diagnosis of MASLD is based on the detection of steatosis of hepatocytes (diagnosed by imaging, biomarkers, or histology) and at least one feature among the following three - overweight/obesity, type 2 diabetes mellitus and metabolic dysregulation. The criterion of metabolic dysregulation is fulfilled when atleast two features among the following are found: increased waist circumference, hypertension, hyperlipidemia, low level of high-density lipoprotein-C (HDL-C), prediabetes, insulin resistance, and subclinical inflammation. These criteria will ensure the identification of a more homogenous disease condition than NAFLD, overcoming the dilemmas and controversies in defining alcohol intake, thereby encouraging new pathophysiological developments and augmenting clinical studies (as elegantly reviewed by Vargas *et al*[25] in this present issue).

LEAN NAFLD

The prevalence of NAFLD showed a rising trend similar to the rising burden of obesity[26,27]. In contrast, lean patients with NAFLD were also detected. In the meta-analysis by Young *et al*[28], 11% and 25% of the general and NAFLD populations, were identified to be "lean NAFLD" respectively[29]. Metabolic profile was more deranged in lean NAFLD patients than healthy controls. These patients also had a higher prevalence of insulin resistance, metabolic syndrome and higher levels of pro-inflammatory mediators[30,31]. On the contrary, lean NAFLD patients have more favorable histologic features than obese NAFLD patients[31].

The ethnicity of the study population should be considered for correctly defining lean MAFLD patients. Body mass index (BMI) cutoffs depending on the ethnicity of the individual have been recommended to define "lean MAFLD". The cutoffs for defining lean MAFLD are BMI < 25 kg/m^2 for Caucasians and < 23 kg/m^2 for Asians[32]. The prevalence of lean NAFLD has been found to be 5%–45% in the Asian population and 5%–20% among Europeans[33]. Further studies are needed to better characterize the newly-defined lean MAFLD patients.

T2DM AND NAFLD

Presence of concomitant T2DM accelerates the disease progression in NAFLD, as patients with concomitant T2DM and NAFLD had higher rates of advanced fibrosis and adverse outcomes compared to NAFLD without T2DM[10]. Furthermore, the concomitant NAFLD and T2DM causes increased liver-related, cardiovascular complications as well as overall mortalities[10]. Other complications of T2DM like diabetic retinopathy, nephropathy, and polyneuropathy, have

been detected more frequently in diabetes patients with coexisting NAFLD[34-36]. Therefore, a novel diagnostic score has been recommended for T2DM patients with NAFLD[37]. Those with an FIB-4 score of more than 1.3 have a higher risk of developing severe disease[38]. Recent NAFLD guidelines recommend that T2DM populations be screened for NAFLD [24].

NAFLD AND METABOLIC SYNDROME

NAFLD is considered as the hepatic manifestation of metabolic syndrome[39]. In the meta-analysis by Ballestri *et al*[40], NAFLD was associated with incident metabolic syndrome in 5-year follow-up. On the other hand, another study by Ma *et al*[41] demonstrated that patients with metabolic syndrome had a higher risk of developing NAFLD. While comparing the new term 'MASLD' with the traditional definition of 'NAFLD', Lin *et al*[42] found higher proportions of metabolic comorbidities in patients with MASLD, emphasizing the impact of positive diagnostic criteria.

DIAGNOSTIC EVALUATION

Liver biopsy is the gold standard to assess disease activity and severity. The severity of liver fibrosis has been identified as the most important prognostic factor and is independently linked with hepatic outcomes in NAFLD patients. Sanyal *et al*[2], in an elegantly done prospective study of 1773 adult patients with NAFLD, found that F3, and F4 fibrosis were associated with increased risk of hepatic complications and death, after adjustment for age, sex, race and diabetes status.

Several non-invasive tests have been developed as diagnostic and prognostic tools in patients of NAFLD as liver biopsy is invasive and less preferred for disease monitoring. Imaging to detect and quantify hepatic steatosis has gained prominence with advances of computerized tomography (liver attenuation index) and magnetic resonance imaging (magnetic resonance imaging proton density fat fraction-MRI-PDFF)[43-45]. Multiparametric MRI, which consists of MR spectroscopy, MR elastography and T1 mapping, has demonstrated high diagnostic accuracy, comparable to liver histology[45,46]. Similar multiparametric CT sequences that can evaluate the hepatic attenuation, liver segmental volume ratio, splenic volume, and liver surface nodularity score, have shown encouraging results as an alternative diagnostic tool to identify advanced fibrosis in NAFLD patients[47]. MRI-PDFF response has been studied as a potential surrogate for histologic improvement after treatment of NAFLD. Several studies have shown a clear correlation between a reduction in MRI-PDFF (usually taken as a ≥ 30% relative reduction) and improvement in the NAFLD activity score, resolution of NASH, and fibrosis[48,49]. Boursier *et al*[50], in a large cohort of 1097 patients, compared the prognostic efficacy of fibrosis index based on 4-factors (FIB4), transient elastography (TE) and liver biopsy. The results showed that FIB4 and TE showed good accuracy for the prediction of liver-related events (LRE), with Harrell's C-indexes > 0.80 [0.817 (0.768-0.866) *vs.* 0.878 (0.835-0.921), respectively, $P = 0.059$], as compared to liver biopsy. The authors proposed a stepwise algorithm to accurately stratify NAFLD patients based on their risk for LRE: compared to patients with "FIB4 < 1.30", those with "FIB4 ≥ 1.30 then TE < 8.0 kPa" had a similar risk of LREs [adjusted hazard ratio (aHR) 1.3; 95%CI 0.3–6.8], whereas the risk of LREs significantly increased in patients with "FIB4 ≥ 1.30 then TE 8.0-12.0 kPa" (aHR 3.8; 95%CI 1.3–10.9), and even more for those with "FIB4 ≥ 1.30 then TE > 12.0 kPa" (aHR 12.4; 95%CI 5.1–30.2).

However, we need to keep in mind a major limitation of using these non-invasive methods to diagnose and monitor hepatic steatosis is that it provides no information on the underlying etiology or associated risk factors.

TREATMENT

As NAFLD is a systemic disease of disordered metabolism, a multi-disciplinary approach is of utmost importance for the treatment. Weight reduction by lifestyle changes and dietary interventions is the cornerstone of treatment in obese and lean NAFLD patients[19,51]. The beneficial effects of lifestyle modifications have been consistently found to be helpful in the resolution of hepatic steatosis in both lean Asian and Caucasian NAFLD patients[51-53]. Medical treatment with glucagon-like peptide receptor agonists, sodium-glucose cotransporter-2 inhibitors, and peroxisome proliferator-activated receptor-γ agonists was able to improve inflammation and fibrosis, as well as reduction in blood pressure, better glycemic control and lipid profile[54-56]. Bariatric surgery is an effective treatment for a select group of patients who are non-responsive to dietary interventions and exercise or unable to lose weight through lifestyle changes. It can improve both histological characteristics of NASH as well as mortality due to cardiovascular complications[57]. Lim *et al*[58] studied the usefulness of endoscopic bariatric therapies such as intragastric balloon, endoscopic sleeve gastroplasty, and duodeno-jejunal bypass liner in reducing weight and found better results as compared to standard medical therapy. With extensive research into the therapeutic options in the pipeline, treatment strategies for NAFLD treatment are promising[59].

FUTURE PERSPECTIVE

Further prospective studies are the need of the hour to develop more accurate diagnostic tools for advanced fibrosis in NAFLD and to explore the underlying pathophysiological mechanisms linking NAFLD with other conditions. More in-depth research on gut microbiota in the etiopathogenesis of NAFLD and its role in the therapeutics is warranted. Most

randomized clinical trials of available drugs do not reflect the proper scenario, due to the limitations of therapeutic targets, drug safety, and other factors. This current review by Vargas in this issue serves as a valuable resource for researchers seeking a comprehensive understanding of NAFLD and tries to address these issues[25].

CONCLUSION

NAFLD must be evaluated as a multisystemic metabolic disorder. It may lead to liver-related complications, thus the need for multidisciplinary screening and disease management cannot be over emphasized. Routine screening for NAFLD is recommended in patients with metabolic syndrome. Lifestyle intervention remains the most important treatment modality. The global pandemic of NAFLD poses significant social and economic burden; thus it is of utmost importance to create widespread awareness in order to make early interventions and achieve better outcome.

FOOTNOTES

Author contributions: Samanta A did the literature review and wrote the original manuscript; Sen Sarma M reviewed and revised the manuscript, did critical analysis; All authors approved the final draft of the manuscript.

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REVIEW

Spectrum of COVID-19 induced liver injury: A review report

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic has caused changes in the global health system, causing significant setbacks in healthcare systems worldwide. This pandemic has also shown resilience, flexibility, and creativity in reacting to the tragedy. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection targets most of the respiratory tract, resulting in a severe sickness called acute respiratory distress syndrome that may be fatal in some individuals. Although the lung is the primary organ targeted by COVID-19 viruses, the clinical aspect of the disease is varied and ranges from asymptomatic to respiratory failure. However, due to an unorganized immune response and several affected mechanisms, the liver may also experience liver cell injury, ischemic liver dysfunction, and drug-induced liver injury, which can result in respiratory failure because of the immune system's disordered response and other compromised processes that can end in multisystem organ failure. Patients with

liver cirrhosis or those who have impaired immune systems may be more likely than other groups to experience worse results from the SARS-CoV-2 infection. We thus intend to examine the pathogenesis, current therapy, and consequences of liver damage concerning COVID-19.

Key Words: Autoimmune liver disease; COVID-19; Clinical manifestation of liver; Drug-induced liver injury; SARS-CoV-2

Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has imposed an unprecedented burden on public health and healthcare globally. It can decompensate pre-existing liver disease or induce acute liver injury. Its presence in hepatocytes directly exhibits cytopathic action and damages the liver because of hypoxia, inflammation, and medication toxicity. The pathophysiology of COVID-19-related liver involvement includes viral cytotoxicity, immunological dysregulation's secondary effect, hypoxia brought on by respiratory failure, ischemia damage from vascular endotheliitis, heart failure, or drug-induced liver injury. This study focuses on the pathophysiology, available treatments, and outcomes of liver injury in relation to COVID-19.

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INTRODUCTION

The continuing infection caused by coronaviruses, which exploded in 2019, has accelerated into a pandemic problem worldwide. The disease is mainly a respiratory tract viral infection caused by newly emerging strains of coronaviruses. More than 60 million confirmed coronavirus disease 2019 (COVID-19) cases, including almost 1.5 million deaths, have been reported globally in 189 countries since its inception up to October 2022[1]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease infection has mainly targeted the respiratory tract system. It can cause severe disease with acute respiratory distress syndrome, which seems to increase its potential fatality in some infected patients[2]. However, lately, the notion that COVID-19 is a systemic infection and inflammatory disease is gaining eye-catching attention, as the disease is showing a systemic feature that affects other visceral organs, including the liver and gastrointestinal tract[3-7]. The chief viral receptor for SARS-CoV-2 infection is angiotensin-converting enzyme 2[8,9]. After virus attachment to the host cell, the host transmembrane serine protease-2 (TMPRSS2) primes the viral S protein [10]. The viral RNA forms two major polyproteins in the host cytosol: protein phosphatase 1 alpha and protein phosphatase 1, which is further converted into 16 non-structural proteins (nsp1 to nsp16)[11]. Phosphatase 1, unlike SARS-CoV-2 infection-induced lung system and myocardial injury, the clinical manifestation of liver organ involvement has been a point of contention since the beginning of the COVID-19 pandemic[3,12-18]. The debate includes potential pathophysiology mechanisms where active viral replication of SARS-CoV-2 in the liver produces liver cytotoxicity and drug-induced liver injury, exacerbating underlying liver disease[19,20]. The incidence of increased concentration of liver transaminase enzymes aspartate aminotransferase and alanine aminotransferase in COVID-19 patients ranges from 2.5% to 76.3%[21-24]. As the liver is humans' primary metabolic and detoxifying organ, the therapeutic efficacy and safety profile could alter the moderate loss of hepatic function. As such, mechanistic insights causing liver injury linked to COVID-19 are required. To date, there is little comprehensive evidence of underlying histopathological changes. In addition to vascular abnormalities that include liver cell necrosis, mild lobular and portal inflammation, unbalanced portal vein branches producing intrahepatic, typically ductular proliferation, and hepatic steatosis (microvesicular) seem to be regularly observed in the livers of diseased patients[25-29]. Although through real-time reverse transcription polymerase chain reaction (PCR), viral RNA has been detected in the liver among major organs excluding the respiratory tract[13], a classic hepatic picture has not yet been reported. Hepatic tropism and direct cytopathic effects are the potential mechanisms for infections associated with liver injury[19,26-29]. S protein is the principal mediator for the entrance of SARS-CoV-2, which interconnects with the host angiotensin-converting enzyme 2 (ACE2) receptor and TMPRSS2 receptors specifically[30]. However, other factors, such as ganglioside[31], can affect how S protein and ACE2 interact. Recently, Ou and colleagues evaluated the capacity of S protein-containing pseudovirions to infect several cell lines. Interestingly, viral vectors encoding the S protein were more effective at transfecting the HuH7 hepatocyte cell line and the Calu3 human lung cancer cell line than the control pseudovirions[32]. As permissive cell types for coronavirus infection are recognized, hepatocyte cell lines such as HuH7 cells have emerged as viable positive controls in SARS-CoV-2 immunostaining[33]. These findings indicate that SARS-CoV-2 infection of human liver ductal teratoma may be possible. Viral replication may occur inside the bile duct epithelium despite the discovery of noticeably greater ACE2; no direct proof of SARS-CoV-2 cholangiocellular infection has been revealed in infectious patients with COVID-19. The detection and identification of SARS-CoV-2 viral RNA or proteins in bile, which are largely generated by hepatocytes and cholangiocytes, are indirect indications of SARS-CoV-2, cholangiocellular infection, and ongoing direct interaction between

biliary fluids with cholangiocellular apical membrane. However, only a single case report indicates SARS-CoV-2 RNA is present in bile[34].Activating hepatic stellate cells is crucial as the primary source of cellular fibrosis in developing chronic liver disorders[35]. Hepatocellular and cholangiocellular damage caused directly or indirectly by COVID-19 may produce a proinflammatory milieu that leads to the activation of hepatic stellate cells and, as a result, the development of fibrosis. Fibrosis has been reported in patients with non-alcoholic fatty liver disease or underlying chronic liver disease (CLD). Long-term follow-up studies are required to establish hepatic fibrosis as a potential long-term side effect of COVID-19, especially in patients with pre-existing liver illnesses, even though the current evidence suggests minor and transient liver damage associated with the virus. SARS-CoV-2 can cause cytopathic consequences such as host lipid metabolism and mitochondrial dysfunction. Hepatic steatosis in patients may be caused by cytokine storm-induced immunopathology, activity, and adverse medication reactions such as corticosteroids. Microvesicular steatosis is often brought on by abnormalities in mitochondrial oxidation, either hereditary or acquired; mammalian target of rapamycin (mTOR), which serves as the primary regulator for autophagy, also stimulates *de novo* lipogenesis[36-40] through mechanisms reliant on viral non-structural protein 6, which is substantially husbanded in SARS-CoV-2 and has been demonstrated in hijacking the autophagy pathway[41-43]. Additionally, it has been discovered that cells infected with Middle East Respiratory Syndrome, HuH7, exhibit hyperactivation of the mTOR pathway, which prevents viral replication when inhibited by the drug rapamycin[44]. Given the most recent findings that SARS-CoV-2 infection limits autophagy[45],it is probable that the mTOR-dependent infection mechanisms of SARS-CoV-2, SARS-CoV, and MERS-CoV are related. Furthermore, it has been demonstrated that interleukin 6 (IL-6) stimulation dramatically boosts mTOR activity[46]. The most common factor in COVID-19-associated acute respiratory distress syndrome (ARDS) is the need for invasive ventilation, substantial doses of positive end-expiratory pressure, and vasoconstrictor therapy due to hemodynamic instability[47-50].

MECHANISM OF SARS-COV-2 INFECTION

The entry of SARS-CoV-2 virus into host cells requires dense glycosylated spike (S) protein containing two functional components, S1 and S2. The S2 subunit controls the fusion of the viral and cell membranes, whereas the S1 subunit controls virus binding to the host cell receptors[51,52]. The serine protease TMPRSS2 must first prime the S protein. TMPRSS2 breaks down the S protein at the S1/S2 and S2 subunit sites[8]. The receptor-binding domain (RBD), composed of about 300 amino acids, is a trimer of separate monomers that comprise the S protein, which is roughly 1300 amino acids long. The RBD on S protein plays a specific role that involves direct participation when host receptors are recognized[53-55]. Zhou *et al*[9] showed that S protein and ACE2 binding are necessary for infection in HeLa cells. Walls and associates also discovered the activity of human ACE2 as a SARS-CoV-2 functional receptor[56]. In a metaphor, the virus S protein can unlock the human body's ACE2 lock. The SARS-CoV-2 S protein has 10-20 times the affinity of ACE2 compared to SARS-CoV, as reported by surface plasma resonance analysis[53]. This is a significant finding that could help explain the virus's high infectivity rate. Many studies have reported the activity of human ACE2 as a SARS-CoV-2 functional receptor[56,57].

HEPATOPATHY/RISK FACTOR

There are insufficient data regarding the influence of hepatitis B on COVID-19, which has a high incidence, particularly in Asia and China. As per the studies, 2% of severe COVID-19 cases involved hepatitis B infection compared to mild COVID-19 cases (0.06%)[58]. Patients who have a history of hepatitis B or C infection are more likely to develop severe hepatitis, similar to the higher susceptibility for developing severe immunodeficiency infection faced by patients with cirrhosis[3,59-61]. Compared to others, patients with liver disease more commonly develop leukopenia and lympho-cytosis, as they suffer from conditions such as leukocytosis with neutrophilia and elevated C-reactive protein[62]. People with related gastrointestinal neoplasms express a higher degree of angiotensin 2 receptors, contributing to the risk of SARS-CoV-2 infection since this enzyme provides an entry for the virus to enter the cells. However, this could be regulated by systemic inflammatory responses and gastrointestinal tract level, as angiotensin 2 modulates this response [63,64]. Monocyte chemoattractant protein-1, known to aggravate COVID-19 and promote steatohepatitis, is higher in the serum of coronavirus-infected patients[18].

ETIOLOGY OF LIVER INJURY IN COVID-19-INFECTED PATIENTS

Viral immunologic injury

SARS-CoV-2 has also been successfully detected in blood and fecal samples from COVID-19 patients, indicating potential intestinal involvement of the virus[65-67]. Through tests with HeLa cells that produced ACE2, Zhou *et al*[9] demonstrated that ACE2 is the cell receptor for the entrance of SARS-CoV-2 into host cells. It was recently found that cholangiocytes might express ACE2 at a level up to 20 times greater than hepatocytes. SARS-CoV-2 may be able to infect cholangiocytes and lead to bile duct dysfunction, as per the expression pattern of ACE2. Viral immunologic damage may be one of the causes of liver injury since cholangiocytes are involved in immune responses and liver regeneration on a multifaceted and crucial level. In individuals with COVID-19, levels of alkaline phosphatase (ALP) and gamma-glutamyl transferase

(GGT), which indicate bile duct damage, do not significantly rise despite the clinical data showing and demonstrating elevated levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) in these individuals. Additionally, the autopsy report revealed no viral inclusions in the liver tissue[68].

Damage of cholangiocytes by SARS-CoV-2

According to research by Qi *et al*[63], the epithelial cells of the bile duct express the ACE2 receptor 20 times more than liver cells[69,70]. This indicates that SARS-CoV-2 may damage and infect bile duct cells directly, which could eventually result in bile duct dysfunction. Epithelial cells for bile ducts are crucial for liver regeneration and the immune system; thus, liver damage may result when SARS-CoV-2 infects them and results in cholestasis[69,70]. Increased concentrations of ALP and GGT are reliable signs of damage to bile duct epithelial cells[71]. More studies are necessary to conclusively connect liver damage to the bile duct cell damage brought on by the SARS-CoV-2 infection. Researchers have hypothesized a potential mechanism for SARS-COV-2-induced liver damage. Infection of liver cells occurs when hepatic parenchymal cells, which are produced from bile duct epithelial cells, compensate for the hyperplasia of ACE2 expression in the liver tissues[72]. Nonspecific liver inflammation can cause an increase in cytokines and inflammatory biomarkers, including IL-2, IL-6, IL-7, and IL-10, interferon gamma (IFN-g) inducible protein 10, and tumor necrosis factor alpha (TNF-α)[12,73], which can cause severe damage (*e.g.*, hepatomegaly, elevated serum transaminase, high bilirubin, hepatic encephalopathy, and even liver failure). In case inflammatory response syndrome worsens without appropriate management, COVID-19 patients may experience multiple organ failure and death[12,74].

Hypoxic injury

The liver is susceptible to cardiovascular abnormalities because of composite vascular supply and substantial metabolic activity. The condition known as ischemic hepatitis, also referred to as hypoxic hepatitis, is frequently found in critically ill patients and is the consequence of underlying circulatory, cardiac, or respiratory failure that can result in passive congestion or reduced hepatic perfusion[75,76]. Systemic stress causes a compensatory reduction in peripheral and splanchnic blood flow, which in turn, causes a reduction in hepatic blood flow, and as a result, hepatocellular hypoxia, particularly in zone 3[12,77]. Cell injury *via* lipid peroxidation can occur when reactive oxygen species are generated by re-exposing ischemic hepatocytes to oxygen in a condition called reperfusion injury[78]. Furthermore, Kupffer cells can trigger the reunion and activation of polymorphonuclear leukocytes by producing cytokines as a response to ischemia [78].

Drug-induced damage to the liver

In the interim, a variety of different medications including antiviral (lopinavir /ritonavir, remdesivir), antibiotic (macrolides), antimalarial/antirheumatic (hydroxychloroquine), immunomodulating (tocilizumab, corticosteroids), and antipyretic (acetaminophen) medications, are being used in clinical studies or an off-label manner. However, most medications (*e.g.*, ritonavir and remdesivir) have already shown hepatotoxic potential. Also included is corticosteroid treatment, which the World Health Organization currently advises for patients with severe SARS-CoV-2 infection[79]. These findings are further supported by the Human Protein Atlas database, which demonstrates that the greatest pattern of ACE2 expression throughout human intestinal cells have a variety of cell types (information accessible at https://www.prote inatl as.org/ENSG0 00001 30234-ACE2/tissue). Additionally, it has been proven that human intestine organoids are susceptible to SARS-CoV and SARS-CoV-2 infections[80]. The most common clinical symptoms, including fever, coughing, exhaustion, and shortness of breath, were highlighted in early clinical trials. Later investigations were able to unearth evidence that COVID-19 also has extrapulmonary manifestations. Because the liver is the primary organ for metabolism and detoxification, it is essential to maintain optimal liver function when utilizing any of the COVID-19 therapeutic modalities. It is well known that liver damage results from hepatic inflammation, including activation of innate immune cells and cytokine production[81]. An old drug called chloroquine has recently been tested in treatments of COVID-19 patients after showing indications of being a potential treatment. The drug's superior efficacy in viral control was well demonstrated when concurrent clinical trials on chloroquine conducted in 10 hospitals across China showed successful inhibition of viral replication[82]. The pharmacodynamics of this drug in treating COVID-19 may show involvement of the arresting of cytokine storms, the activation of CD8 T cells or prevention of endocytosis-mediated uptake of the virus[83,84]. The COVID-19 virus primarily affects the lungs[74]. However, it can also harm the liver through a variety of mechanisms, including an disorganized immune response, virus-related liver cell damage, drug-induced liver injury (DILI), and ischemic liver dysfunction in the context of multiorgan failure[85]. Patients with cirrhosis and those with impaired immune systems may be more likely to experience negative consequences after contracting SARS-CoV-2[21]. The direct cytopathic impact of COVID-19, DILI, an uncontrolled immunological response, or sepsis are only a few of the possible causes of liver damage[86]. In COVID-19 patients who also experience diarrhea, SARS-CoV-2 RNA has been found in blood and stool samples, indicating that the liver is likely implicated in the etiology of this illness [67]. The underlying condition might become worse because of COVID-19. CLD increases the risk of death, especially in critically ill patients, by causing hepatic decompensation or acute-on-chronic liver failure[56,86,87]. In severe COVID-19 infections, liver damage is more frequently caused by an inflammatory cytokine storm[86,88] than by the virus itself in a direct cytotoxic manner[86]. While in the final stage of SARS, the continued interaction between the lung and systemic inflammation causes multiorgan vascular dysfunction and a cytokine storm, and prothrombotic factors aggregate and produce thrombosis due to the bone marrow and liver acute phase responses[89,90].The human body's primary organ for drug metabolism is the liver. The medications used to treat COVID-19 individuals may harm the liver. According to Kulkarni *et al*[21], there is drug-induced liver damage as frequently as 25.4% of the time. In the United States, antimalarial drugs, including chloroquine and hydroxychloroquine, have received emergency authorization to treat COVID-19. However, because hydroxychloroquine concentrates in the liver, individuals with hepatitis or other hepatic illnesses, as

well as those taking other medications known to be hepatotoxic, should exercise caution[91]. Along with antimalarial drugs, antiviral drugs such as lopinavir-ritonavir, remdesivir, and favipiravir have been utilized to treat COVID-19. According to one case study, remdesivir was the drug that produced the most instances of hepatotoxicity, with 23% of patients reporting elevated levels of liver enzymes linked to the drug[92]. A 50-year-old man who developed ARDS and died from severe COVID-19 had his first COVID-19-related autopsy. The liver's autopsy revealed minor lobular and portal activity, as well as moderate microvesicular steatosis. The SARS-CoV-2 virus or liver damage brought on by drugs is thought to be the source of the injury. There was an increase in proinflammatory CCR6+T-helper 17 (Th17) in CD4 T cells and cytotoxic granules in CD8 cells. Hepatocyte dysfunction may also be caused by decreased CD4 and CD8 cell numbers[93]. The clinical findings of patients with and without a history of non-alcoholic fatty liver disease (NAFLD) were compared. The results showed that patients with NAFLD had a longer viral shedding time (P = 0.0001) and a greater probability of disease development. According to research published by Sonzogni and associates[94], a hepatic biopsy from one of these patients revealed microvesicular steatosis along with overactivation of T cells, which raised the potential for collateral damage of the liver caused by virally induced cytotoxic T cells. The main finding of the study revealed that patients with severe and non-severe COVID-19 may be at combined risk for elevated blood ALT levels. In adult patients with severe and non-severe COVID-19, the secondary outcomes included the risk of the variant parameters namely elevated AST, hyperbilirubinemia, and hypoalbuminemia. The amount of GGT from the included prior studies was also evaluated using a pooled mean difference (MD). Serum ALT or AST values over 40 U/L were deemed to be high. As opposed to hypoalbuminemia, defined as a serum albumin level below 40 g/L, hyperbilirubinemia is defined as a total bilirubin (TBIL) level greater than 17 mmol/L[95]. Immune-mediated injury, or ischemic hepatitis, can result from a significant systemic inflammatory response brought on by COVID-19. Current therapies include lopinavir/ritonavir, hydroxychloroquine, and remdesivir, all of which have the potential to be hepatotoxic and increase the risk of DILI[96, 97]. The underlying chronic hepatitis B may reactivate while using tocilizumab or other immunosuppressive options. Although it has been hypothesized that viral replication within the infected hepatocytes might directly induce cytotoxicity, SARS-CoV-2 viral inclusions have not been found in the liver[68]. The clinical signs and symptoms of COVID-19 might be anything from asymptomatic to respiratory failure. Fever (50%) and cough (38%) are the symptoms that patients experience most frequently. Patients have also mentioned experiencing other symptoms such as headaches, nausea, vomiting, and diarrhea[98,99]. Inflammatory cytokines including IL-1, IL-6, IL-8, and TNF-α increase COVID-19 severity. When there is a serious infection, abnormal liver enzyme and bilirubin levels are discovered, which makes it challenging to treat individuals with liver disease who are also on immunosuppressants, notably cirrhosis or autoimmune hepatitis[100-102]. Conversely, investigations have indicated a relationship between elevated LDH levels, creatinine kinase, and myoglobin in critically ill COVID-19-infected patients. Therefore, it is proposed that elevations of aminotransferase levels might also be due to other conditions apart from liver damage. Nevertheless, COVID-19 infection could cause myositis similar to that caused by severe flu. A liver biopsy of a patient who died due to COVID-19 infection revealed the presence of microvesicular steatosis as well as mild lobular and portal inflammation[17,103,104]. Purely hepatocellular and cholestatic types of drug-induced liver damage are clinical conditions. Patients with COVID-19 who have abnormal liver function are typically described as having hepatocellular damage. The primary signs and symptoms of COVID-19 are fever, cough, exhaustion, and dyspnea. Because acetaminophen is a common constituent in antipyretic medications, some COVID-19 patients may have a history of using them. These substances are known to be typical medications that can directly poison hepatocytes. The use of several patented Chinese medications to prevent COVID-19 infection may potentially induce liver damage. Recent liver pathology findings from analyzing COVID-19 patients point to the possibility of drug-induced liver damage, as the data revealed the presence of mild lobular inflammation and moderate microvascular steatosis[97]. Antiviral medications such as ribavirin, in addition to altering liver function, may also induce or worsen tissue hypoxia through hemolysis, which might raise blood liver enzyme levels. Before receiving COVID-19 medication, patients with chronic liver diseases such as hepatitis B or C may already have increased transaminase levels, which might increase their chance of developing drug-induced liver damage. As a result, individuals with COVID-19 who also have basic liver disorders as comorbidities are clinically treated with antipyretic medications, traditional herbal remedies, or antiviral medications. Multiple proinflammatory cytokines and inflammatory indicators, including TNF, IL-2, IL-6, IL-7, IL-18, granulocyte-colony stimulating factor, IFN, and ferritin, can be abundantly released when a cytokine storm occurs[12]. Fulminant and deadly hypercytokinemia may start a series of events that result in tissue damage or organ failure, including liver failure[105].

Autoimmune liver disease

The clinical effect of pre-existing immunosuppression on the severity of COVID-19 is still an intricate topic. The hazards that some illness groups confront are a source of worry. For instance, individuals with rheumatoid diseases and inflammatory bowel diseases have been linked to greater disease severity while using maintenance corticosteroids and thiopurines, respectively[106,107]. However, the effect of immunosuppression on the course of the illness in patients who have had solid organ transplantation, such as liver transplantation (LT), resembles that of non-immunosuppressed persons (described below)[108,109]. Additionally, interval meta-analyses have confirmed that immunosuppressed individuals do not have a noticeably higher chance of contracting severe SARS-CoV-2 infections[110,111]. SARS-CoV-2 is a member of the same family as SARS-CoV and MERS-CoV, the Coronaviridae. They are pathogenic and have a similar structure to the genomic sequence of SARS-CoV-2, which is 80% and 50% identical to that of SARS-CoV and MERS-CoV, respectively[51]. One week after the start of the illness, the presence of severe symptoms, including dyspnea and hypoxemia, might be a marker of severe pneumonia, which can result in ARDS, multiple organ dysfunction syndromes, and even mortality[112]. Due to its similar genetic lineage B, the new coronavirus reacts to the same receptor as both beta-coronaviruses[113]. The RBD, a ligand that interacts with the host cell surface receptor, is present in the glycoprotein (S protein) located on the virion envelope S, allowing for the fusing of membranes, viral penetration, and viral

multiplication[113,114]. Although the target receptor is mainly expressed in type II pneumocyte of the lungs, the predominance of extrapulmonary symptoms suggests that SARS-CoV-2 infection may potentially impact other organs. It has been established by transcriptomics and immunohistochemical investigations that the lower respiratory tract, heart, lungs, ileum, esophagus, kidney, and bladder contain the largest percentage (>1%) of ACE2 receptors. Other organs such as the liver, stomach, brain, pancreas, arteries, endothelium, breast, uterus, oral and nasal mucosa, and ovary have lower ACE2 expression[115-117]. The majority of SARS-CoV-2 infection risk factors are associated with metabolic syndrome. As a vital organ for lipid metabolism, the liver is a critical factor in determining metabolic disorders and glucose metabolism. As a result, several studies have related severe COVID-19 to metabolic dysfunction-associated fatty liver disease (MAFLD)[12]. Even when compared to the meta-analysis statistics described before[28], patients with underlying hepatic problems were older and afflicted with numerous additional comorbidities, such as hypertension (68%) or diabetes (48%). The previously mentioned SARS-CoV-2 target receptor has variable distribution in the liver. Several published reviews have determined its presence in cholangiocytes and lack in Kupffer or sinusoidal endothelial cells[118,119]. It has been discovered that, among those over 65, sex has no bearing on the likelihood of this complication[120,121]. The connection between low testosterone and clinical outcomes, likewise fatty liver and atherosclerosis, metabolic syndrome, type 2 diabetes, and obesity, is one hypothesis that has been proposed. Female sex hormones have a preventive effect against these diseases in younger women[122]. Individuals with chronic hepatitis C, alcoholic liver cirrhosis, alcoholic liver damage, and chronic hepatitis B were shown to have the lowest association. By contrast, those with non-alcoholic liver disease and non-alcoholic cirrhosis had the strongest association. Additionally, SARS-CoV-2-infected individuals who also had pre-existing liver illnesses showed greater hospitalization and mortality rates than other patient groups[123]. Age, having non-liver cancer, and having higher baseline blood creatinine levels are all risk factors for patient mortality [124]. Immunosuppressants used to lower transplant rejection risk may make patients more susceptible to COVID-19, but they may also reduce the uncontrolled inflammatory response that results from SARS-CoV-2 infection. Additionally, long-term immunosuppressant usage may lengthen the viral shedding period, extending the duration of the communicable period[125]. For patients with acute liver failure, a high Model for End-Stage Liver Disease score, or hepatocellular carcinoma (HCC) at the Milan criteria's top limits, most societies advise against transplantation. Both organ donors and receivers should be checked for SARS-CoV-2 infection as an additional precaution[126]. SARS-CoV-2 vaccines are being developed at an unprecedented rate. Since the start of the pandemic, 126 new vaccines have been put into clinical development, as per the World Health Organization (WHO) COVID-19 vaccine tracer. WHO has given the go-ahead for seven of them for commercial usage. BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna), based on the mRNA encoding SARS-CoV-2 spiny glycoprotein variations, are the most widely used COVID-19 vaccines. Other vaccines include ChAdOx1 nCov-19 (AZD1222, the Oxford-AstraZeneca) and Ad26.COV2.S (Johnson & Johnson/Janssen) is an adenoviral vector-based vaccine. A recombinant human adenovirus type 26 vector encoding the SARS-CoV-2 S protein is used in the Johnson & Johnson/Janssen vaccination. Conversely, the Oxford-AstraZeneca vaccine is an adenoviral vector that is replication-free and carries a full-length, codon-optimized gene that encodes the SARS-CoV-2 S protein in chimpanzees. Since none of the three vaccinations contain live viruses, they cannot even trigger viral replication in immunocompromised people[127]. The COVID-19 vaccine should be taken into consideration for patients with hepatocellular carcinoma (HCC) who are receiving locoregional or systemic therapy without delaying their course of therapy[128-130]. Similarly, those with chronic liver disease who use antiviral medicines for the treatment of hepatitis B or hepatitis C should not stop taking those medications while getting the COVID-19 vaccination. Hepatopathy has already been linked to the severity of illness brought on by two additional extremely pathogenic coronavirus strains: MERS-CoV and SARS-CoV-1. Many factors affect the pathogenesis including hypoxic hepatitis (secondary to respiratory failure), hepatic congestion associated with mechanical ventilation (high levels of positive end-expiratory pressure (PEEP), virally induced intrahepatic cytotoxic T cells and Kupffer cells, and drug toxicity. Hepatic impairment has been linked to the most severe COVID-19 instances when thrombocytopenia, activated coagulation, and fibrinolysis are present[74]. In COVID-19 patients, abnormalities in the liver's biochemistry have been observed. ALT and AST show moderate increases that range from 14% to 53%[12,74,120] and are frequently seen in these anomalies. Patients with more severe symptoms than those with mild to moderate symptoms, particularly those who require admission to an intensive care unit, may have higher rates of transaminase increase[131]. The SARS-CoV-2 gene was the target of PCR testing to diagnose COVID-19 in samples of the nasal or pharynx collected before admission. All patients underwent at least one chest computed tomography scan after admission. These requirements must be satisfied for release from the hospital: two negative reverse transcriptase-PCR findings for SARS-CoV-2 respiratory samples obtained at least 24 h apart, remission of respiratory symptoms, improvement in lung inflammation, and normal body temperature for at least 3 d[132,133]. A fatty liver was seen in 40.0% of all mild cases[17]. Hepatic dysfunction should be of concern, according to growing research, as it is prevalent in COVID-19 patients[134]. As per meta-analysis, 27.4% of COVID-19 patients had liver dysfunction[135]. As per the WHO report dated March 5, 2021, there have been 115289961 COVID-19 cases documented globally. Where the Americas made up the majority (44%), Europe came in second (34%), and Southeast Asia came in third (12%)[8,136]. Except for individuals under 1-year-old, children with COVID-19 often only have minor symptoms affecting the upper respiratory tract[6]. The most common technique of diagnosis for COVID-19 infection is PCR testing using a nasal swab sample. At the same time, presumptive diagnoses can also be made using clinical, laboratory, and imaging data[8]. Direct invasion and cytokine storms are the two key phases in the pathogenic processes of SARS-CoV-2. Direct invasion is the phase of SARS-CoV-2 infection where the virus penetrates target cells by binding to the ACE2 receptor through the viral structural S protein[137]. Pneumocytes, gastrointestinal epithelia, vascular endothelium, the liver, and nasal and bronchial epithelial cells all have the ACE2 receptor[138,139]. Type 2 transmembrane serine protease (TMPRSS2) in host target cells promotes viral uptake, particularly in alveolar epithelial type II cells[93]. Some infected individuals may undergo cytokine storms, an extrapulmonary systemic hyperinflammation syndrome[12]. Interleukin (IL-2, IL-6, IL-7, Il-10) and TNF-α are just a few of the cytokine types that may see an increase in levels as a result of a

cytokine storm[140]. A cytokine storm also increases inflammatory biomarkers such as granulocyte colony-stimulating factor, IFN-g inducible protein 10, and monocyte chemo-attractant protein 1[141]. Significantly increased cytokine levels are a serious issue since they can lead to serious illness[142]. Serum ferritin, IL-6, and IL-10 were found to be powerful predictors of severe COVID-19 illness by a meta-analysis[23]. The most prevalent pre-existing liver diseases in COVID-19 patients are cirrhosis, HCC, autoimmune hepatitis, chronic hepatitis B, chronic hepatitis C, MAFLD, alcohol-related liver disease, and autoimmune hepatitis[21,143,144]. Certain underlying hepatic conditions may impact the prognosis for COVID-19. It may be the underlying mechanism because MAFLD is a proinflammatory hypercoagulable condition linked to severe illness and thrombosis in COVID-19 patients[145]. The best course of action for people with end-stage chronic liver disease or immediate liver failure is LT[146,147]. The primary biochemical markers used in the diagnosis of liver damage include ALT, AST, TBIL, ALP, GGT, albumin, and prothrombin time (PT)[148].

Gastrointestinal damage caused by lung infection

The potential gastric complications have been summarized in Figure 1. Immunity and chronic enteritis depend on CD4+ T cell infiltration of the intestinal mucosa. The entrance of CD4+ T lymphocytes into small intestinal cells is known to be facilitated by the C-C chemical receptor type 9 (CCR9)[149]. CCR9+ CD4+ T lung-derived cells expanded after viral infection, according to Wang *et al*[18]. Chemokine (C-C motif) ligand 25 can be incorporated by the small intestinal epithelium[150], which, therefore, encourages the recruitment of CCR9+ CD4+ T cells into the small intestine[151]. It damages the intestinal flora's balance and the gut immune system. As a result of the high levels of IL-17A produced, Th17 cell polarization and neutrophil recruitment were induced in the gut[152]. As a result, various gastrointestinal symptoms, such as diarrhea and intestinal immunological damage, might manifest. Additionally, intestinal inflammation may cause intestinal flora and cytokines to enter the circulation and travel to the lungs, influencing the immune system[153,154]. However, there are times when these antiviral immunological processes manage to get beyond the regulatory system, as shown in some patients. It ultimately helps to cause viral infection-induced multi-organ failure, which includes liver failure. Significant tissue and organ damage can also result from a host immune system's overreaction when stimulated. Significant tissue and organ damage can also result from an overreaction of the host immune system caused by the activation of a systemic inflammatory state brought on by elevated cytokine production. The latter condition, called a "cytokine storm", is recognized to harm tissue significantly[18]. Due to their advanced age and pre-existing medical conditions such as diabetes, asthma, and cardiovascular disease, COVID-19 patients experience more severe consequences, which increases their death rate. Through multisystem inflammation, COVID-19 can harm the liver directly or indirectly. As a result, pre-existing liver illnesses may raise patients' risk of developing severe COVID-19[155]. The three primary causes of COVID-19-induced liver injury are ischemia and hypoxia. Other considerations include pre-existing liver illness (such as hepatitis steatosis, cholangitis, thrombosis, Kupffer cell proliferation, and liver dysfunction), severe inflammatory responses/sepsis, and the direct cytotoxic action of the virus on cholangiocytes (through ACE2 receptors)[156]. Individuals with severe COVID-19 are at a significant mortality risk for hypoxic liver injury (HLI), which is not an uncommon occurrence. HLI is mainly caused by lung and heart failure and is related to immune-mediated inflammation. Patients with HLI are at a significant risk of death due to numerous organ failures. Comparing HLI instances to non-HLI cases, there is a statistically significant increase in the levels of (TBIL), (CRP), procalcitonin and IL-6. Additionally, compared to non-HLI patients, HLI patients had a considerably lower median survival time[157]. Elevated levels of ferritin, CRP, IL-6, LDH, and cytokine storm (known as cytokine release syndrome) are the hallmarks of this condition[158]. Damage in the liver is triggered by dysfunctional monocytes and macrophages[159]. Hepatotoxicity can be treated successfully with hydroxychloroquine, either alone/or in combination with lopinavir/ritonavir, remdesivir, azithromycin, umifenovir, darunavir, baricitinib, IFN-b, and imatinib. These medications are now often used for off-label COVID-19 therapy in several nations due to their quick accessibility[160].

COVID-19 AND LIVER-ASSOCIATED CLINICAL FEATURES

According to reports from China, patients who recuperated from severe COVID-19 symptoms experienced symptoms including pigmentation and a darker complexion. The primary factor for pigmentation and darkening of the skin is multiple organ damage, particularly liver disease[161,162]. Through three distinct pathways, liver dysfunction, aberrant liver function, and liver injury-poor liver function can readily change pigmentation. When the amount of estrogen rises, liver dysfunction prevents the inactivation of estrogen; it reduces the inhibition of thiamine on tyrosinase, which then enhances the transformation of tyrosine into melanin[163]. When the liver cannot properly metabolize the melanocyte-stimulating hormone released by the anterior pituitary gland, the body produces more melanin[164,165]. Liver damage increases blood iron levels, and if that blood is given to the facial skin, it may create a darkened face[166,167].The most prevalent comorbidities experienced by the patients in a clinical review that included 331 critically ill COVID-19 patients were hypertension (136 cases, or 41.1%), CHD (66 cases, or 19.9%), and diabetes (60 cases, or 18.1%). The majority of the patients' treatments included inhaling oxygen (262 cases, or 79.2%), antibacterial therapy (256 cases, or 77.3%), adjuvant corticosteroid therapy (211 cases, or 63.7%), gamma globulin (146 cases, or 44.1%), mechanical ventilation (120 cases, or 36.3%), and muscle relaxants (37 cases, or 11.2%). A total of 273 (82.5%) of the 331 critically ill patients received antiviral medication including oseltamivir, arbidol, lopinavir/ritonavir, ganciclovir, and IFN. According to an investigation, 36 (13.2%) of the patients in this group experienced liver damage complications[168]. This research suggests that lopinavir may make ritonavir more likely to cause liver damage or vice versa. The reactive metabolites and drug-induced oxidative stress are thought to play a role in the molecular mechanism of hydroxychloroquine-induced liver damage, or the inflammatory processes after the viral infection itself may have an *ad hoc* or synergistic impact[169]. Azithromycin, hydroxy-

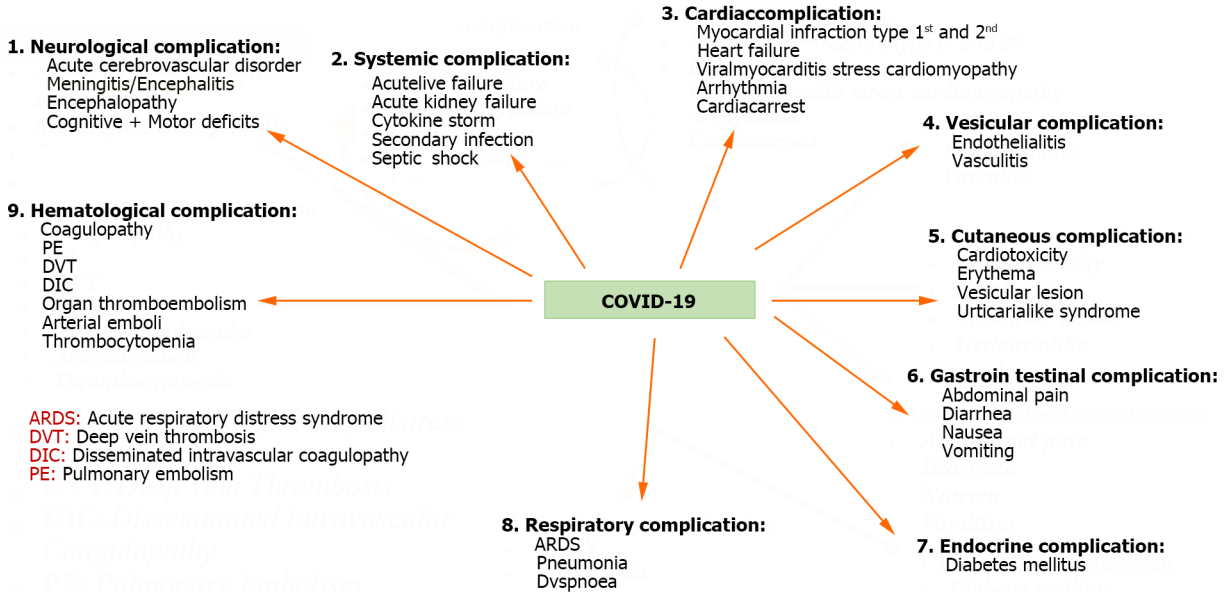

Figure 1 Potential complications due to coronavirus disease 2019. COVID-19: Coronavirus disease 2019.

chloroquine, and lopinavir/ritonavir use are linked to many liver damage pathways, including drug-induced oxidative stress[170]. Due to the many medications involved, providing a consistent molecular idea suited for all pharmaceuticals under consideration is impractical. However, the molecular mechanisms through which a single medication, such as acetaminophen, damages the liver through cytochrome P450, particularly its isoform 2E1, are well understood and may be applied to individuals who take an excessive amount of the drug.

Contrary to the majority of other medicines that generate unanticipated idiosyncratic liver disease, acetaminophen's intrinsic liver harm is predictable. Polypharmacy, a well-documented therapeutic strategy employed for COVID-19 patients (for instance, patients who were treated with up to 18 different medicines), poses a more significant risk for DILI than the use of a single drug from a molecular perspective. If two or more hepatotoxic medicines were administered concurrently for therapy, polypharmacy increased the incidence of DILI by a ratio of up to six[171]. SARS-CoV-2 can cause an asymptomatic infection or a condition that might be fatal[172,173]. Since the COVID-19 pandemic began in 2019, there have been worries that people with pre-existing CLD may be more likely to experience worse health outcomes after contracting SARS-CoV-2. This is particularly significant considering that advanced age, obesity, diabetes, and severe COVID-19 and CLD share risk factors[68,174]. Progressively rising levels of ALT, AST, ALP, and LDH were found during hospitalization in the first case of COVID-19 reported in the United States.

By contrast, the bilirubin level with PT remained normal[62]. Males are more likely than females to experience liver impairment, according to Xie *et al*'s[57] analysis of liver function in patients not receiving critical care. Fifty-two patients from the COVID-19 trial who required mechanical breathing or help with at least 60% of the inspired oxygen fraction were included in the research. According to the research, 29% of the patients had liver damage, 15% had acute renal damage, and another 15% had cardiac damage[174]. The liver damage caused by COVID-19 is hepatocellular rather than cholestatic, and as a result, it primarily manifests as increases in ALT, AST, and LDH levels. Hepatocellular damage markers such as AST are also linked with mortality risk in COVID-19 patients[175].

HEMATOLOGICAL AND HEPATIC VARIATIONS

Changes in blood cellularity are observed during the early onset of the disease. A study with 1099 participants showed that 83.2%, 36.2%, and 33.7% of the patients had lymphopenia, thrombocytopenia, and leukopenia, respectively. These patients showed a rise in ALT and AST values, which indicated liver damage, particularly in severe COVID-19 cases[3,58,62,176]. It is speculated that the virus's attachment to ACE2 can directly cause cytopathic damage, which can lead to liver damage. Patients with COVID-19 also have cholangiocytes with high ACE2 expression. In addition to an extended PT, hypoproteinemia and coagulation abnormalities are further COVID-19 hepatic symptoms. This phenomenon may even be secondary to the use of hepatotoxic medicines as the cause of acute viral hepatitis, which is thought to be the source of viral tropism in the liver tissue[172]. In disseminated intravascular coagulation conditions, an increase in the D dimer and thrombocytopenia was observed in 36.2%-46.4% of critically ill patients, and a poorer prognosis was identified[172].

THERAPEUTIC RECOMMENDATIONS FOR COVID-19: VACCINES, LABEL, AND OFF-LABEL MEDICATIONS FOR PATIENT CARE

Classical antimalarial drugs include chloroquine, aminoquinolines, and hydroxychloroquine, which are polymerase inhibitors. The drug kills the malaria parasite by accelerating the formation of poisonous heme within the parasite through heme polymerase inhibition. It is believed that the function of medicines in treating COVID-19 is to stop the virus from entering host cells by limiting the glycosylation of host receptors and by inhibiting the generation of viral proteins through endosomal acidification. The WHO recommends the use of hydroxychloroquine or chloroquine together with lopinavir/ritonavir for COVID-19 therapy, regardless of the severity and length of the disease. On the other hand, regardless of the severity of the disease, remdesivir and systemic corticosteroids are suggested as prospective pharmacological options for conditional use in routine care of hospitalized COVID-19 patients (WHO/2019-nCoV/therapeutics/2020.1). The WHO recognizes this type of off-label pharmaceutical usage as being nation-specific. In several nations, clinicians provide COVID-19 therapeutic options that have yet to receive official approval (MEURI; http://www.who.int/). COVID-infected patients have been treated with off-label, compassionate-use treatments such as IFN and the repurposed medication Kaletra, which is an authorized combination of the virus, steroids, anti-IL-6 inhibitors, and protease inhibitors lopinavir and ritonavir, chloroquine, and azithromycin[177].

Coronaviruses are positive-sense single-stranded RNA viruses with an envelope measuring 80-220 nm in diameter. The virus is known as a coronavirus because it has an envelope measuring 20-nm-long spikes that resemble the sun's corona when seen under an electron microscope. Both humans and animals can become ill as a result of the infection. Among the RNA viruses that are currently known, it has the largest genome[178]. The coronavirus nucleoprotein (N) covers the RNA genome to create a coiled tubular shape. The viral envelope (E) that surrounds this helical nucleocapsid contains two or three structural proteins, including the S structural protein, which serves as the target for neutralizing antibodies, and the matrix protein (M), which is incorporated in the envelope. Numerous beta-coronavirus strains also include hemagglutinin esterase. There are four structural proteins, namely N, E, M, and S and RNA-dependent RNA polymerase (RdRp), which are encoded by the five essential genes in coronaviruses. The highly conserved arrangement of these genes is 5'-RdRp-S-E-M-N-3'[179].

It was observed that 51.1% of all confirmed cases were males. Eighty percent of the confirmed cases that were reported either had either no pneumonia or mild to moderate. In comparison, 15% showed severe pneumonia and 6% required critical care due to respiratory failure, shock, and multiple organ failure. The COVID-19 fatality rate is 3.8% across all of China, with fatality rates of 5.8% in Wuhan City and 0.7% across the remainder of mainland China. Old age (60 years and above) and medical comorbidities, namely hypertension, diabetes mellitus, cardiovascular disease, chronic pulmonary illness, or cancer, are risk factors for acquiring severe pneumonia or dying from it. Laboratory testing found leukopenia, lymphopenia, and mildly raised C-reactive protein in COVID-19 cases, but patients having severe pneumonia showed higher levels of leukocytes, neutrophils, and creatinine kinase. According to computed tomography of the chest, both lung fields exhibit a ground glass appearance, interstitial infiltration, or numerous patchy consolidations[180]. Some individuals had the rapid onset of severe pneumonia, pulmonary edema, acute respiratory distress syndrome, acute respiratory failure, and multiple organ failure. Chen *et al*[163] were the first to detect abnormal liver enzymes in infected patients. Forty-three cases (43.4%) of Wuhan's confirmed patients had elevated blood levels of the enzymes AST, ALT, and lactic dehydrogenase. Except for one instance, which had extremely high levels of aminotransferases, most had a modest increase in aminotransferase. None, however, was seen to have liver failure or apparent intrahepatic cholestasis [181]. The biggest subfamily of S viruses in the Nidovirales family is the coronaviruses. In the past 20 years, the virus has been responsible for three major outbreaks, including the most recent pandemic that the SARS-CoV-1 brought on. In 2002, Guangdong Province in China had the first outbreak of the 21st century. SARS-CoV-1 formed a severe form of SARS and caused 8098 fatalities (9.6%) worldwide[182-186]. The lung is the main organ affected by pneumonia caused by COVID-19. Typical respiratory symptoms, including dyspnea, coughing up sputum, exhaustion, ARDS, respiratory failure, and even death, affect the majority of COVID-19 patients. Contrarily, extrapulmonary clinical manifestations can affect a variety of other organs, including the cardiovascular system (*i.e.* acute coronary syndrome, arrhythmias, pericarditis, myocarditis,) the kidneys (acute kidney injury and acute tubular necrosis) and the liver[187].

ABNORMALITIES IN LIVER FUNCTION TEST PREVALENCE AMONG COVID-19 PATIENTS

There are few studies particularly investigating the clinical characteristics of liver failure in COVID-19 patients. These investigations revealed a rising trend in the liver enzyme levels seen in individuals who were severely ill or who did not survive. The percentage of patients with elevated ALT, AST, and GGT levels was 82%, 75%, and 72%, respectively. According to a recent finding, the incidence of abnormal liver test results was 76.3% and that of liver damage was 21.5% among hospitalized COVID-19 patients[188]. In that research, 26.7% of patients with abnormal liver tests indicated a tendency toward developing severe pneumonia within 2 wk of hospitalization, and individuals had higher levels of abnormal liver tests. Patients diagnosed with severe COVID-19 had considerably more liver damage than those with non-severe COVID-19. The scientists also noted that individuals with hepatocyte types had noticeably increased chances of suffering from severe COVID-19. In a different investigation, the levels of ALT, AST, GGT, ALP, and TBIL were significantly greater in dead patients than in healthy ones[173].

POSSIBLE IMPACT OF DRUGS ON LIVER FUNCTION IN COVID-19 PATIENTS

It is hypothesized that COVID-19 individuals who use certain medications may suffer from liver damage. For instance, individuals with COVID-19 may have liver damage as a result of taking various medications including antibiotics, antivirals, and antipyretics, as well as analgesics and traditional Chinese medicine[174]. It was observed that medications like ACE inhibitors (ACEis) and angiotensin II receptor blockers (ARBs) may impair liver functioning in COVID-19 patients. In research, participants who used ACEis or ARBs throughout their hospitalization had higher liver enzyme levels; however, the rise was not statistically significant in those who were not hospitalized[189]. Serum GGT, a possible cholangiocyte damage diagnostic marker, is up to 72% higher in individuals with severe COVID-19[173,185]. According to a preliminary investigation, the ACE2 receptor is highly expressed in cholangiocytes[189]. These data suggest that SARS-CoV-2 may bind to cholangiocytes that are ACE2-positive and cause liver damage[188].

Nevertheless, liver tissue from a patient who passed away from COVID-19 did not contain any viral inclusions[190]. A significant factor in the liver damage brought on by COVID-19 may be dysfunctional control of the innate immune response. The following potential processes could cause COVID-19 patients' livers to become damaged: (1) immune-mediated inflammation including cytokine storm and hypoxia caused by pneumonia; (2) direct cytotoxicity due to active viral replication in the liver cells; (3) drug-induced liver damage, including the potential hepatotoxicity of uminefovir in patients with severe COVID-19, concerning antiviral medications like lopinavir/ritonavir, chloroquine as remdesivir, and tocilizumab; (4) patients who have a history of chronic hepatic illness are more susceptible to acquiring hepatic damage from this viral infection; and (5) reactivation of pre-existing hepatic disease hepatitis B virus reactivation could occur as a result of using biological medications such as tocilizumab and baricitinib, which have the potential to cause liver impairment. Another unanswered question is whether SARS-CoV-2 infection worsens cholestasis in an individual or with underlying cholestatic hepatic disorders. More mechanistic research is needed to better understand how viruses enter and replicate in liver cells and the possible liver effects of the prescriptions used to treat COVID-19[188].

MANAGEMENT AND PREVENTION

Preventing liver damage in COVID-19

All COVID-19 patients should have their liver biochemical markers monitored, including ALT/AST, albumin, bilirubin, and PT, for possible risk of liver damage. For instance, if serum AST and LDH levels increase while the ALT level is normal, skeletal muscle or myocardial damage should be diagnosed rather than a liver injury because chronic liver illness is a significant medical burden among older COVID-19 patients; doctors are encouraged to give attention to the treatment of pre-existing liver disease. Antiviral medications for the treatment of chronic hepatitis B should not be stopped to prevent the virus from reactivating. However, anti-HBV medicines should also be considered for patients receiving glucocorticoid therapy[12,13,18,172].Those receiving glucocorticoids or immunosuppressants as part of their treatment for autoimmune liver disease should be closely monitored because of their compromised immune systems. People with cirrhosis also require close monitoring for the development of complications and secondary infections[15,18]. Due to the ability of the new coronavirus to trigger a cytokine storm and a sequence of immunological responses, certain COVID-19 patients may quickly advance to multiple organ failure or death, septic shock, and acute respiratory distress syndrome. Therefore, prompt treatment in severe instances is necessary to avoid any additional liver damage. During COVID-19 clinical management, a variety of antiviral drugs, nonsteroidal anti-inflammatory drugs, traditional Chinese herbs, and glucocorticoids may be used. As such, it is advisable to streamline treatment and minimize the use of redundant medication types, doses, and durations to lower the risk of drug-induced liver injury[191].

COVID-19 liver injury management

The primary COVID-19 treatments currently employed include intensive care, treating hypoxemia with oxygenation assistance/mechanical ventilation, providing continuous renal replacement therapy for cytokine storm syndrome, maintaining sufficient blood volume, and other supportive therapies (Table 1). These therapies are essential for preventing and treating multiple organ failures, including liver damage[192].

RESULTS AND DIRECTIONS FOR THE FUTURE

The COVID-19 pandemic has caused considerable setbacks in several healthcare services, particularly the management of CLD, and has been a historically severe worldwide health disaster. According to Besur *et al*[179], the pandemic delayed CLD screening and frequent follow-up appointments, which had an impact on CLD prevention and treatment and worsened the prognosis for CLD patients. Late detection of CLD consequences such as HCC may have an impact on these patient's clinical outcomes. Social isolation practices have increased the likelihood that CLD patients may have decompensation, mental health impairment, and malnutrition[186]. Additionally, individuals with COVID-19 reported experiencing recurrent gastrointestinal issues[74]. In individuals with severe COVID-19, research found an abnormally high level of aminotransferase that may not have a hepatic origin[76]. The study shows a consistent link between the severity of COVID-19 and liver damage, although the mechanisms behind this damage are still unknown, given the multifaceted nature of the condition. One possible condition is hepatocyte apoptosis[187]. It was noted that while Frank's steatohepatitis symptoms were only present in two instances, macrovesicular steatosis, which displays a fat distribution

Table 1 Future directions and preventative strategies for coronavirus disease 2019

Aspect	Actions
Prevention	Monitor liver biochemical markers (ALT/AST, bilirubin, prothrombin time and albumin, prothrombin time) to detect liver damage
	Differentiate liver injury from other conditions (*e.g.*, skeletal muscle or myocardial damage)
	Focus on the treatment of pre-existing patients with liver disease
	Consider continuation of antiviral medications for chronic hepatitis B to prevent reactivation
	Consider anti-HBV medications for patients receiving glucocorticoid therapy
	Cautiously monitor the COVID-19 course in patients with autoimmune liver disease on glucocorticoids or immunosuppressants
	Intensively monitor individuals with cirrhosis for complications and secondary infections due to immunocompromised state
	Reduce the risk of drug-induced liver impairment by streamlining the treatment and avoiding redundant pharmaceutical types, doses, and durations
Management	Provide intensive care and supportive therapies to prevent and treat patients with multiple organ failure, including liver damage
	Correct hypoxemia with oxygenation support or mechanical ventilation
	Continuous renal replacement therapy for cytokine storm syndrome
	Maintain adequate blood volume
	Monitor liver enzymes and other liver function markers regularly
Implications	The COVID-19 pandemic has impacted the management of CLD and delayed screening and follow-up appointments
Future directions	Social isolation practices may lead to decompensation, mental health impairment, and malnutrition in CLD patients
	COVID-19 can cause liver damage, potentially through direct harm, immune-mediated hepatotoxicity, or cytokine storm
	Liver involvement may be associated with the severity of COVID-19
	Obesity and comorbid conditions like diabetes or hypertension increase the risk of liver disease and worsen SARS-CoV-2 infection
	Liver dysfunction is a potential risk factor for mortality in COVID-19 patients
	Liver cells may be directly infected by SARS-CoV-2, leading to liver dysfunction
	Histological characteristics of liver infection include significant apoptosis and binuclear hepatocytes

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CLD: Chronic liver disease; COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

unusual for NAFLD, was widespread in patients (75%). COVID-19 may have exacerbated steatosis in some individuals. This discovery is in line with those made public by previous investigations. For instance, a team connected to the Centers for Disease Control revealed that 50% of the livers from autopsies under study had steatosis[188]. ACE2 and TMPRSS2 are primarily used by SARS-CoV-2 as the docking and entrance receptor on host cells for cellular entry[189]. Additionally, it has been proposed that myositis, rather than liver damage, maybe the cause of the high aminotransferase level in COVID-19 individuals[191]. A reliable predictor of death was hypoalbuminemia[192]. Even at therapeutic quantities, acetaminophen, a common medication used to treat COVID-19 symptoms, can affect aminotransferase levels[193]. Compared to individuals without NAFLD, patients with NAFLD showed quicker disease development and a longer viral shedding time[194]. Obese patients with NAFLD also showed an increased risk for severe disease[195]. High PEEP may cause comparable hemodynamic changes in the liver of mechanically ventilated patients[176,187]. Most reports and research concentrate largely on the causes and side effects of cardiovascular damage due to the Kawasaki-like presentation of multisystem inflammatory syndrome in children (MIS-C)[188,174]. Recently, the assessment of COVID-19 severity or MIS-C presentation has incorporated liver involvement and the use of liver enzymes as potential prognostic markers [175]. Adults who have comorbid conditions, including obesity, diabetes, or hypertension, may develop non-alcoholic hepatosteatosis (fatty liver disease). The condition worsens patients' SARS-CoV-2 infection[176]. According to research published in September 2020 by Zhou *et al*[186], younger children were more commonly affected by liver involvement in COVID-19 instances than older children. Young age-related liver immaturity is thought to be the cause[182]. Acute COVID-19 cases were thought to be caused by direct liver damage from hepatotropic viral invasion because of the reported ACE2 receptors on the surface of liver and bile duct epithelial cells[183]. By contrast, MIS-C[184] has a precise immune-mediated hepatotoxicity mechanism. When the COVID-19 infection is severe, "cytokine storm" and multiorgan dysfunction are types of liver involvement where cytokines encourage the increase and release of liver enzymes[185]. The progression of the reported situation is comparable to sepsis-associated liver dysfunction, in which increased levels of

hepatic markers and bilirubin, as well as reduced synthesis function, result in hypoproteinemia and coagulation abnormalities[195]. Additionally, as part of the cytokine storm, IL-6 participation in COVID-19-related liver failure has been demonstrated[176]. The subfamily of SARS-CoV-2 has four genera, namely alpha-coronavirus, beta-coronavirus, gamma-coronavirus, and delta-coronavirus, according to genomic and phylogenetic studies[172]. Early in December 2019, Wuhan, China, reported the first case of pneumonia with a previously unidentified origin after being identified as a new beta-coronavirus using high-throughput sequencing research; the case was designated SARS-CoV-2. The WHO formally proclaimed the SARS-CoV-2 virus a pandemic of worldwide concern following its sudden global outbreak[148]. Early liver impairment in COVID-19 individuals raises their mortality risk. Cholestasis was detected in 151 patients (42.5%) and hepatocellular damage in 101 patients (28.5%). It was shown that severely sick individuals were more likely to have liver dysfunction[149]. In all, 9.6% of the chosen group had an elevated ALP level > 150 U/L and a cholestatic pattern of liver damage. High levels of ALT/AST, GGT, ALP, and TBIL were found to be the aberrant liver functions that were observed in a different investigation of cholestatic liver damage carried out at the Shanghai Public Health Clinical Center from January 20 to December 31, 2020[150]. The most frequent cancer is HCC, nevertheless. Patients with HCC also have underlying CLD, such as chronic hepatitis B or C virus infection, NAFLD, and alcoholic liver damage[151]. According to reports, COVID-19 infections are very likely to occur in cancer patients. A study in a Chinese hospital showed that 28 of 1276 confirmed COVID-19 cases had cancers, with two having HCC[152]. SARS-CoV-2 infection of liver cells may be directly connected to liver dysfunction in COVID-19 patients. About 2% to 10% of COVID-19 patients who had diarrhea contain SARS-CoV-2 RNA in their blood and stool, suggesting the possibility of exposure to the liver virus. The upper respiratory tract, lung tissue, and liver cholangiocytes are considered the main target sites for SARS-CoV-2 and SARS-CoV due to their affinity for the ACE2 receptor. In these tissues, the virus multiplies and shows symptoms[183] and the reported conspicuous cytopathy. Disturbed levels of liver enzymes, an elevated alveolar-arterial oxygen gradient and GGT level, a reduced level of albumin, and the presence of circulating CD4+ T cells and B lymphocytes are all indicators of SARS-CoV-2 infection and significant apoptosis with binuclear hepatocytes are the main histological characteristics of COVID-19 liver infection[195].

CONCLUSION

COVID-19 has been shown to impact additional organs in addition to the respiratory system, with the liver being one of the most often afflicted organs. Several factors, such as virus-associated immunological liver injury, direct cholangiocyte destruction resulting in liver injury, hypoxia injury, DILI, and autoimmune liver disease, can result in liver damage.

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FOOTNOTES

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REVIEW

Multifaceted roles of lymphatic and blood endothelial cells in the tumor microenvironment of hepatocellular carcinoma: A comprehensive review

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Abstract

The tumor microenvironment is a complex network of cells, extracellular matrix, and signaling molecules that plays a critical role in tumor progression and metastasis. Lymphatic and blood vessels are major routes for solid tumor metastasis and essential parts of tumor drainage conduits. However, recent studies have shown that lymphatic endothelial cells (LECs) and blood endothelial cells (BECs) also play multifaceted roles in the tumor microenvironment beyond their structural functions, particularly in hepatocellular carcinoma (HCC). This comprehensive review summarizes the diverse roles played by LECs and BECs in HCC, including their involvement in angiogenesis, immune modulation, lymphangiogenesis, and metastasis. By providing a detailed account of the complex interplay between LECs, BECs, and tumor cells, this review aims to shed light on future research directions regarding the immune regulatory function of LECs and potential therapeutic targets for HCC.

Key Words: Lymphatic endothelial cells; Blood endothelial cells; Hepatocellular carcinoma; Tumor microenvironment

Core Tip: Lymphatic and blood endothelial cells are important components of stromal cells in the tumor microenvironment. Besides their essential function in the formation of tumor draining blood and lymphatic vessels, they can activate various signaling pathways to promote tumor development and metastasis. This review discusses lymphangiogenesis and angiogenesis, and summarizes the current knowledge on common markers of lymphatic and blood endothelial cells and their roles in tumor metastasis, particularly in hepatocellular carcinoma. Based on the available evidence, researchers are attempting to discover new targeted therapies for the prevention of tumor progression.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a prevalent form of cancer worldwide, particularly in Asia where the majority of cases are reported. According to the World Cancer Report released in GLOBOCAN 2020, there were an estimated 905677 new cases of HCC globally, with 72.5% of those occurring in Asia[1]. Liver cancer accounts for a significant proportion (8.3%) of cancer-related deaths[2]. Metastasis, the spread of cancer cells to other parts of the body, is the primary cause of mortality in patients with solid tumors. While surgical resection and liver transplantation are common treatment options for HCC, several other approaches (*e.g.*, transhepatic arterial chemoembolization, microwave ablation, targeted drugs, and immunotherapy) are also employed. However, the emergence of resistance to drugs, such as sorafenib and lenvatinib [3], has prompted an investigation into alternative treatment strategies. Lymphatic and blood vessels are the primary routes for metastasis. Healthy tissues and solid tumors consist of two distinct regions, namely the parenchyma and the stromal region[4]. The term tumor microenvironment (TME) refers to the area where tumor cells reside, including the stromal region. It is a complex milieu comprising non-malignant cells, such as lymphatic endothelial cells (LECs), blood endothelial cells (BECs; also termed vascular endothelial cells), mesenchymal cells, pericytes, immune cells, as well as the extracellular matrix (ECM) and inflammatory mediators they secrete.

Pan-cancer analysis has revealed that the regulation of the TME significantly impacts tumor invasion. Studies have extensively investigated the effects of immune cells and inflammatory mediators secreted by stromal cells on the TME. For example, it has been demonstrated that CD8+ T cells and natural killer T cells cooperatively promote liver damage and carcinogenesis through interaction with hepatocytes in a non-alcoholic steatohepatitis-mouse model[5]. In addition, in human glioblastoma multiforme, macrophage-associated phosphoglycerate kinase 1 (PGK1) phosphorylation promotes aerobic glycolysis and tumorigenesis. CD8+ cytotoxic T cells kill tumor cells by granule exocytosis and Fas ligand-mediated (FasL-mediated) apoptosis. They induce cytotoxicity by secreting interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα). Research using mouse melanoma models has shown that promoting fatty acid catabolism improves the ability of CD8+ tumor-infiltrating lymphocytes to delay tumor progression. In lung adenocarcinoma, hypoxia upregulates C-C motif chemokine ligand 28 (CCL28) to recruit regulatory T (Treg) cells, which are involved in the immune escape of tumor cells. However, Treg cells suppress effector T cells, including cytotoxic T cells[6-10]. Mesenchymal stem cells secrete hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), prostaglandin E2 (PGE2), and transforming growth factor β (TGFβ), which inhibit cytotoxic activity and differentiation of T helper 1 cells. Interleukin-10 (IL-10) and PGE2 secreted by mesenchymal stem cells in the TME impair dendritic cell maturation, thereby reducing T cell activation[4]. In HCC, the action of IL-4, IL-13, and IL-10, and activation of toll-like receptors diminish antigen-presenting activity[11]. TGFβ and thymic stromal lymphopoietin (TSLP) inhibit T cells and promote T cell skewing towards a T helper 2 phenotype, respectively. Cancer-associated fibroblasts (CAFs) also secrete inflammatory cytokines, including CXC-chemokine ligand 8 (CXCL8), IL-4, and IL-6, further suppressing T cell activity. Of note, several chemokines secreted by CAFs in the TME inhibit immune cells: CXCL12 repels T cells; CXCL13 recruits B cells; and CCL2, CCL3, CCL4, and CCL5 recruit myeloid cells, including macrophages and myeloid-derived suppressor cells, and ECM[12]. ECM secreted by stromal cells in the TME also significantly influences anti-tumor immune responses. The role of LECs and BECs (representative stromal cells in the TME) in inhibiting tumor-associated lymphangiogenesis and neoangiogenesis has not been fully elucidated. In HCC, colorectal carcinoma, and breast invasive carcinoma, it has been shown that immune invasion is highly correlated with the expression of LECs and BECs. Previous studies have revealed associations between the presence of LECs and BECs in the TME and immune invasion in colorectal and breast cancer[13]. However, research studies on the role of LECs and BECs in HCC remain limited. Previous investigations have demonstrated interactions between LECs, BECs, and liver injuries. Chronic inflammation in the liver can induce the proliferation of LECs by promoting the production of chemoattractant cytokines. An increased number of LECs has been positively correlated with disease severity. The quantity of LECs is increasing during idiopathic portal hypertension, hepatitis C virus-associated cirrhosis, and primary biliary cirrhosis. Seemingly, changes in LECs reflect the type of peripheral inflammation[14]. The levels of bacterial products, such as lipopolysaccharide (LPS), are increased in cirrhosis; these products activate nuclear factor-κB (NF-κB) in LECs. Consequently, they upregulate the expression of prospero homeobox 1 (PROX1) and vascular endothelial growth factor receptor 3 (VEGFR-3). TGFβ1 is released in the TME of HCC

to increase the expression of CD105 in BECs, thus enhancing the invasion and metastasis of liver cancer cells by inducing neoangiogenesis. This comprehensive review aims to provide valuable insights into the characteristics, effects, and intricate interactions of LECs and BECs in the TME. The article specifically focuses on their roles in tumor development, metastasis, and potential therapeutic interventions in HCC.

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TUMOR LYMPHANGIOGENESIS AND ANGIOGENESIS

Lymphangiogenesis

Lymphangiogenesis refers to the formation of new lymphatic vessels, and is closely associated with tumor metastasis[15,16]. In the TME, various lymphangiogenic growth factors contribute to the proliferation and morphological changes of LECs, thereby facilitating lymphangiogenesis (Figure 1).

Vascular endothelial growth factor-C: Vascular endothelial growth factor-C (VEGF-C) is one of the most potent stimulating factors for LEC growth. Studies have shown that VEGF-C is correlated with lymphangiogenesis, lymph node metastasis, and worse prognosis in patients with tumors. In murine models of human cancer, experiments involving supplementation with or blocking of VEGF-C have demonstrated its great importance[17,18]. VEGF-C binds to the receptor tyrosine kinase VEGFR-3, along with the less potent ligand VEGF-D[19,20]. This binding induces a series of downstream signaling events, including the activation of protein kinase C-dependent (PKC-dependent) pathways such as p42/p44 mitogen-activated protein kinase (MAPK) and AKT phosphorylation[19]. These signaling pathways promote the survival, growth, and migratory ability of LECs. Blocking VEGFR-3 effectively inhibits VEGF-C-induced lymphangiogenesis and tumor progression, thereby highlighting the central role of the VEGF-C/D-VEGFR-3 axis in LEC growth [21].

Neuropilin 2: Neuropilin 2 is another lymphangiogenic growth factor, a type 1 transmembrane glycoprotein highly expressed by LECs. It forms a complex with VEGFR-3 upon binding with VEGF-C/D, leading to the activation of VEGFR-3 and subsequent enhancement of lymphangiogenesis[22-24].

Fibroblast growth factor: Fibroblast growth factor receptor-3 (FGFR-3) has been identified as a novel PROX1 target gene. PROX1 induces the expression of the IIIc isoform, which is also the major isoform of FGFR-3 expressed in LECs. FGF-1 and FGF-2 promote the proliferation, migration, and survival of cultured LECs without involvement of blood endothelial cell growth factor receptor-3[25-27]. In mouse corneal tissue which lacks vascular and lymphatic vessels, FGF-2 directly acts on LECs to promote proliferation and migration *via* activation of the FGFR-1–mediated signaling pathway[26].

Sphingosine-1-phosphate: Sphingosine-1-phosphate (S1P) acts as a lymphangiogenic mediator in LECs. It induces migration, sprouting, capillary-like tube formation, and intracellular calcium mobilization in cultured human LECs *in vitro* and in a Matrigel plug assay *in vivo*[28]. In a murine model of breast cancer metastasis[29], S1P, suppressed by SK1-I, the specific sphingosine kinase 1 (a critical role in producing S1P and mediating tumor-induced lymphangiogenesis) inhibitor, reduced metastases to lymph nodes and lungs, and decreased overall tumor burden.

HGF: HGF plays a dual role in lymphangiogenesis. On one hand, HGF overexpression in transgenic mice or its intradermal delivery induces lymphatic vessel hyperplasia, indicating its direct involvement in lymphangiogenesis. On the other hand, experiments using prostate and breast tumor mouse models revealed that HGF can also promote the expression of VEGF-C/D, indirectly contributing to lymphangiogenesis[30,31]. Moreover, in oral squamous cell carcinoma, HGF significantly enhanced the proliferation, migration, invasion and tube formation of LECs; this process could be inhibited by downregulating the expression of c-Met, the receptor of HGF[32].

Platelet-derived growth factor: The platelet-derived growth factor (PDGF) family induces lymphatic vessel expansion independently of the VEGF-C/D/VEGFR-3 pathway. PDGF-BB, a member of this family, acts as a direct lymphangiogenic factor. Overexpression of PDGF-BB in a syngeneic fibrosarcoma tumor mouse model promoted tumor lymphangiogenesis and lymphatic metastasis, which could be reduced by blocking PDGF receptors (PDGFR). *In vitro*, PDGF-BB stimulated MAPK activity and the motility of isolated LECs[33].

Angiopoietins: Angiopoietins (ANGPTs) (Ang1, Ang2, and Ang3/Ang4) and their receptors Tie1 and Tie2 are involved in blood vessel maturation and patterning. However, they also play a role in lymphangiogenesis. Overexpression of ANGPTs promotes lymphangiogenesis in adult tissue *in vivo*, as observed in experimental pancreatic cancer models[34,35]. Holopainen *et al*[36] demonstrated that Ang2 blockade attenuated tumor lymphangiogenesis, dissemination of tumor cells *via* the lymphatic vessels, lung metastasis, and colonization of the lungs by tumor cells.

Adrenomedullin: High levels of adrenomedullin (AM) have been reported in several types of tumors in humans[37,38].

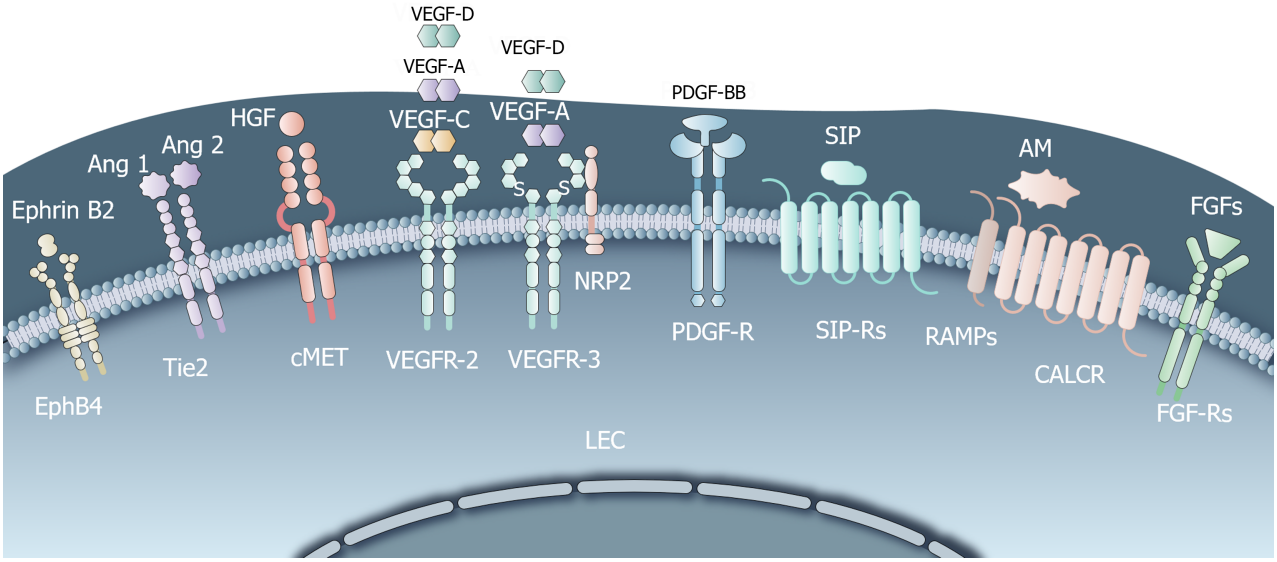

Figure 1 Common lymphangiogenesis-mediating receptors. Vascular endothelial growth factor receptor C (VEGF-C), fibroblast growth factors, hepatocyte growth factor (HGF), angiopoietins, adrenomedullin promote the survival, growth, and migratory ability of lymphatic endothelial cells (LECs); Neuropilin 2 forms a complex with VEGFR-3 upon binding with VEGF-C/D, enhancing lymphangiogenesis; Sphingosine-1-phosphate induces migration, sprouting, capillary-like tube formation of LECs; HGF, platelet-derived growth factor are directly involved in lymphangiogenesis. HGF indirectly promotes VEGF-C/D expression, contributing to lymphangiogenesis. LEC: Lymphatic endothelial cell; VEGF: Vascular endothelial growth factor receptor; HGF: Hepatocyte growth factor; S1P: Sphingosine-1-phosphate; AM: Adrenomedullin; FGFs: Fibroblast growth factors; PDGF: Platelet-derived growth factor.

In a mouse model of lung carcinoma, AM overexpression has been correlated with increased tumor- and lymph node-associated lymphangiogenesis, as well as distant organ metastasis[39]. Berenguer-Daizé *et al*[40] and Fritz-Six *et al*[41] and found that histologic examination of anti-AM antibody-treated tumors showed evidence of disruption of tumor vascularity, with depletion of vascular, LECs, and pericytes, and increased LEC apoptosis. Another important finding was that anti-AM antibody potently blocks tumor-associated lymphangiogenesis, but does not affect established vasculature and lymphatic vessels in normal adult mice.

Angiogenesis

In 1971, Folkman[42] hypothesized that angiogenesis is essential for the development and growth of solid tumors beyond a size of 2-3 mm^3. Subsequent evidence supported the notion that solid tumors rely on angiogenesis for sustained growth [43]. Angiogenesis involves the formation of new blood vessels from existing vasculature in disease. This process differs from vasculogenesis, the *de novo* formation of new blood vessels from endothelial progenitors[44]. Numerous studies have shown that metabolic stress, such as hypoxia, low pH, or hypoglycemia, as well as the immune and inflammatory response, can stimulate tumor angiogenesis[44,45].

Among these factors, hypoxia is a primary driver of tumor angiogenesis, leading to increased expression of VEGF and other angiogenesis stimulators from hypoxic cells[46]. Hypoxic tumor cells can activate the angiogenesis pathway by regulating pro-angiogenic genes through the hypoxia-inducible factor (HIF) pathway. Hypoxia occurs when there is insufficient oxygen reaching the tissue, often resulting from a mismatch between the demand for tumor growth and the supply of oxygen and nutrients[47,48]. Distinguished from angiogenesis under normal physiological conditions, tumor blood vessels exhibit immaturity and impaired functionality, including excessive permeability, poor perfusion, and increased hypoxia[49]. These effects are attributed to the secretion of abnormal levels of growth factors by tumor and stromal cells, among which VEGF plays a key role.

Markers of LECs and BECs

LECs and BECs exhibit specificity and sensitivity in expressing positive markers, while maintaining resistance to biological and chemical agents during histological processing. We did not search for markers that meet the above criteria; in actual practice, we often labeled specific cells with two or more markers. Common markers have been listed in Table 1.

PROX1: The transcription factor PROX1 is regarded as a constitutive marker of LECs due to its pivotal role in lymphangiogenesis[50,51]. It is consistently located in the nuclei of all LECs, regardless of their physiological or pathological state [52]. Recent studies showed that PROX1 could inhibit the proliferation of HCC cells, and reduced PROX1 expression was associated with poor prognosis of HCC[53]. Research has demonstrated that PROX1 can enhance tumor lymphangiogenesis in both breast cancer and melanoma[54,55]. This finding highlights the significance of PROX1 in promoting the formation of new lymphatic vessels within tumors, thereby facilitating the spread of cancer cells through the lymphatic system. In glioblastoma, overexpression of PROX1 enhanced the growth and proliferation of primary and implant focal tumor cells, and this invasive growth potential is regulated by activation of the NF-κB signaling pathway[56].

Table 1 Common Markers of Lymphatic endothelial cells and Blood endothelial cells

Marker	Definition	Expressed on cells	Addition	Types of cancer	Ref.
PROX-1	An evolutionarily conserved class of atypical homeodomain proteins	LECs	Located in the nucleus and promoting lymphangiogenesis	Breast cancer; Melanoma; Glioblastoma	[50-56]
PDPN	A transmembrane mucin type O-glycoprotein	LECs	Functioning in the downstream of PROX-1	Angiosarcomas;Melanoma; Colorectal carcinoma; Breast cancer; Osteosarcoma	[57-60]
LYVE-1	One of the hyaluronan-binding glyco-protein receptors	LECs	Acting with VEGFR and PDGFR in the LECs	Oral oncogenesis; Lung cancer	[62-66]
VEGFR-3	An receptor tyrosine kinase	LECs/BECs	Functioning by activating RAS/RAF-1/MEK/ERK signaling pathway	Gastric cancer; Intrahepatic cholangiocarcinoma; Colorectal carcinoma	[67,68]
CD31	One of the immunoglobulin superfamily	LECs/BECs	Promoting tumor angiogenesis by regulating TME indirectly	Breast cancer; Melanoma; Gastric cancer	[71,73-75]
CD105	Homodimeric transmembrane glycoprotein, a coreceptor for ligands of the TGF-β family	BECs	Also called Endoglin	Esophageal squamous cell carcinoma; Colorectal carcinoma	[76-79]

BECs: Blood endothelial cells; LECs: Lymphatic endothelial cells; LYVE-1: Lymphatic vessel endothelial hyaluronan receptor 1; PDGFR: Platelet-derived growth factor receptor; PDPN: Podoplanin; PROX-1: Prospero homeobox 1; VEGFR: Vascular endothelial growth factor receptor; TME: Tumor microenvironment.

Podoplanin: The transmembrane glycoprotein podoplanin (PDPN) was initially identified on podocytes; it is also expressed on LECs, but not on BECs[57]. The anti-D2-40 antibody is a commonly used commercial antibody that specifically targets a fixation-resistant epitope of PDPN[58]. This antibody is widely utilized to detect and study PDPN expression in various research and diagnostic applications. Notably, both PROX1 and PDPN are mucin-type transmembrane proteins expressed in LECs. It has been hypothesized that PDPN functions downstream of PROX1[59]. Furthermore, it appears that PDPN expression is regulated by PROX1 in LECs at the transcriptional level[60]. Hence, both PROX1 and PDPN are excellent markers for the identification of LECs[58-61].

Lymphatic vessel endothelial hyaluronan receptor 1: The integral membrane glycoprotein lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) acts as a homologue of the CD44 hyaluronan receptor. Although it is not a signaling receptor, it is involved in cell interactions. It has been shown that the expression of LYVE-1 in tumors promotes lymphangiogenesis and facilitates the transfer of tumor cells to lymph nodes[62]. Studies have suggested that LYVE-1 induces signals indirectly through the tyrosine kinase Src and crosstalk with growth factor receptor tyrosine kinase-linked receptors. Furthermore, co-immune precipitation studies have indicated that LYVE-1 physically associates with VEGFR and PDGFR in the LEC plasma membrane[61]. While LYVE-1 is predominantly expressed in mature LECs, it is absent in some LECs; notably, it is also expressed in certain BECs. Consequently, the use of LYVE-1 alone as a marker for LECs can be challenging[63-66].

VEGFR-3: The receptor tyrosine kinase VEGFR-3, also termed fms-related receptor tyrosine kinase 4 (FLT4), plays a crucial role in both tumor angiogenesis and lymphangiogenesis. Binding of VEGF-C/D to VEGFR-3 triggers dimerization and transphosphorylation of the receptor, thereby activating the RAS/RAF-1/MEK/ERK signaling pathway and ultimately promoting lymphangiogenesis[67,68]. Sorafenib[69] and lenvatinib[70], which are widely used VEGFR-3 inhibitors, have demonstrated effectiveness as monotherapies. Recently, Paillasse *et al*[68] investigated EVT801, a novel selective VEGFR-3 inhibitor that specifically targets VEGFR-3-positive tumors and tumors with a VEGFR-3-positive TME. The results showed that EVT801 was effective in these settings without causing side effects, such as hypertension. The efficacy of EVT801 was correlated with the expression levels of VEGFR-3. HCC is primarily driven by angiogenesis, which is influenced by both tumor cells and the microenvironment. As a result, anti-vascular therapies have become increasingly popular for the treatment of HCC. For example, sorafenib is a VEGFR inhibitor that blocks the VEGF pathway to inhibit tumor angiogenesis. However, the effectiveness of sorafenib is limited; thus, this agent can only be used to treat advanced HCC. Therefore, it is likely that other unknown angiogenic mechanisms are involved in this process.

CD31: CD31, commonly termed platelet and endothelial cell adhesion molecule 1 (PECAM-1), is a widely used pan marker for endothelial cells. It has been utilized in various studies to isolate BECs[71]. CD31 belongs to the immunoglobulin (Ig) superfamily and is expressed on platelets, leukocytes, and endothelial cells. It is highly expressed at intercellular junctions on endothelial cells[72]. DeLisser *et al*[73] found that CD31 acts as a mediator of the late progression of metastatic tumors, driving advanced metastatic progression. Their experiments suggested that CD31-null mice had reduced tumor cell proliferation in non-vascularized, pre-angiogenic lesions[74]. These results indicated that CD31 may function as a modulator of the TME, rather than through direct stimulation of angiogenesis. However, CD31 is not a specific marker for BECs in the TME, as it is also expressed on the surface of normal cells, such as hematopoietic and

immune cells (*e.g.*, platelets, neutrophils, monocytes, megakaryocytes, natural killer cells, and some T cells)[75]. Therefore, researchers have identified other markers to more precisely distinguish BECs.

CD105: The transmembrane glycoprotein CD105, alternatively referred to as endoglin (ENG), is expressed on the surface of endothelial cells. It is a component of the TGFβ receptor complex. CD105 is involved in modulating TGFβ signaling to promote endothelial cell proliferation[76]. Sakurai *et al*[77] reported that CD105 is related to malignant tumor properties and prognosis in esophageal squamous cell carcinoma, and may be useful as a marker of angiogenesis. Additionally, increased CD105 expression has been observed in aggressive and metastatic colorectal cancer[78,79].

ROLE OF LYMPHANGIOGENESIS AND ANGIOGENESIS IN CANCER PROGRESSION

Role of lymphangiogenesis and angiogenesis in tumor metastasis

An increase in the number and density of LECs in and around tumor masses creates more opportunities for contact between these cells and tumor cells. Lymphatic vessels provide a relatively comfortable environment for tumor cells within the tumor mass, offering better survival conditions compared with the bloodstream due to lower hydrodynamic stress. This aids in their survival during lymphatic metastasis[80]. Moreover, an increased number of functional lymphatic vessels can lead to better drainage of lymphatic fluid and lower interstitial pressure. This, in turn, can result in increased blood perfusion and nutrient supply to tumor cells, promoting their growth and proliferation[21]. Migration of tumor cells along LECs and lymphatic vessels is a crucial step in lymphatic metastasis. LECs facilitate this process by secreting chemokines that attract tumor cells expressing the corresponding receptors, such as C-C motif chemokine receptor 7 (CCR7) and C-X-C motif chemokine receptor 4 (CXCR4)[81]. The chemokine gradient towards tumor-draining lymph nodes is generated by the flow of interstitial fluid, ensuring the unidirectional migration of tumor cells along lymphatic vessels and into tumor-draining lymph nodes[82]. Notably, LECs may utilize distinct mechanisms (Figure 2) to attract tumor cells along collecting lymphatic vessels or into the sinus system of the lymph node[83].

CCL1/CCR8 axis: The CCL1/CCR8 axis plays a pivotal role in metastasis to lymph nodes. Proinflammatory mediators, including TNF, IL-1β, and LPS, increase the production of CCL1 by LECs and enhance the migration of tumor cells towards LECs. CCR8, the receptor for CCL1, is highly expressed in human malignant melanomas. Blocking CCR8 or CCL1 can inhibit the migration of tumor cells towards LECs. Additionally, this axis is involved in recruiting Treg cells into the tumor niche and converting CD4+ T cells into Treg cells[83,84].

CXCL12/CXCR4 or CXCR7 axis: CXCL12 is a key regulator of tumor progression with two receptors, namely CXCR4 and CXCR7. Overexpression of CXCL12 is associated with increased risk and poor prognosis in several common types of cancer, including HCC, colorectal carcinoma, and breast invasive carcinoma. Chronic hypoxia-induced increase in CXCR4 expression stimulates cancer cell proliferation and leads to the migration of LECs into lymphatic vessels, thus facilitating the invasion of cancer cells into adjacent tissues and organs. Epithelial–mesenchymal transition (EMT) is considered an important process in tumor metastasis. In CRC, LPS (normally produced by the microbiota) use NF-κB signaling, which can suppress apoptotic signaling, to induce CXCR4 expression in tumor cells. This process promotes EMT and metastasis. VEGF enhances the effect of CXCL12 on LECs by increasing CXCR4 expression on endothelial cells[85-91].

CCL21/CCR7 axis: CCL21 is mainly produced by LECs and interacts with CCR7 on immune cells, such as dendritic and T cells, playing a crucial role in their migration to lymph nodes. Tumor cells expressing CCR7 can also utilize this mechanism to enter lymphatic vessels for lymphatic metastasis. The CCL21/CCR7 axis induces EMT in tumor cells, upregulates the expression of matrix metalloproteinases (MMPs) by activating the ERK1/2 pathway, and promotes cancer cell proliferation. It can also promote LEC proliferation by activating the AKT pathway and the AKT/ERK1/2 pathway in tumor cells. Tumor cells expressing CCR7 can sense the gradient of CCL21 concentrations in lymphatic vessels, and migrate from low to high concentrations into those vessels[82,92].

The Ang/Tie axis is involved in regulating vascular development, vascular homeostasis, pathological inflammation, and angiogenic responses[93,94]. In addition to the above mentioned abnormal growth factors, the Ang/Tie axis also plays an important role in tumor angiogenesis. Tie is the receptor for Ang, including Tie1 and Tie2. Tie1 is an orphan receptor that does not bind to Ang; in contrast, Tie2 is expressed in BECs, pericytes, monocytes, and macrophages, and binds to members of the Ang family. The Ang family mainly includes Ang1, Ang2, Ang3, and Ang4. Although Ang1 and Ang2 bind to Tie2 with similar affinity, they exert different regulatory effects on BECs. Ang3 and Ang4 have been rarely studied thus far; hence, data on their characteristics and functions are inconclusive. Ang1, expressed in pericytes and smooth muscle cells, binds to Tie2 in a paracrine manner and phosphorylates Tie2 to maintain vascular stability and survival of BECs. Ang2 is expressed only in BECs; it acts on BECs in an autocrine manner, and is a partial competitive antagonist of Ang1 for Tie2. Qian *et al*[95] found that, by blocking the phosphorylation of Tie2, Ang1 affects downstream phosphatidylinositol-3-kinase (PI3K) and the growth factor receptor bound protein 2 (GRB2) signaling pathway. These effects inhibit the proliferation and migration of BECs, and result in an incomplete tumor vascular basement membrane and intercellular space. Consequently, circulating tumor cells pass through leaky tumor blood vessels and tumor external lumen, thereby facilitating tumor metastasis.

Role of LECs and BECs in tumor metastasis

The process of tumor cell dissemination through either blood or lymphatic vessels is complex. Moreover, the pathways

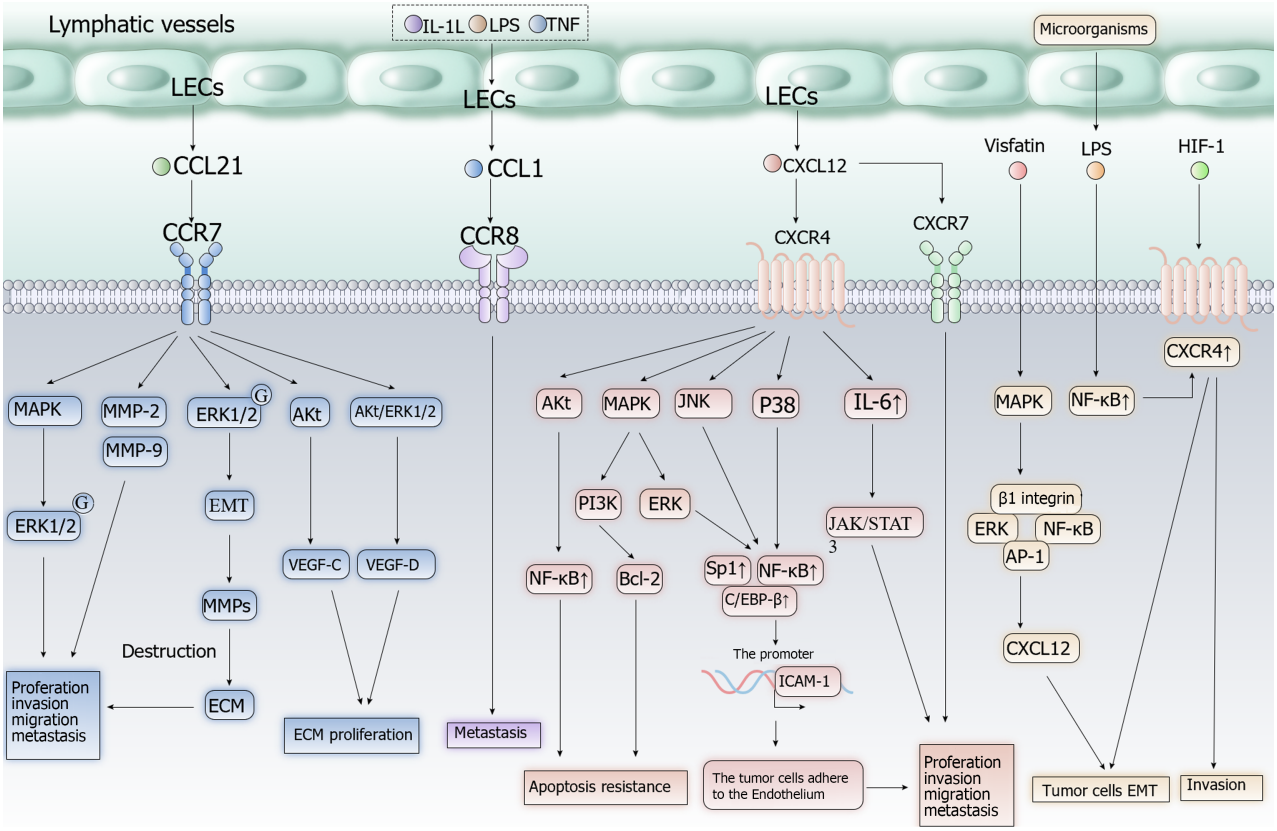

Figure 2 Cell signaling pathways of CXCL12/CXCR4, CCL21/CCR7, CCL1/CCR8 axis in tumor cells. Proinflammatory mediators increase the production of CCL1 by lymphatic endothelial cells (LECs) and enhance the migration of tumor cells towards LECs. CCL1/CCR8 Axis is involved in recruiting Treg cells into the tumor microenvironment and converting CD4+ T cells into Treg cells. CXCR4 expression caused by products of the microbiota and chronic hypoxia stimulates tumor proliferation and migration of LECs into lymphatic vessels. Vascular endothelial growth factor receptor can enhances the effect of CXCL12/CXCR4 Axis. The CCL21/CCR7 axis induces epithelial-mesenchymal transition and promotes proliferation of tumor cells, LECs and extracellular matrix. Akt: Serine/threonine kinase; AP-1: Activated protein 1; Bcl-2: Anti-apoptotic gene; ECM: Extracellular matrix; EMT: Epithelial-mesenchymal transition; ERK: Extracellular signal-regulated kinase; HIF-1: Hypoxia-inducible factor 1; ICAM-1: Intercellular adhesion molecule 1; IL-1L: Interleukin 1L; IL-6: Interleukin 6; JAK/STAT: Janus kinase/signal transducer and activator of transcription; JNK: Jun N-terminal kinase; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; MMP: Matrix metalloproteinase; NF-κB: Nuclear factor kappa B; PI3K: Phosphatidylinositol-3-kinase; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor receptor.

preferred by tumor cells remain partly understood. Several factors can influence this preference, including characteristics specific to the tumor cells themselves, the TME, and the newly formed vasculature[96]. In terms of the TME, factors that attract tumor cells into blood vessels or lymphatic vessels should be considered. These factors include inflammation, host hematopoietic precursors, and soluble factors (*e.g.*, chemokines, growth factors, and soluble receptors). Studies have shown that gene expression profiles can differentiate between LECs and BECs, highlighting their distinct physiological functions and potential as metastatic pathways for tumor cells[97]. Besides their direct association with the development of cancer, BECs are one of the sources of CAFs[98]. The heterogeneous group of CAFs is the main inducer of migration and invasion of cancer cells. Nutrition and oxygen are provided to HCC by discontinuous BECs, thereby contributing to its growth and development. BECs are also involved in intravasation, allowing HCC cells to translocate into the blood vessel lumen[99].

Additionally, the specific coreceptors expressed by tumor cells play a role in determining whether they migrate through LECs or BECs, as these cells express different receptors and signaling molecules. Furthermore, the choice between lymphangiogenesis and angiogenesis may depend on the balance of different inducers in the local TME[100]. The selection of a specific route for dissemination may also depend on various factors, such as the structural and mechanical properties of blood vessels, the expression of adhesion molecules, the secretion of chemokines, and the activity of specific signaling pathways.

SPECIAL FOCUS ON LECS AND BECS IN THE TME OF HCC

The role of LECs and BECs in HCC has also been gradually explored. Preliminary results have implied a correlation between lymphangiogenesis and cancer prognosis. Apart from its well-established functions (*i.e.*, processing of gut-derived nutrients, clearance of toxins, and bile production), the liver is also considered a lymphoid organ. For example, hepatic stellate cells and liver sinusoidal endothelial cells exhibit antigen-presenting and immunomodulatory functions to

create a tolerant microenvironment. Therefore, LECs in the TME of HCC participate in the nearby arising immune responses[14,101].

Chronic inflammation in the liver can induce the production of chemoattractant cytokines and activate NF-κB in LECs, leading to upregulation of PROX1 and VEGFR-3 expression[102]. This, in turn, increases the sensitivity to VEGF-C/D, thus influencing lymphangiogenesis. It has been confirmed that high expression of VEGF-C in patients with HCC is associated with poor prognosis[103]. Lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1+) cells have been identified in the tumor-surrounding environment of human HCC samples. Liver tumors expressing VEGF-C/D are more likely to spread within the liver, resulting in poorer outcomes and reduced patient survival[104,105].

HCC is a solid tumor with a high degree of capillarization and arterialization[106,107]. Thus, angiogenesis also plays a critical role in its development and metastasis. HCC-released TGFβ1 promotes the expression of CD105 in BECs, acting as a promoter of tumor angiogenesis[108]. CD105, in turn, enhances the invasion and metastasis of liver cancer cells by increasing VEGF expression and inducing neoangiogenesis[109]. Researchers are actively searching for additional anti-angiogenic targets. For instance, the sphingosine-1-phosphate receptor 1 (S1PR1), which binds to bioactive molecule S1P involved in angiogenesis, may serve as an important target for suppressing angiogenesis in HCC. Inhibiting S1PR1 shows promise as an approach to anti-tumor therapy against HCC[110,111].

CONCLUSION

In this manuscript, we chiefly discussed the current knowledge regarding tumor lymphangiogenesis and angiogenesis. In addition, we summarized the markers of LECs and BECs, as well as their roles in tumor metastasis (especially in HCC).

LECs can play a significant role in the TME by producing growth factors to sustain tumor cells or present tumor antigens to the immune cells. Nevertheless, there is a need to reveal the cellular and molecular mechanisms involved in these processes. Furthermore, several distinct subpopulations of LECs have been identified, which may fulfill diverse types of functions. Further studies are required to investigate whether subpopulations of LECs could be involved in different aspects of anti-tumor immunity and related to the sequential steps of tumor metastasis. Further investigation is warranted to examine the possibility of preventing tumor progression by removing LECs that promote tumor cell metastasis and by preserving LECs that inhibit tumor cells. Thus far, the mechanisms underlying the inhibitory effect of BECs on anti-tumor immunity remain unclear. Blockage of this process can avoid tumor development by regulating self-immunity without the occurrence of intolerable complications. Hence, it is important to investigate the mechanisms involved in this process. Additionally, high-risk factors of HCC, such as chronic infection with hepatitis B virus or nonalcoholic steatohepatitis, are linked to different mechanisms. Therefore, the distinct functions of the TME components during the development of HCC warrant further research. It is currently established that BECs and LECs can play a markedly more complex role than merely offering nutrition and forming the conduits of tumor cell metastasis. Thus, additional research is required to decipher the mechanisms involved. Such work may result in the development of effective therapies targeting BECs and LECs.

FOOTNOTES

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REVIEW

Quantitative hepatitis B core antibody and quantitative hepatitis B surface antigen: Novel viral biomarkers for chronic hepatitis B management

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Abstract

The management of hepatitis B virus (HBV) infection now involves regular and appropriate monitoring of viral activity, disease progression, and treatment response. Traditional HBV infection biomarkers are limited in their ability to predict clinical outcomes or therapeutic effectiveness. Quantitation of HBV core antibodies (qAnti-HBc) is a novel non-invasive biomarker that may help with a variety of diagnostic issues. It was shown to correlate strongly with infection stages, hepatic inflammation and fibrosis, chronic infection exacerbations, and the presence of occult infection. Furthermore, qAnti-HBc levels were shown to be predictive of spontaneous or treatment-induced HBeAg and HBsAg seroclearance, relapse after medication termination, re-infection following liver transplantation, and viral reactivation in the presence of immunosuppression. qAnti-HBc, on the other hand, cannot be relied on as a single diagnostic test to address all problems, and its diagnostic and prognostic potential may be greatly increased when paired with qHBsAg. Commercial qAnti-HBc diagnostic kits are currently not widely available. Because many methodologies are only semi-quantitative, comparing data from various studies and defining universal cut-off values remains difficult. This review focuses on the clinical utility of qAnti-HBc and qHBsAg in chronic hepatitis B management.

Key Words: Quantitative hepatitis B core antibody; Quantitative hepatitis B surface antigen; Chronic hepatitis B management; Novels viral biomarkers

Core Tip: It is possible to employ a quantitative hepatitis B virus (HBV) core antibody (qAnti-HBc) level as a predictor of therapeutic response, recurrence after hepatitis B surface antigen (HBsAg) loss, and HBsAg seroclearance. Just like other newly identified biomarkers, qAnti-HBc is not a stand-alone diagnostic test that can solve every issue. The information that is now available indicates that it may have a much higher diagnostic and prognostic effectiveness when combined with quantitative HBsAg. Further research involving larger and more variable patients is required to assess the actual usefulness of these biomarkers.

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INTRODUCTION

Hepatitis B virus (HBV) is a chronic infection that affects 250 million people globally, 10% to 15% of whom develop chronic liver disorders such as chronic hepatitis B (CHB), cirrhosis, and hepatocellular carcinoma (HCC). The incidence of cirrhosis and HCC can now be greatly decreased by attaining both HBV DNA undetectable and hepatitis B surface antigen (HBsAg) clearance with or without hepatitis B surface antibody (HBsAb) appearance, known as functional cure[1-3]. However, the effectiveness of currently available medications, such as nucleoside or nucleotide analogs (NAs) and interferon (IFN), is still insufficient when taken as a single medication. The likelihood of prolonged HBsAg loss or seroconversion in CHB patients has significantly increased because of newly discovered novel techniques that switch to or add on pegylated-interferon-alpha (PEG-IFN-α) to ongoing NA therapy[4-7]. After 48–96 wk of medication, the loss rate in patients with quantitative HBsAg (qHBsAg) < 1500 IU/mL, with or without early on-treatment HBsAg reduction, can range from 22% to 58%. Moreover, around 40% to 80% of these patients still test positive for HBsAg even after experiencing PEG-IFN-α side effects[8-11]. Thus, it is essential to develop novel markers or techniques to accurately pinpoint the patients who have the best chance of achieving HBsAg clearance while receiving PEG-IFN-α-based treatment. Two innovative indicators for patients with CHB are the quantitative anti-hepatitis B core antibody (qAnti-HBc) and qHBsAg. Research has demonstrated that in CHB patients, qAnti-HBc levels are much greater during the immune clearance and reactivation stages of natural history than they are during the immunological tolerance and low replication phases[12-14]. In patients who are new to treatment, there is a close correlation between the qAnti-HBc levels and serum ALT levels, as well as the degree of hepatic inflammation. Over the course of NA medication, there was a steady decrease in these levels[15,16]. With regard to qHBsAg, it is utilized to track antiviral medication response, forecast recurrence following treatment termination, and calculate the risk of HCC[17-19]. It is yet unknown, nevertheless, if qAnti-HBc and qHBsAg levels in NA-suppressed CHB patients undergoing NA therapy alone or in conjunction with PEG-IFN-α can forecast HBsAg clearance. The purpose of this review was to evaluate the kinetics of serum qAnti-HBc and qHBsAg levels in CHB patients who were treated with NAs alone or with PEG-IFN-α as an adjuvant to continuous NA therapy. Additionally, the significance of these two new biomarkers in the context of treatment response prediction, the occurrence of recurrence subsequent to the loss of HBsAg, and the phenomenon of HBsAg seroclearance are considered.

LIFE CYCLE OF HBV

The HBV belongs to the family *Hepadnaviridae* and is an enveloped DNA virus. The antigen for HBV, which is now known as surface antigen but was once named "Australia antigen" according to research published in 1965 by Blumberg and colleagues, was initially found in an Australian aborigine[20]. Independent reports of this antigen's existence in hepatitis patients date back to 1968 from Prince, Okochi, and Murakami[21,22]. Dane and colleagues used an electron microscope to visualize the viral particles in 1970[23]. In the blood of individuals who have contacted the infection, at least three different types of HBV particles have been identified: filament structures of varying length with a diameter of 22 nm, spherical structures measuring 42 nm, and those with a diameter of 22 nm. A nucleocapsid made up of the viral genome DNA, viral polymerase (Pol), and the hepatitis B core protein (HBc) is encased in a lipid membrane containing three different viral surface antigens (HBs): large (L-HBs), middle (M-HBs), and small (S-HBs). These 42 nm particles are also known as Dane particles. Subviral particles (SVPs), which are non-infectious due to their absence of nucleocapsid, are among the 22 nm particles seen in patient serum, which are significantly more prevalent. Moreover, various non-infectious particles, such as envelope-less particles (naked nucleocapsids), those carrying viral RNA, and enclosed particles without a viral genome, are now known to be created during infection[24].

The relaxed-circular DNA (rcDNA) that makes up the HBV genome is around 3.2 kb long and has an incomplete plus strand and a full minus strand. Four overlapping open reading frames (ORFs), C, P, S, and X, are encoded by the viral genome and are the source of functional viral proteins. From C, HBc and related proteins like E antigen (HBe) and 22-kDa pre-core protein (p22cr) are produced; from P, Pol is produced; from S, three different types of surface antigens are produced: L-HBs, M-HBs, and S-HBs; and from X, HBV X protein (HBx) is produced. In infected cells, rcDNA is transformed into covalently closed circular DNA (cccDNA). CccDNA then generates HBV RNAs, namely 3.5 kb, 2.4 kb,

2.1 kb, and 0.7 kb, which are transcribed from various promoters in the HBV. The protein product from C and P is generated by a 3.5-kb RNA; L-HBs are translated by a 2.4-kb RNA, and M-HBs and S-HBs are synthesized by a 2.1-Kb RNA; HBx is produced by a 0.7-kb RNA. The resulting HBc protein product first combines to form a dimer *via* its N-terminal domain. Subsequently, it self-assembles to form an icosahedral capsid made up of 90 or 120 dimers, which includes the 3.5-kb viral pregenomic RNA (pgRNA) linked to Pol (more on this below). The 3.5-kb preC mRNA, which has a prolonged 5' upstream region of the C gene and is translated into HBe, is then cleaved at the C-terminus of the resulting protein. The largest HBV protein, Pol, is made up of four domains, each of which has a distinct enzymatic function: (1) The spacer domain, whose function is unclear; (2) the terminal protein (TP) domain, which is vital for binding to pgRNA and acts as a protein primer to start minus-strand DNA synthesis; (3) the reverse transcriptase (RT) domain, which has DNA elongation activity for both reverse transcription and DNA-dependent DNA polymerization; and (4) the ribonuclease H (RNaseH) domain, which digests pgRNA following reverse transcription. The C-terminal S region of all three HBs proteins is identical. Furthermore, an expanded area at the N-terminus of the S region, known as the preS2 region, is carried by M-HBs and L-HBs. At the N-terminus of the preS2 and S sections, L-HBs also have an extension (preS1 region) that is crucial for receptor binding during viral entry. The multifunctional protein HBx contributes to the development of HCC associated with HBV and stimulates the creation of viruses at several stages, including transcription and replication. By using these viral proteins in conjunction with host-derived stimuli, HBV multiplies in host cells[25,26].

ORIGIN AND DEGRADATION OF CIRCULATED HBsAg

Serum HBsAg is made up of cccDNA and integrated HBV DNA. The former can exist in either a compacted, transcriptionally inactive state or a relaxed, transcriptionally active state. Infectious HBV particles are generated and released into the blood, as are noninfectious SVPs[27]. The latter is HBV DNA that has been incorporated into various regions of the hepatocyte chromosome. Because the integrated DNA lacks a typical circular shape, it can only produce S- and M-HBsAg, and the spherical SVPs released into the blood cannot synthesize pregenomic RNA (pgRNA) or other viral proteins[28,29]. SVPs contain 99.99% of the HBsAg in the blood. The majority of blood HBsAg in CHB patients with a full virological response or zero hepatitis B e-antigen (HBeAg) derives from integrated HBV DNA rather than cccDNA[30,31]. HBV DNA that is transcriptionally active and integrated is present throughout the liver and generates broad HBsAg independent of HBV replication. Infected hepatocytes control HBsAg secretion by a number of breakdown mechanisms, including endoplasmic reticulum-mediated proteolysis and autophagy. Furthermore, the degradation of M- and L-HBsAg is aided by the unique proteolytic mechanisms of the proteasome, ubiquitin, and proteome-independent mechanisms[32-34]. When hepatocytes are infected, the activity of the degradation pathways increases, demonstrating that HBsAg renewal is implicated in the synthesis of SVP and virus.

PATTERN OF HBsAg SEROCLEARANCE

With a yearly incidence of about 1%, spontaneous HBsAg seroclearance is an uncommon occurrence[35]. The cumulative rates of spontaneous HBsAg seroclearance after 10 and 25 years, respectively, were 8.1% and 44.7%, according to research with 1076 CHB patients[36]. The crude incidence rate of HBsAg loss was 1.6% per 100 person-years, according to a prospective follow-up of 1240 patients with negative HBeAg who had not received treatment for 5.5 years. More significant correlations between HBsAg seroclearance and quantitative HBsAg levels, non-Asian race, older age, inactive HBsAg carriers, HBV genotype A, and lower HBV DNA were also discovered[37]. Instead of directly interacting with cccDNA, NAs only inhibit HBV DNA. Once they are phosphorylated, they work as antimetabolites by being similar enough to nucleotides to be incorporated into growing DNA strands; but they act as chain terminators and stop viral DNA polymerase. As a result, NAs find it extremely challenging to stop the synthesis of HBV particles and their antigens. In 26614 person-years of entecavir (ETV) or tenofovir dipivoxil (TDF), the 10-year cumulative loss rate of HBsAg was just 2%, according to a new large multicenter cohort study that included 4769 CHB patients[38]. HBsAg loss may result after discontinuing NAs in accordance with the recommendations' withdrawal criteria, notwithstanding the possibility of virological recurrence, clinical relapse, or worsening of the liver disease[39,40].

DURABILITY AND CLINICAL OUTCOMES OF HBsAg SEROCLEARANCE

Without focusing on the removal of integrated HBV DNA and cccDNA, functional cure is more closely associated with the restoration of liver function, particularly the specific immune function against HBV, *via* the maximal long-term suppression of HBV replication. There is still a significant difference between a functional cure and a full recovery. The length of functional cure following medication withdrawal and the improvement of long-term outcomes are also critical indicators of complete cure, in addition to the detection of integrated HBV DNA and cccDNA. In a retrospective cohort study, 4568 CHB patients with HBsAg clearance were included; of them, 793 had undergone NA treatment and 60 underwent interferon (IFN) therapy. Fifty-four individuals (2.9%), including 49 who had spontaneous HBsAg clearance and five males over 50 receiving NA treatment, developed liver cancer over a median follow-up of 3.4 years. At one, three, and five years, the cumulative incidence rates of HCC were 0.9%, 1.3%, and 1.5%, respectively. Within five years,

no patient receiving PEG-IFN-α developed liver cancer. Two independent predictors of the likelihood of HCC in patients with HBsAg clearance were sex and age[41]. In addition, 7124 CHB patients with HBsAg loss were also evaluated. There were 1207 cases of NA-induced clearance and 5917 cases of spontaneous clearance. There was no discernible difference in the incidence of HCC at 1.6% and 1.3%, respectively, after an average follow-up of 4.3 years[41]. Additionally, to determine the HBsAg status of each end point and the composite end point, Anderson *et al*[42] conducted a systematic review and meta-analysis of rate ratios (RR) using a random-effects model independently. They also reported the incidence of liver decompensation, liver transplantation, HCC, and all-cause mortality. 188316 patients with chronic HBV infection and 1486081 person-years (PY) of follow-up evaluation were included in the 28 studies whose data they evaluated; 26 of the studies contained data on HCC, 7 on liver decompensation, and 13 on liver transplantation and/or death. For both the HBsAg seroclearance group and the HBsAg-persistent group, the composite event rates were 2.45/1000 PY and 0.19/1000 PY, respectively. In the HBsAg seroclearance group, the pooled relative risks (RRs) for liver decompensation, HCC, liver transplantation, and/or mortality and the composite endpoint were 0.28, 0.30, and 0.22, respectively. Within subgroups of various research or patient characteristics, no variations in RR estimations were noted. They discovered a substantial correlation between improved patient outcomes and seroclearance of HBsAg. It is uncertain if individuals with CHB who acquired HBsAg seroclearance naturally or as a result of anti-viral treatment had similar clinical outcomes. Several studies examined the risk of HCC, hepatic decompensation, and recurrence of HBsAg positive in CHB patients who cleared HBsAg spontaneously or after anti-viral treatment with NAs alone, IFN-α alone, or IFN-α combination with NAs. Table 1 summarizes these clinical results[43-62].

Recently, Vittal *et al*[63] also conducted a meta-analysis comprised of 57 papers throughout the globe which recruited 2 prospective population-based studies, 22 prospective cohort studies, and 33 retrospective cohort studies. Out of the 258744 HBsAg-positive patients in total, 63270 (24.4%) had HBsAg loss. There were ten studies with patients who lost HBsAg spontaneously, twelve with patients who lost HBsAg as a result of therapy, twenty-two with patients who lost HBsAg as a result of both treatment and spontaneous means, and thirteen without reporting the method of HBsAg loss. They discovered that HBsAg loss is linked to a lower likelihood of developing HCC as well as other significant clinical outcomes such as hepatic decompensation, liver cirrhosis, and death from all causes. The use of HBsAg seroclearance as the major end point of trials for individuals with persistent HBV infection is supported by the consistent outcomes across various study types and patient subpopulations studied. Additionally, Song *et al*[64] also conducted a meta-analysis to assess the HCC incidence and durability of HBsAg seroclearance following therapy discontinuation. They used a random-effects model to analyze the data. In the end, 38 studies and 43924 patients were included. The pooled recurrence rate of 6.19% demonstrated the durability of HBsAg seroclearance. Recurrence rates following various seroclearance techniques, as well as rates across recurrence kinds and geographical areas, did not differ significantly. The rate of recurrence was considerably lower after anti-HBs seroconversion. Compared to HBsAg-positive patients, those who had HBsAg seroclearance had a noticeably decreased incidence of HCC. Following HBsAg seroclearance, the pooled incidence of HCC was 1.88%; among patients without cirrhosis at baseline, this rate was 0.76%. Additionally, Liu *et al*[65] conducted a meta-analysis and systematic review of 28 studies encompassing 34952 individuals who had HBsAg seroclearance. Despite HBsAg seroclearance, the aggregate pooled percentage indicated that 2.29% of CHB patients will develop HCC. The pooled percentage of HCC development in HBsAg seroclearance patients without cirrhosis or HCV co-infection was 1.55%. Furthermore, individuals who had cirrhosis or were older than 50 years at the time of HBsAg seroclearance had a markedly increased chance of developing HCC. However, as compared to chronically positive HBsAg, seroclearance of HBsAg was substantially linked to a lower risk for HCC. They concluded that while HBsAg seroclearance can dramatically lower the risk for HCC, certain CHB patients may still develop HCC following HBsAg seroclearance. Because HBsAg loss is linked to a more persistent reduction of viremia, the physicians favored using it as the surrogate endpoint of therapy rather than HBV DNA.

QHBSAG

qHBsAg tests have been used for the detection of hepatitis B infection since the 1970s. The expression of HBsAg occurs at an early stage in the viral life cycle. There has been a recent surge in interest in the measurement of HBsAg and its potential as a predictor of therapy results. Various commercially available qHBsAg assays have been developed, including Elecsys HBsAg II Quant by Roche Diagnostics and ARCHITECT QT by Abbott Laboratories. Both options are offered at a reasonable price, have undergone standardization procedures, and use an automated two-step chemiluminescence method. In the ARCHITECT QT test, the HBsAg, paramagnetic microparticles, and acridinium-labeled anti-HBs conjugate are combined in a sandwich configuration. The chemiluminescent outcomes are assessed using relative light units and then converted into an HBsAg quantification. The Elecsys HBsAg II Quant test employs a specific technique in which HBsAg, streptavidin-coated microparticles, and biotin-ruthenium-labeled monoclonal antibodies are combined in a sandwich configuration. The mixture is subjected to a provided voltage while it is drawn into a measuring cell, resulting in the generation of ruthenium chemiluminescence. This chemiluminescence may then be quantified using a photomultiplier. The automated dilution feature has the potential to elevate the detection limit of both assays to levels above 50000 IU/mL. The dynamic ranges of the tests are 0.05–250 IU/mL for the ARCHITECT QT assay and 0.05–130 IU/mL for the Elecsys HBsAg II Quant assay. The HISCL HBsAg test has been recently developed for the quantification of HBsAg using the chemiluminescence enzyme immunoassay (CLEIA) technique, using the CDP-Star® chemiluminescent substrate. The biotinylated monoclonal antibodies targeting HBsAg derived from mice exhibit a particular reaction with HBsAg present in the sample. These antibodies subsequently attach themselves to streptavidin-coated magnetic particles. Following the process of bound/free (B/F) separation, the monoclonal antibodies (mouse) tagged with alkaline

Table 1 Summary of the incidence of hepatocellular carcinoma, hepatic decompensation, and hepatitis B surface antigen seroreversion of chronic hepatitis B patients with hepatitis B surface antigen seroclearance

Ref.	Country/region	Study design	*n*	HBsAg seroreversion (%)	Hepatic decompensation (%)	HCC incidence (%)
Spontaneous HBsAg seroclearance						
Yip *et al*[43], 2023	Hong Kong	Retrospective	7942	6.1	1.3	1.1
Yip *et al*[44], 2021	Hong Kong	Retrospective	5917	7	-	1.6
Choi *et al*[45], 2021	South Korea	Retrospective	1624	1.2	-	2.2
Park *et al*[46], 2021	South Korea	Retrospective	984	-	10.9	1.4
Yeo *et al*[47], 2020	Multicenter	Retrospective	11264	1.3	-	-
Song *et al*[48], 2019	China	Prospective	652	-	-	1.2
Zhu *et al*[49], 2018	China	Prospective	348	-	-	0.2
Chen *et al*[50], 2016	Taiwan	Retrospective	312	-	-	1.3
NAs treatment HBsAg seroclearance						
Yip *et al*[44], 2021	Hong Kong	Retrospective	1207	7.7	-	1.3
Choi *et al*[45], 2021	South Korea	Retrospective	348	5.5	-	4
Kim *et al*[51], 2020	South Korea	Retrospective	276	3.6	-	2.9
Yip *et al*[52], 2019	Hong Kong	Retrospective	376	1.4	-	0.5
Suarez *et al*[53], 2019	Spain	Retrospective	69	1.5	-	1.5
Sun *et al*[54], 2019	China	Retrospective	54	-	-	0
Chi *et al*[55], 2017	Multicenter	Retrospective	70	3.7	-	0
Chen *et al*[50], 2016	Taiwan	Retrospective	110	-	-	0.9
IFN-α alone or combined with NAs treatment HBsAg seroclearance						
Li *et al*[56], 2022	China	Prospective	231	8.2	-	-
Chen *et al*[57], 2021	China	Prospective	48	6.3	-	0
Wu *et al*[58], 2021	China	Prospective	68	-	-	0
Pan *et al*[59], 2021	China	Prospective	376	17.3	-	0.3
Wu *et al*[60], 2020	China	Retrospective	238	5.9	-	0
Choi *et al*[61], 2020	Canada	Retrospective	65	1.9	-	4.4
Li *et al*[62], 2019	China	Prospective	176	13.4	-	0.6

CHB: Chronic hepatitis B; HBsAg: Hepatitis B surface antigen; HCC: Hepatocellular carcinoma; INF-α: Interferon-alpha; NAs: Nucleoside analogues.

phosphatase (ALP) exhibit specific binding to the HBs antigen on a microparticle. Subsequently, the ALP present on the membrane protein (MP) cleaves the CDP-Star® substrate, resulting in the formation of an energetically excited intermediate that gives rise to a luminous signal. The assay samples were evaluated at their original concentration using an analytical measurement range spanning from 0.03 to 2500 IU/mL, without the need for any preliminary dilution. Nevertheless, the system is equipped with an automatic dilution feature to address instances when HBsAg concentrations are very high. Additionally, a Lumipulse HBsAg-HQ test was created to assess HBsAg concentrations. The measurement of HBsAg was conducted using the two-step sandwich assay technique, using a fully automated chemiluminescent enzyme immunoassay system known as Lumipulse G1200, manufactured by Fujirebio, Inc. The samples underwent pretreatment using a solution containing a surfactant in order to disrupt HBV particles. This process aimed to detach HBsAg from HBsAg-anti-HBs complexes and denature epitopes into a linear form. The detection of linearized HBsAg was accomplished by using two monoclonal antibodies that target exterior structural areas, namely determinant "a," as well as an internal epitope that served as a capture reagent. Additionally, two monoclonal antibodies linked to ALP were used as detectors. During the test methods, 100 μL of blood serum and/or plasma samples, together with 20 μL of pretreatment solution, were subjected to incubation with monoclonal antibodies that bind to ferrite microparticles. This incubation took place at a temperature of 37 °C for a duration of 10 min. Following the completion of the automated washing process, a volume of 250 μL of ALP-labeled antibodies was introduced and then incubated at a temperature of 37°C for a duration of 10 min. Following the washing procedure, a volume of 200 μL of the substrate solution, AMPPD, was introduced and then incubated at a temperature of 37 °C for a duration of 5 min. The measurement of chemiluminescence intensity was conducted in order to determine the concentration of HBsAg by comparing it to a standard curve. The assay included a range of HBsAg values from 0.005-150 IU/mL. In cases where the concentration exceeded this range, retesting was conducted using a 100, 200 or 1000-fold dilution of the samples. The development of iTACT-HBsAg occurred in 2016, using monoclonal-antibody-coated ferrite particles as the solid phase *via* the coupling approach with chemical linkers. The particle solution was prepared by suspending ferrite particles coated with antibodies in a diluent specifically designed for particle suspension. The detection reagent consisted of ALP-linked monoclonal antibodies that were produced using the hinge approach and then purified by chromatography using a HiLoad 16/600 Superdex 200 pg column. The preparation of the conjugate solution included the process of diluting the ALP conjugate in the conjugate diluent. The iTACT-HBsAg test was conducted using the LUMIPULSE PRESTO II, an automated CLEIA instrument manufactured by Fujirebio. Fifty μL of samples were subjected to incubation with a 30 μL acidic pretreatment solution for a duration of 6.5 min at a temperature of 37 °C. The samples that had been pre-treated were thereafter combined with a 50-μL solution containing particles and incubated for a duration of 8 min at a temperature of 37 °C. Following the washing of the ferrite particles with Lumipulse Presto washing buffer, an incubation period of 8 min at 37 °C was conducted with the conjugate solution. Subsequently, the relative luminous intensity was assessed by incubating the particles for 4 min at 37 °C with 200 μL of substrate solution (Table 2)[66-71].

QHBSAG AND FUNCTIONAL CURE

Chtourou *et al*[72] conducted a study to assess the diagnostic utility of qHBsAg in detecting HBV functional cure among 174 Tunisian patients with HBeAg-negative CHB infection over a 1-year prospective follow-up period. During the follow-up period, it was found that 21.6% of patients with a low initial viral load (< 2000 IU/mL) and 19.5% of patients with an intermediate viral load (2000–20000 IU/mL) experienced an increase in their HBV DNA levels above 2000 and 20000 IU/mL, respectively. Notably, significant fluctuations in viral load were observed in 61.1% of patients at 6-month intervals. Among the 174 patients, 89 (51.1%) belonged to inactive carriers, 33 (19%) to patients with negative HBeAg CHB, and 52 (29.9%) to patients with an indeterminate state. Out of the 14 patients who underwent a liver biopsy, seven exhibited moderate-to-severe liver disease. The combination of HBV DNA <2 000 IU/mL and qHBsAg < 832 IU/mL effectively ruled out CHB in 98.4% of cases. Furthermore, a qHBsAg cutoff point of < 100 IU/mL, coupled with an annual decline of >0.5 Log 10 IU/mL, proved to be a reliable predictive marker for achieving a functional cure in CHB patients. It was discovered that individuals with HBeAg-negative chronic HBV infection, namely those with genotype D, had significant short-term changes in HBV DNA levels. Therefore, by using the qHBsAg cutoff value of 832 in conjunction with the HBV DNA cutoff value of 2000, it becomes feasible to effectively rule out CHB in the majority of patients. The authors proposed doing an initial assessment of patients by measuring ALT, HBV DNA, and qHBsAg levels. In instances where values fall below the established thresholds, it may be appropriate to conduct further monitoring *via* the use of ALT and qHBsAg tests only.

Coffin *et al*[73] conducted a retrospective review of clinical outcomes in a broad community of patients with CHB who obtained functional cure, defined as HBsAg loss. The study compared these patients to people with different levels of HBsAg using the quantitative HBsAg assay. A total of 844 individuals diagnosed with CHB were included in the study. Among these patients, 237 (28.0%) tested negative for HBsAg, while 190 (22.5%) had a qHBsAg level ranging from 1 to 100 IU/mL. Additionally, 91 (10.8%) patients had a qHBsAg level between 100 and 500 IU/mL, 54 (6.4%) had a qHBsAg level between 500 and 1000 IU/mL, and 272 (32.2%) had a qHBsAg level beyond 1000 IU/mL. In general, a total of 682 individuals, accounting for 80% of the sample, had documented HBeAg status at the final follow-up. The predominant proportion, namely 87.0%, exhibited a negative HBeAg status. Furthermore, it is worth noting that 54% of the participants in the study, namely 461 out of 844 individuals, had previously had antiviral medication. The patients who received treatment exhibited a reduced likelihood of developing cirrhosis or HCC in comparison to the individuals who did not get treatment. The HBsAg-loss group had a lower prevalence of cirrhosis in comparison to the HBsAg-positive group, with rates of 5.7% and 10.9%, respectively. Additionally, the HBsAg-loss group showed a lower incidence of HCC at 0.9%

Table 2 Summary of the characteristics of quantitative hepatitis B surface antigen assays from several companies

Test names	Companies	Principle techniques	Detection methods	Pretreatment procedure	Range of detection	On-board dilution
Elecsys HBsAg II	Roche Diagnostic	Sandwich technique, 2 capture mAbs, and a mixture of mAbs and polyAbs detection	ECLIA (ruthenium)	None	0.05–130 IU/mL	1:400 with buffered negative human serum
Architect HBsAg QT	Abbott Laboratories	Sandwich technique, capture mAbs, and polyAbs detection	CMIA (acridinium)	None	0.05–250 IU/mL	1:500 with recalcified negative human plasma
Sysmex HISCL HBsAg	Sysmex Corporation	Sandwich technique, capture mAbs, and mAbs detection	CLEIA (CDP-Star)	None	0.03–2500 IU/mL	Auto dilution
Lumipulse G HBsAg-Quant	Fujirebio Incorporation	Sandwich technique, 2 capture mAbs and 2 detections mAbs	CLEIA (AMPPD)	Yes, to disrupt viral particles and dissociate HBsAg from HBsAg-anti-HBs complexes	0.005–150 IU/mL	1:100, 1:200 or 1:1000 with NaCl and Tris buffer
iTACT-HBsAg	Fujirebio Incorporation	Sandwich technique	ICT-CLEIA (AMPPD)	Yes, to inactivate anti-HBs, releases the antigen from the immune complexes, and to disassociate the antigen polymers into monomers	0.0005–113 IU/mL	N/A

AMPPD: Adamantane; CMIA: Chemiluminescent microparticle immunoassay; CLEIA: Chemiluminescent enzyme immunoassay; ECLIA: Electrochemiluminescence immunoassay; ICT-CLEIA: Immune complex transfer-chemiluminescent enzyme immunoassay; iTACT: Immunoassay for total antigen including complex *via* pretreatment; mAbs: Monoclonal antibodies; polyAbs: Polyclonal antibodies; N/A: Not associated.

compared to the HBsAg-positive group at 6.2%. The predominant group of patients who had HBsAg loss consisted of individuals who had not received treatment, were negative for HBeAg, exhibited undetectable levels of HBV DNA, and demonstrated normal liver enzyme levels. The presence of low-level qHBsAg (< 100 IU/mL) was seen in individuals who had HBsAg loss, as determined by testing conducted within one year following HBsAg seroclearance. The investigators concluded that individuals with CHB who had antiviral medication and experienced a reduction of HBsAg had a reduced likelihood of developing cirrhosis and HCC. There was no observed correlation between the amount of qHBsAg and the presence of hepatic fibrosis. Individuals who successfully attained HBsAg loss had low-level qHBsAg during a period of one year following seroclearance.

In order to compare the efficacy of two novel assays — iTACT-hepatitis B surface antigen (iTACT-HBsAg) and iTACT-hepatitis B core-related antigen (iTACT-HBcrAg) — with 120 HBsAg-negative and anti-HBc-positive individuals, Wong *et al*[74] conducted a study in which 556 serial sera were collected from 96 CHB patients who had HBsAg seroclearance as confirmed by standard assays. Sixty seronegative people who tested negative for HBsAg, antiHBc, and anti-HBs made up the control group. They discovered that 154/418 (36.8%) of the samples obtained following seroclearance could detected HBsAg. In samples taken before and after seroclearance, HBcrAg was found in 78.3% and 65.9% of the samples, respectively. Following seroclearance, the detectability rates of HBsAg and HBcrAg gradually dropped. Ten years following seroclearance, detectable HBsAg and HBcrAg were still present in 20.4% and 64.5% of the patients, respectively. 66 individuals (71%) had HBsAg and/or HBcrAg that could be detected. Eleven (9.2%) and four (3.3%) of the 120 HBsAg-negative and anti-HBc-positive people showed detectable HBsAg and HBcrAg, respectively. In the controls, HBsAg and HBcrAg were not detected. They concluded that the iTACT tests found that >70% of patients had low levels of HBsAg and/or HBcrAg even ten years after seroclearance, indicating that CHB patients who had seroclearance may still have low levels of HBV protein expression. Research on the clinical implications of detectable viral proteins following seroclearance in relation to the progression of the disease and HBV reactivation is warranted.

Seroclearance of HBsAg remains infrequent in CHB infections. ALT rise during acute flares of CHB (AFOCHB) indicates an increasing immune response aimed at clearing the virus. Hui *et al*[75] postulated that there is a correlation between severe AFOCHB and a more significant decrease in qHBsAg levels, as well as a higher rate of HBsAg seroclearance. A total of 75 patients diagnosed with severe AFOCHB and having ALT levels 10 times higher than the upper limit of normal (ULN) were selected. These patients were then matched with a control group based on age and sex in a 1:2 ratio. QHBsAg levels were assessed throughout the flare and yearly until the final follow-up. They discovered that the rate of HBsAg seroclearance was greater in the severe AFOCHB group compared to the control group (11.8% *vs* 5.0%, $P = 0.04$), even though the former group had a somewhat higher baseline median qHBsAg (3127 IU/mL *vs* 1178 IU/mL, $P = 0.076$). In comparison to the control group, the severe AFOCHB group had a higher yearly decrease in qHBsAg levels (-242.4 IU/mL/year vs. -47.3 IU/mL/year, $P = 0.002$). HBsAg seroclearance was independently linked with increasing age ($P = 0.049$), decreased baseline qHBsAg ($P = 0.002$), and severe AFOCHB ($P = 0.014$). Nevertheless, the overall incidence of HCC was much greater in the severe AFOCHB group compared to the control group (15.8% *vs* 1.9%, $P < 0.001$). Caviglia *et al*[76] examined the clinical significance of qHBsAg in a real-world group of CHB patients receiving NAs therapy in a specialized medical facility in North-West Italy. A retrospective enrollment was conducted for a total of 101 patients with CHB (86 of whom were HBeAg-negative) who were having treatment with NAs. The level of HBsAg was assessed at four time points: baseline (T0), 6 months (T1), 12 months (T2), and the final follow-up (FU). The median

follow-up period was 5.5 years, ranging from 3.2 to 8.3 years. After the follow-up period, 11 patients had a loss of HBsAg, resulting in an annual incidence rate of 1.8%. The initial levels of HBsAg were substantially higher in patients who did not experience an HBsAg decrease compared to those who had a functional cure (3.46 *vs* 1.11 Log IU/mL, $P < 0.001$). The reduction in HBsAg from T0 to T2 was substantially different between the two groups of patients. The first group had a decline of 0.05, ranging from -0.04 to 0.13 Log IU/mL, while the second group had a decline of 0.38, ranging from 0.11 to 0.80 Log IU/mL. This difference was statistically significant, with a *P*-value of 0.002. The stratified cross-validation study demonstrated that the combination of baseline HBsAg and ΔHBsAg T0–T2 had a very high accuracy in predicting HBsAg loss, with a C statistic of 0.966. These findings confirm the effectiveness of qHBsAg in predicting HBsAg seroclearance in Caucasian CHB patients undergoing antiviral treatment.

Participants in the retrospective analysis were CHB patients who had not previously had entecavir medication but who had received at least two years of consecutive treatment in order to determine the usefulness of qHBsAg kinetics during long-term entecavir treatment. Abdominal sonography, liver biochemistry, and HBV DNA were used for patient follow-up at 3- to 6-month intervals. Patients who tested positive for HBeAg had their levels checked every three to six months until the findings stopped coming back positive. We measured serum qHBsAg levels at baseline, one year, and five years. Biopsies of the liver, imaging tests, or the presence of portal hypertension in the patient's symptoms led to the diagnosis of liver cirrhosis. Histological analysis or research using dynamic imaging techniques confirmed the presence of hepatocellular cancer. Patients who tested positive for HBeAg had an earlier virological response, biochemical response, and HBeAg seroconversion when their baseline qHBsAg levels were lower; patients who tested negative for HBeAg but did not have cirrhosis had an earlier virological response. Despite the gradual reduction in qHBsAg levels seen following long-term entecavir therapy, patients with higher baseline HBsAg levels and those who tested positive for HBeAg but did not have cirrhosis had the fastest qHBsAg decline rates in the first year. Rapid HBsAg fall was not associated with better clinical outcomes; rather, it was dependent on lower HBsAg levels to begin with. Results showed that baseline HBsAg is a good predictor of response to therapy. When trying to make sense of qHBsAg changes, it's important to take both the levels and the rates of reduction into account with the patient's illness condition[77]. The objective of the study conducted by Ma *et al*[78] was to determine the factors that might predict HBsAg seroconversion in patients with CHB who are undergoing antiviral medication. They investigated the blood levels of quantitative pg-RNA, HBcrAg, and HBsAg as potential predictors. A cohort of 335 patients on antiviral treatment was included in the study. Out of them, only 23 patients achieved seroconversion for HBsAg. Additionally, a random selection of 138 individuals without seroconversion of HBsAg was made from the remaining 312 patients. The samples from a total of 161 patients were tested at various time intervals. The reduction in titers of pg-RNA, HBcrAg, and HBsAg from baseline to 6 months and baseline to 12 months was denoted as Δpg-RNA, ΔHBcrAg, and ΔHBsAg, respectively. Subsequently, Δpg-RNA, ΔHBcrAg, and ΔHBsAg were used as predictors for HBsAg seroconversion. They discovered that a total of 6.9% of the patients achieved seroconversion of HBsAg after a median duration of 3.61 years of therapy. The receiver operating characteristic (ROC) analysis was used to predict the seroconversion of HBsAg. At 6 months, a ΔHBsAg of 0.64 Log10 IU/mL yielded an area under the curve (AUC) of 0.886, indicating a strong predictive ability. Similarly, at 12 mo, a ΔHBsAg of 1.45 Log10 IU/mL resulted in an AUC of 0.939, indicating a high level of predictive accuracy. These values were shown to have the highest Youden's index, indicating optimal predictive performance. The present study used the Kaplan-Meier approach to compare the frequencies of HBcrAg conversion between two groups of patients: 23 individuals who had HBsAg conversion and 138 individuals who did not. The analysis revealed a statistically significant difference between the two groups at the point of antiviral cessation. Based on the findings, ΔHBsAg has the potential to serve as a useful indicator for identifying patients who are likely to achieve seroconversion *via* adherence to antiviral medication. This is of significant importance in attaining the objective of a functional cure, or perhaps a clinical cure.

To estimate the length of treatment required to produce a functional cure, Cho *et al*[79] performed a study to look at the on-treatment dynamics of qHBsAg during entecavir therapy. A linear mixed model was used to evaluate the kinetics of qHBsAg reduction in 410 individuals from a cohort of 1009 CHB treatment-naïve patients initiated on entecavir. Based on baseline liver cirrhosis, HBeAg positivity, and HBeAg seroclearance, the variation in the kinetics of qHBsAg was ascertained. With a median age of 48 years, 213 (52.0%) and 217 (66.1%) male patients were among the 410 patients. Over a median follow-up of 53.5 months, there was a gradual but steady decline in the qHBsAg level. For HBeAg (+) and HBeAg (-) patients, the anticipated log qHBsAg values as a function of time during entecavir therapy were 3.4773 - 0.0039 × months and 3.1853 - 0.0036 × Months, respectively. For HBeAg-positive patients in this study, the expected time to clearance quantitative HBsAg was more than 74.1 years, whereas for HBeAg-negative individuals, it was 73.5 years. Without considering the occurrence of liver cirrhosis or HBeAg seroclearance, the estimated period to attain a functional cure is a lifetime. According to mathematical modeling based on a long-term follow-up of patients with CHB using entecavir, clearing HBsAg takes decades of therapy. Therefore, to obtain a functional cure in individuals treated with entecavir, lifetime medication is required. For patients who test positive for HBsAg, HBeAg seroconversion represents a significant spontaneous shift and therapy endpoint. It is also a requirement for HBsAg loss or functional cure. In a retrospective analysis, Wang *et al*[80] used serum qHBsAg and quantitative hepatitis B core-related antigen (qHBcrAg) to predict seroconversion in HBeAg-positive patients receiving NA treatment. A retrospective analysis was done on 118 HBeAg-positive individuals (genotypes A–G) at various time points. ROC analysis was used to estimate the predictive potential of the on-treatment levels of qHBsAg and qHBcrAg, with cut-off values set by maximizing Youden's index. A median of 39 months of therapy resulted in HBeAg seroconversion in around 36.4% of patients. Regarding the HBV DNA treatment kinetics, there were differences in qHBsAg and qHBcrAg between HBeAg seroconverters and non-seroconverters. For HBeAg seroconversion, a combination of qHBsAg and qHBcrAg had the highest predictive value: at 6 months, a combination of 3.9 Log10 IU/mL and 5.7 Log10 U/mL of qHBsAg and qHBcrAg had a sensitivity of 71.4%, a specificity of 79.5%, a PPV of 65.2%, and an NPV of 83.8%, with an AUROC of 0.769; at 12 months, a combination of 3.8 Log10 IU/mL and HBcrAg 5.5 Log10 U/mL had a sensitivity of 73.7%, a specificity of 79.5%, a PPV of 63.6%, and an NPV

of 86.1%, with an AUROC of 0.807. As the care of CHB shifts to medicines that give functional cures, they concluded that qHBsAg and qHBcrAg may be utilized to identify individuals who are unlikely to attain treatment endpoints.

Although the clearance rate is quite low, HBsAg clearance reflects a clinical cure. The objectives of the study by Cao *et al*[81] were the assessing the viability and safety characteristics of pegylated interferon α-2a (PEG-IFNα-2a) as a treatment alternative for dormant HBsAg carriers. A therapy group of 102 participants and a control group consisting of 42 subjects were formed from the 144 inactive HBsAg carriers who were recruited. Subjects in the therapy group with HBV DNA < 20 IU/mL and 20 IU/mL ≤ HBV DNA < 2000 IU/mL, respectively, received PEG-IFNα-2a and PEG-IFNα-2a in combination with adefovir dipivoxil. There was a maximum of 96 wk in total for treatment. The therapy effectiveness was assessed using the seroconversion rates and HBsAg clearance at therapeutic weeks 48 and 96. According to protocol analysis, the therapy group's HBsAg clearance and seroconversion rates were 29.8% and 20.2% at week 48 and grew to 44.7% and 38.3% at week 96, respectively. Nonetheless, in weeks 48 and 96, the HBsAg clearance rate in the control group was 2.4%, and no patient attained seroconversion. HBsAg clearance was well predicted by the qHBsAg levels and variations at week 12 and week 24 of the therapy, as well as by the rise of ALT at week 12. The side effects matched those of therapy for long-term hepatitis B patients. They deduced that PEG-IFNα-2a-based therapies might attain greater amounts of HBsAg clearance, as measured by qHBsAg and seroconversion, and these treatments posed no risk to inactive HBsAg carriers.

QANTI-HBC

In HBV low-to-medium epidemic locations, anti-HBc has historically been used as a blood screening test and is thought to be an indication of prior or current HBV infections. Interestingly, most people with chronic or even cured HBV infection often have anti-HBc levels beyond the top limit of commonly used qualitative anti-HBc tests. In order to accurately establish the differences in anti-HBc levels across patients or the changes that occur with the course of the diseases, studies on the usefulness of anti-HBc levels in chronic HBV infection need repeated dilutions or assays with large dynamic ranges. Qualitative tests cannot determine these differences[82]. When it comes to CHB infections, those with active hepatitis in the immune-active phase usually have 10-fold greater levels of qAnti-HBc than people in the immune-tolerant or inactive-carrier phases when hepatitis is not present[83]. In individuals with CHB, there is a positive link between serum ALT activity and qAnti-HBc, which suggests that the latter may be indicative of hepatic inflammation, according to many studies. Furthermore, it has been shown that qAnti-HBc favorably correlates with the liver biopsy-determined histological severity of hepatic inflammation. Surprisingly, research has shown that qAnti-HBc is linked to the severity of fibrosis and, when combined with other factors, may enhance the efficacy of non-invasive indices. However, the connection between fibrosis degree and qAnti-HBc is often smaller than the correlation with inflammation, indicating that the former relationship may be partly explained by the correlation between qAnti-HBc and liver inflammation[84-87]. The gold standard for identifying liver inflammation is a liver tissue biopsy, yet since it is invasive, it is not often performed. ALT, a frequently used marker for hepatocellular damage, has a risk of being ignored since moderate to severe hepatic inflammation is present in 20%-30% of individuals with normal ALT. Several studies have looked at the additional role of qAnti-HBc in identifying immune activation status and inflammation in HBV-positive persons with normal ALT levels[88,89]. Zhang *et al*[90] found that in 1376 untreated CHB patients with normal ALT, there was a dose-responsive association between the degree of liver inflammation and qAnti-HBc. The qAnti-HBc cut-off value for identifying moderate and severe inflammation was around 4.5 Log10 IU/mL. The investigation was conducted across many centers. This study also found a correlation between the reduction of liver histological inflammation and the drop in qAnti-HBc after NAs administration. Furthermore, Feng *et al*[91] similarly observed that substantial liver damage was associated with high qAnti-HBc in CHB patients with normal ALT; however, the study's cut-off value (3.7 Log10 IU/mL) seemed to be lower than the previous study. More specifically, a high level of qAnti-HBc was also observed to be related to considerable liver inflammation in populations of HBeAg-negative chronic HBV-infected individuals with normal ALT, which was evaluated by Yao *et al*[92].

The current qAnti-HBc assays are based on competitive/inhibitory, indirect, or double-antigen sandwich immunoassay technology and are available as enzyme-linked immunosorbent assays (ELISA), chemiluminescent microparticle immunoassays (CMIA), or CLEIA. Table 3 summarizes the techniques and units used to quantify anti-HBc. The development of the double-antigen sandwich immunoassay for qAnti-HBc was first established in 2010. This particular assay has been shown to exhibit enhanced sensitivity and specificity compared to other methods. However, it should be noted that the quantitation range of this test is very limited. The commercially available ELISA, which is based on the double-antigen sandwich immunoassay, may now be obtained from Wantai Biological Pharmacy Enterprise Company in Beijing, China. This ELISA can be calibrated against the WHO International Standard, allowing findings to be provided in IU/mL. The study indicated a linear detection range of 2–5 Log IU/mL, with a lower limit of quantitation of 0.1 IU/mL[93,94]. Lumipulse G Anti-HBc-N, a fully automated two-step sandwich CLEIA developed by Fujirebio in Tokyo, Japan, is another often-used test. The present methodology involves the automated reporting of anti-HBc IgG levels as the cut-off index (COI), which is determined by calculating a multiple of the cut-off value derived from calibration data. The lowest limit of quantification was declared by the manufacturer as 1 COI. However, it is possible to calibrate the data using the WHO International Standard in order to acquire IU/mL. The lower limit of detection was 0.5 IU/mL. In comparison to other approaches using chemiluminescence for detecting qAnti-HBc, this particular method exhibited superior levels of sensitivity, specificity, and a wider linear dynamic range[95]. Furthermore, for the purpose of determining the quantities of anti-HBc in serum, a straightforward and quick fluorescence point-of-care test based on a lateral flow immunoassay technique has been developed. The method used is a competitive time-resolved fluoroimmun-

Table 3 Summary of the characteristics of quantitative hepatitis B antibody assays from several companies

Test names	Companies	Principle techniques	Detection methods	Range of detection
Anti-HBc QN[93]	Wantai BioPharm	Double-antigen sandwich	ELISA	0.1–25 IU/mL
Anti-HBc HQ[94]	United Medical Instruments	Fully automated two-step sandwich	CMIA	100–100000 IU/mL
Lumipulse G HBcAb-N[95]	Fujirebio Incorporation	Fully automated two-step sandwich	CLEIA	0.5–50 IU/mL
Europium III chelate microparticles HBcAb[96]	Guangzhou Darui Biotechnology	Rapid fluorescence POCT	LFIA	0-640 IU/mL

CLEIA: Chemiluminescent enzyme immunoassay; CMIA: Chemiluminescent microparticle immunoassay; ELISA: Enzyme-linked immunosorbent assay; HBcAb: Hepatitis B core antibody; HQ: High value quantitative detection; LFIA: Lateral flow immunoassay; POCT: Point-of-care test; QN: General value quantitative detection.

oassay which utilizes microparticles of carboxylate-modified polystyrene Europium III chelate as a reporter. The fluorescence is measured using a portable TRF strip reader. The test demonstrates a very low detection limit, as well as rapidity with a reported time of 15 min and cost-effectiveness[96].

QANTI-HBC AND FUNCTIONAL CURE

In NA-suppressed CHB patients undergoing PEG-IFN-α add-on treatment, Wang *et al*[97] studied qAnti-HBc and qHBcrAg as predictors for HBsAg clearance. Following at least a year of NA therapy, 74 CHB patients with HBV DNA suppression (HBV DNA < 20 IU/mL) and quantitative HBsAg (qHBsAg) < 1500 IU/mL were included. While 59 individuals got PEG-IFN-α add-on treatment, 15 patients persisted on NA monotherapy. Every 12 or 24 wk, respectively, the add-on and NA-alone groups' serum qAnti-HBc and qHBcrAg levels were measured. They discovered a negative correlation between the length of previous NA treatment and baseline blood qAnti-HBc but not qHBcrAg levels. Both qAnti-HBc and qHBcrAg levels decreased after 48 wk of therapy, and in the add-on and NA groups, 17/59 (28.81%) and 0/15 (0%) of patients, respectively, achieved HBsAg clearance. Patients in the add-on group with baseline qAnti-HBc < 0.1 IU/mL had a substantially greater percentage of HBsAg clearance (52.63%). Baseline qAnti-HBc was shown to be an independent predictor of HBsAg loss using logistic regression analysis, but not qHBcrAg. The combination of qAnti-HBc and qHBsAg exhibited a greater predictive value for HBsAg clearance, according to an examination of the ROC curve. In NA-suppressed CHB patients undergoing PEG-IFN-α add-on treatment, they determined that a combination of qHBsAg and baseline qAnti-HBc levels would be a superior prediction technique for HBsAg clearance. During chronic HBV infection, the HBeAg-negative state is associated with a wide range of clinical conditions, from inactive carriers with overall survival comparable to HBV-non-infected individuals to active chronic hepatitis with significant hepatic necroinflammatory activity and rapid progression to cirrhosis. The danger of transitioning from an inactive carrier status to an active infection is always unexpected. Current antiviral therapy may change the course of an ongoing infection by halting fibrosis development and lowering the risk of end-stage consequences. Thus, distinguishing true inactive carriers from individuals with persistently low viremic active infection (HBV DNA > 2000 IU/mL with normal ALT) and predicting the potential of HBV DNA undetectability and HBsAg seroclearance are critical. Unlike in HBeAg-positive phases, a lower level of qAnti-HBc is linked with a better clinical result in HBeAg-negative phases[98]. Wu *et al*[99] conducted a study to investigate the potential of qAnti-HBc level as a biomarker for predicting the recurrence of CHB patients who had HBsAg clearance after antiviral therapy. Sixteen instances of recurrence and fifty-seven cases of non-recurrence out of the 73 patients with HBsAg clearance were included. Before and after treatment, the qAnti-HBc level was measured using a recently developed double-sandwich immunoassay. The predictive power of qAnti-HBc levels for recurrence was assessed using logistic regression analysis. They discovered that both the recurrence and non-recurrence groups' post-treatment qAnti-HBc levels were much lower than those from before therapy. Furthermore, compared to the non-recurrence group, the recurrence group's falling trend was much larger.

In 60 cases of CHB patients treated with PEG-IFN-α2a plus NAs antiviral therapy previously, Lin *et al*[100] investigated the potential role of qAnti-HBc in predicting HBsAg clearance (41 cases in the clearance group and 19 cases in the non-clearance group). The qAnti-HBc levels of the patients were measured using the double antigen sandwich technique at baseline, 24, 48, 72, and 96 wk. When antiviral treatment was prolonged, qAnti-HBc levels in both the clearance and non-clearance groups showed a progressive downward trend. However, at baseline and at subsequent detection time points during the antiviral treatment, the levels in the clearance group were significantly higher than those in the non-clearance group. At week 24, multivariate logistic regression revealed a reduction in both the baseline qAnti-HBc level and qHBsAg. The greatest independent predictor of HBsAg clearance among them was the baseline qAnti-HBc level; its sensitivity and specificity for predicting HBsAg clearance over 3.40 Log10 IU/mL were 56.1% and 89.5%, respectively. To increase prediction accuracy, integrated predictors were constructed using the logistic regression model as a guide. The combined factor 3 had the best predictive value out of all of them. 80.5% and 78.9%, respectively, were the sensitivity and specificity. Furthermore, the HBsAg clearance predicted rate had reached 94.12%-100%, and the combined index — which

is the combination of any two or more indices based on the baseline qAnti-HBc level—had further increased the predictive value. Lou *et al*[101] conducted a study to employ biomarkers to assess HBV reinfection in patients following orthotopic liver transplantation. They enlisted 79 individuals who had liver transplants, and biomarker levels were measured at various time periods. They discovered that 42 instances had HBsAg loss with a median time of 65.2 mo following liver transplantation, while 37 patients had HBsAg recurrence with a median time of 8.8 mo. The highest Youden's index values at the start of the study were 4.25 Log10 IU/mL for qAnti-HBc and 2.82 Log10 IU/mL for qHBsAg, with areas under the curves of 0.685 and 0.651, respectively. The Kaplan-Meier technique revealed that the levels of qHBsAg and qAnti-HBc were significantly different between the two groups. Furthermore, the Cox regression model verified the predictive efficacy of qAnti-HBc at baseline. They hypothesized that lower pre-transplantation qAnti-HBc levels are linked to HBV re-infection. The baseline concentration of qAnti-HBc in patients obtaining liver transplantation is a potential predictor of HBV recurrence and may be used to guide antiviral therapy for HBV infection.

In individuals receiving antiviral therapy, baseline anti-HBc levels may be predictive of HBeAg seroconversion. A greater baseline concentration of qAnti-HBc was a robust predictor of HBeAg seroconversion in a retrospective investigation of 231 patients treated with pegylated interferon and 560 receiving NAs, outperforming both HBV DNA and ALT levels. Patients receiving NAs therapy had a much higher reduction in anti-HBc levels following treatment than those getting pegylated interferon, which might be attributed to interferons' stronger pleiotropic effects on immune activation [102]. QAnti-HBc levels at the end of treatment may also be indicative of viral recurrence after stopping NAs therapy. Indeed, after controlling for age, HBeAg status, and length of consolidation therapy, high levels of qAnti-HBc at the end of treatment were linked with a lower probability of clinical relapse. Further stratification of anti-HBc levels at the completion of therapy revealed that levels of 1000 IU/mL were linked with the lowest likelihood of clinical relapse (21% at year 4), compared to values between 100 and 999 IU/mL (50% at year 4) and 100 IU/mL (85% at year 4)[103].

CONCLUSION

Anti-HBc IgM and IgG antibodies are the most common serological markers of HBV infection. Anti-HBc IgM is positive during the inflammatory phase of the liver but turns negative during the recovery stage; hence, it may assist in identifying acute HBV infection or acute exacerbation from quiescent CHB. Anti-HBc IgG is a marker for present and prior HBV infection that may last a lifetime. The double-antigen sandwich approach was utilized to quantify anti-HBc antibodies, and the detection indicated the sum of IgG and IgM antibodies. HBsAg reveals the transcriptional activity of both cccDNA and integrated HBV DNA, and the continued seroclearance of high-sensitivity or ultra-sensitive qHBsAg may signify a complete cure of CHB to some extent. HBV antigens, particularly HBsAg, are also implicated in the immunopathogenesis of hepatitis B. Thus, HBsAg loss may dramatically improve aberrant immune function, which may simplify the clearance of remaining viruses such as cccDNA and integrated HBV DNA. Some outstanding concerns must be resolved. It is uncertain if continuous HBsAg loss indicates that cccDNA and integrated HBV DNA are fully inactive, preventing HBsAg expression, or that the majority of them are removed. Moreover, a growing number of novel medications that suppress HBV and improve host immune responses have been established in numerous clinical studies. Beyond the prognostic and diagnostic utility of qAnti-HBc levels, the actual process remains unknown. The level of qAnti-HBc is recognized as a surrogate measure of HBV-specific adaptive immune response activity. However, the increased immune response is a double-edged sword since it produces severe liver damage in an effort to eliminate the infection. HBV core antibody quantification is a novel, non-invasive biomarker that may be utilized to address a variety of diagnostic challenges. It may offer useful information on viral activity, disease advancement, and therapy responses. qAnti-HBc, like other recently used biomarkers, cannot be relied on as a single diagnostic test to address all problems. Based on existing evidence, it is clear that when paired with qHBsAg, its diagnostic and prognostic efficacy may be considerably increased. More studies with larger and more diverse patient groups are needed to evaluate the true utility of these biomarkers.

FOOTNOTES

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REVIEW

Molecular mechanism of nanomaterials induced liver injury: A review

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Abstract

The unique physicochemical properties inherent to nanoscale materials have unveiled numerous potential applications, spanning beyond the pharmaceutical and medical sectors into various consumer industries like food and cosmetics. Consequently, humans encounter nanomaterials through diverse exposure routes, giving rise to potential health considerations. Noteworthy among these materials are silica and specific metallic nanoparticles, extensively utilized in consumer products, which have garnered substantial attention due to their propensity to accumulate and induce adverse effects in the liver. This review paper aims to provide an exhaustive examination of the molecular mechanisms underpinning nanomaterial-induced hepatotoxicity, drawing insights from both *in vitro* and *in vivo* studies. Primarily, the most frequently observed manifestations of toxicity following the exposure of cells or animal models to various nanomaterials involve the initiation of oxidative stress and inflammation. Additionally, we delve into the existing *in vitro* models employed for evaluating the hepatotoxic effects of nanomaterials, emphasizing the persistent endeavors to advance and bolster the reliability of these models for nanotoxicology research.

Key Words: Nanoparticles; Hepatotoxicity; Oxidative stress; Inflammation; Autophagy

Core Tip: This comprehensive review explores nanoparticle-induced hepatotoxicity, focusing on diverse nanomaterials (*e.g.*, silver nanoparticles, carbon nanotubes) and their impacts on hepatic function. It categorizes nanoparticles, discusses exposure routes, and highlights hepatotoxic mechanisms. The review emphasizes the need for comprehensive assessments, understanding, and responsible practices in nanotechnology to guide future research for the development of safer nanomaterials.

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INTRODUCTION

In the rapidly advancing field of nanotechnology, the utilization of nanomaterials has become widespread across various industries, promising groundbreaking applications in medicine, electronics, and environmental science. Among the myriad potential benefits, the unique physicochemical properties of nanoparticles (NPs) have enabled remarkable achievements, from targeted drug delivery systems to innovative diagnostic tools. However, this surge in nanomaterial applications has brought forth concerns regarding their safety, particularly in the context of hepatotoxicity[1]. This comprehensive review aims to delve into the intricate landscape of nanoparticle-induced hepatotoxicity, exploring the diverse range of nanomaterials and their impacts on hepatic function. We will navigate through recent findings on prominent nanomaterials, including silver nanoparticles, carbon nanotubes, quantum dots, and gold nanoparticles, shedding light on the complex mechanisms underlying their hepatotoxic effects[2-4]. By examining the interplay between nanoparticles and liver cells, such as hepatocytes and Kupffer cells, this review seeks to provide a nuanced understanding of the potential risks associated with nanomaterial exposure.

NPs are classified into four main groups based on structural morphology: organic, inorganic, carbon-based, and composite[1,5]. Organic nanoparticles, derived from compounds like proteins and lipids, exhibit non-toxic and biodegradable properties, making them suitable for drug delivery, imaging, biosensors, and cancer treatment[5,6]. Inorganic nanoparticles, including metal-based, metal oxide-based, ceramic, and semiconductor nanoparticles, offer tailored electrical, optical, and magnetic properties for applications in biomedical science, catalysis, and imaging[5,7]. Quantum dots, semiconductor nanoparticles with size-dependent optoelectronic properties, find applications in electronic and biomedical industries[8,9]. Carbon-based nanoparticles, such as graphene, fullerenes, and carbon nanotubes, demonstrate unique structural configurations and are utilized in electrical and photonic devices, biomedical sciences, and nanocomposites[10,11]. Composite nanoparticles integrate different components, leading to unique physical and chemical properties, with three main categories: simple hybrid, core or shell structured, and multifunctional quantum nanoparticles, applied in electronics, optoelectronics, and biomedical sciences[12].

To ensure the safety of NPs within the human body, understanding their exposure route is crucial[13-15]. NPs can be orally exposed through food, drinks, supplements, or nanomedicines, with absorption occurring in organs like the stomach and small intestine. Factors like size, charge, and concentration influence absorption, with NPs under 100 nm diameter taken up directly through endocytosis in the small intestine. Inhalation is another exposure route, with NPs deposited in different regions of the respiratory tract, potentially translocating to other organs. Elimination of NPs from the lungs is complex and depends on physicochemical properties. Dermal exposure, through cosmetics and medications, is facilitated by the skin's permeability to nanoscale particles. Skin penetration varies based on factors like particle size and skin condition. Overall, understanding exposure routes is vital for assessing NP-induced toxicity and ensuring their safe utilization.

Various NPs exert hepatotoxic effects, with silica nanoparticles (SiNPs) showing size-dependent liver injury, synergies with other toxins, and impacts on cholesterol biosynthesis. Nickel oxide nanoparticles (NiO-NPs), tungsten trioxide nanoparticles (WO3 NPs), and copper oxide nanoparticles (Nano-CuO) induce oxidative stress-related liver damage, apoptosis, and genotoxicity[2,16-18]. Integrative omics analyses identify key proteins and disrupted metabolic pathways in SiNP-induced hepatotoxicity[19]. Zinc oxide (ZnO-NPs), titanium dioxide (TiO2NPs), magnesium oxide (MgO-NPs), aluminum oxide (Al_2O_3NPs), chromium oxide (Cr_2O_3-NPs), and iron oxides (IONPs) exhibit diverse hepatotoxic mechanisms, including oxidative stress, endoplasmic reticulum (ER) stress, inflammation, and disruptions in metabolism [20-24] (Figures 1 and 2). Carbon nanotubes (CNTs) induce hepatotoxicity through inflammatory responses and oxidative stress, with variations in toxicity based on type and administration method[4]. Copper sulfide/cadmium sulfide nanoparticles (CuS/CdS-NPs), cobalt nanoparticles, and nanoclay particles also induce oxidative stress-mediated apoptosis and acute hepatotoxicity[25,26]. Various nanomaterials, such as nanocellulos, polystyrene nanoparticles, chitosan nanoparticles, hydroxyapatite nanoparticles, quantum dots, and gold nanoparticles, display hepatotoxicity through disrupted redox balance, altered metabolism, necrotic cell death, and impaired mitochondrial function[8,9,27-29]. The complexity of nanoparticle-induced hepatotoxicity highlights the need for comprehensive assessments and understanding for safe use.

In conclusion, this review not only synthesizes existing knowledge but also highlights critical gaps in understanding nanoparticle-induced hepatotoxicity. By proposing recommendations for future research, we aim to guide the scientific

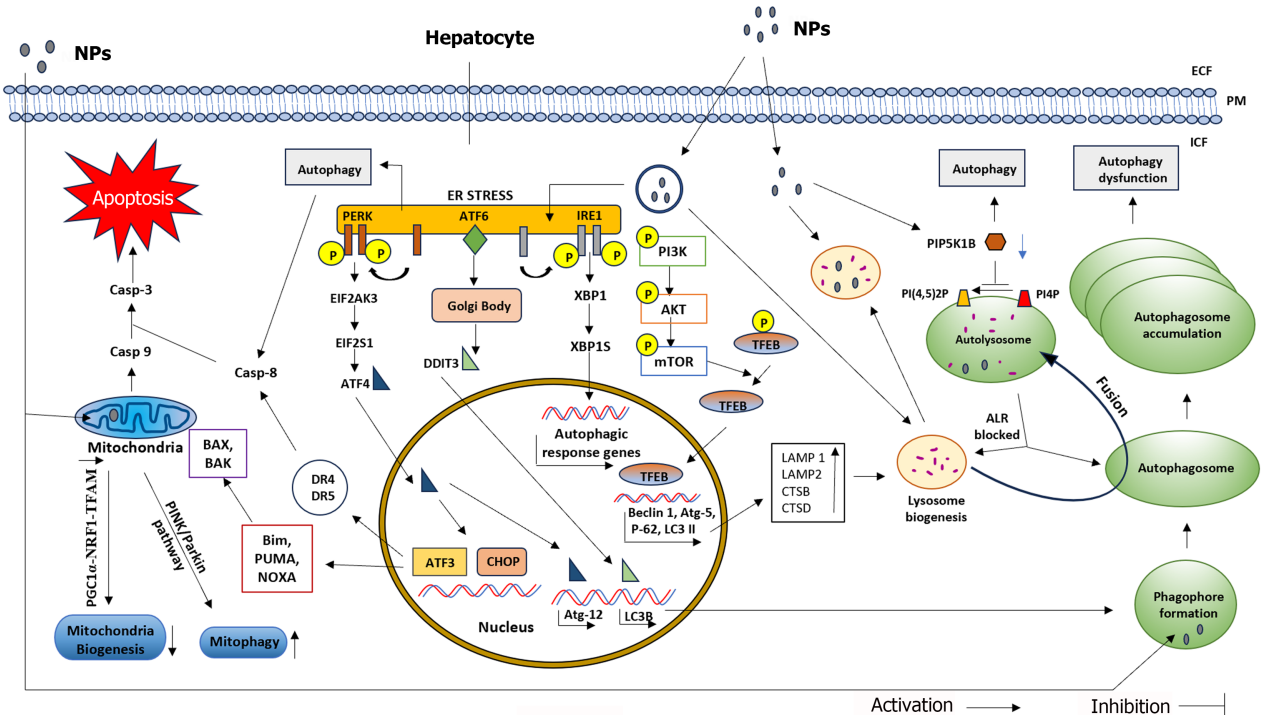

Figure 1 Diagram showing NPs induced hepatotoxicity through crosstalk between endoplasmic reticulum stress, oxidative stress, autophagic and apoptotic pathways. AKT: Protein kinase B; ALR: Autophagic lysosome reformation; ATF 3/4/6: Activating transcription factor 3/4/6; Atg 5/12: Autophagy related gene 5/12; BAK: Bcl-2 homologues antagonist/killer; Bax: Bcl-2-associated X-protein; Bim: Bcl-2 interacting mediator of cell death; Casp 3/8/9: Caspase 3/8/9; CHOP: C/EBP Homologous Protein; CTSB: Cathepsin B; CTSD: Cathepsin D; DDIT3: DNA damage inducible transcript 3; DR: Death receptor; ECF-Extra cellular fluid; EIF2AK3: Eukaryotic translation initiation factor 2-alpha kinase 3; EIF2S1: Eukaryotic translation initiation factor 2 subunit 1; ICF-Intra cellular fluid; IRE1: Inositol-requiring enzyme type-1; LAMP1/2: Lysosome-associated membrane protein 1/2; LC3B-Microtubule-associated proteins 1A/1B light chain 3B; LC3II: LC3-phosphatidylethanolamine conjugate; mTOR: Mammalian target of rapamycin; NOXA: Phorbol-12-myristate-13-acetate-induced protein 1; NPs- Nanoparticles; NRF1: Nuclear factor erythroid 2-related factor 1; P 62-Ubiquitin-binding protein p62; P: Phosphate; Parkin-Parkin RBR E3 ubiquitin-protein ligase; PERK: Protein kinase RNA like endoplasmic reticulum kinase; PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PI(4,5)2P: Phosphatidylinositol 4,5-bisphosphate; PI3K: Phosphatidylinositol 3-kinase; PI4P: Phosphatidylinositol 4-phosphate;PINK: PTEN induced kinase; PIP5K1B: Phosphatidylinositol-4-phosphate 5 kinase type 1 beta; PM: Plasma membrane; PUMA- p53 upregulated modulator of apoptosis; TFAM: Mitochondrial transcription factor A; TFEB: Transcription factor EB; XBP1/1S: X box binding protein-1/1S.

community toward developing safer nanomaterials and fostering responsible practices in nanotechnology. As the field continues to evolve, this exploration into nanotoxicology endeavors to contribute to the ethical and sustainable advancement of nanotechnology.

MAJOR TYPES AND APPLICATIONS OF NANOPARTICLES

Nanoparticles are categorized into four groups based on structural morphology: Organic, inorganic, carbon-based, and composite. Some of the most important types of nanoparticles are listed below:

Organic nanoparticles

Organic nanoparticles, derived from compounds like proteins, carbohydrates, lipids, and polymers, encompass micelles, dendrimers, liposomes, nanogels, polymeric NPs, and ferritin[6]. Generally non-toxic and biodegradable, they may have a hollow core, such as liposomes, and are sensitive to thermal and electromagnetic radiation. Formed through non-covalent interactions, these labile organic NPs are easily cleared from the body. Nanospheres or nano-capsules, common polymeric forms, collectively referred to as labeled polymorphic NPs, possess properties like a high surface area to volume ratio, stability, inertness, ease of functionalization, and unique optical, electrical, and magnetic behaviors, making them suitable for applications in drug delivery, imaging, biosensors, and cancer treatment[30].

Inorganic nanoparticles

Inorganic nanoparticles, devoid of carbon atoms, are hydrophilic, non-toxic, and biocompatible, providing high mechanical strength and stability. Precise control over size, shape, and composition allows researchers to design nanoparticles with tailored electrical, optical, and magnetic properties for targeted biomedical applications[5,7].

Metal-based nanoparticles: Metal-based nanoparticles, derived from various metals through disruptive or constructive methods and typically ranging in size from 10 to 100 nm, including aluminum (Al), cadmium (Cd), cobalt (Co), copper

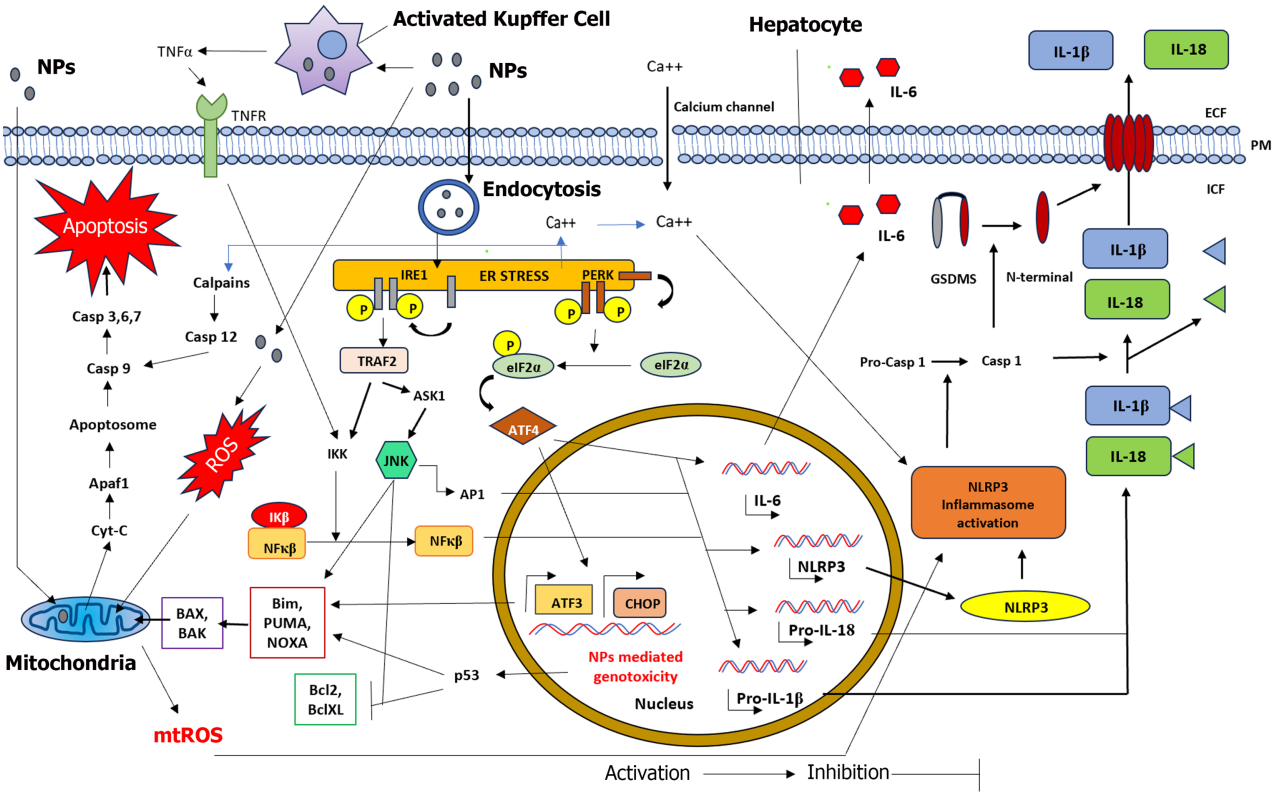

Figure 2 Diagram showing nanoparticles induced hepatotoxicity through inflammatory pathway and its crosstalk with oxidative stress, endoplasmic reticulum stress and apoptotic pathways. AP1: Activator protein 1; Apaf-1: Apoptotic peptidase activating factor 1; ASK1: Apoptosis signal-regulating kinase 1; ATF3/4: Activating transcription factor 3/4; BAK: Bcl-2 homologues antagonist/killer; Bax: Bcl-2-associated X-protein; Bcl2: B-cell lymphoma 2; BclXL: B-cell lymphoma-extra-large; Bim: Bcl-2 interacting mediator of cell death; Ca++: Calcium ion; Casp 1/3/6/7/9/12: Caspase 1/3/6/7/9/12; CHOP: C/EBP Homologous Protein; CytC: Cytochrome C; eIF2α: Eukaryotic initiation factor 2 alpha; GSDMS: Gasdermins; IKK: IκB kinase; Iκβ: Inhibitor of nuclear factor kappa beta; IL-6/18: Interleukin 6/18; IL-1β: Interleukin 1 β; IRE1: Inositol-requiring enzyme type 1; JNK: Jun N-terminal kinase; mtROS: Mitochondrial reactive oxygen species; NFkβ: Nuclear factor kappa beta; NLRP3: NOD-like receptor protein 3; NOXA: Phorbol-12-myristate-13-acetate-induced protein 1; NPs- Nanoparticles; p53: Tumor suppressor protein p53; PERK: Protein kinase RNA like endoplasmic reticulum kinase; Pro-Casp 1: Pro- Caspase 1; Pro-IL-18: Pro- Interleukin 18; Pro-IL-1β: Pro- Interleukin 1 β; PUMA- p53 upregulated modulator of apoptosis; ROS: Reactive Oxygen Species; TNFR: Tumor necrosis factor receptor; TNFα: Tumor necrosis factor alpha; TRAF2: TNF receptor associated factor 2.

(Cu), gold (Au), iron (Fe), lead (Pb), silver (Ag), and zinc (Zn), exhibit unique optoelectrical properties due to localized surface plasmon resonance[31,32]. Specifically, alkali and noble metals like Cu, Ag, and Au, when utilized in nanoparticle construction, show significant absorption in the visible region of the solar spectrum[33]. The synthesis of metal nanoparticles with specified facets, sizes, and forms necessitates controlled conditions, and their advanced optical properties make them versatile across various research domains[5,34]. These nanoparticles, distinguished by their small dimensions and surface properties, including pore size, surface charge, *etc.*, find applications in biomedical science, such as cancer treatment, disease diagnostics, radiation enhancement, drug delivery, and gene transport[35].

Metal oxide based nanoparticles: Metal oxide nanoparticles result from modifying the properties of metal-based nanoparticles. These nano-scale metal oxides find diverse applications in fluorescence, optical sensors, catalysts, biomedicine, gas sensors, and fuel cell anode materials[22,36-38]. Various synthesis methods, including inert gas condensation, co-precipitation, and lithography, have been used, but traditional methods often lack control over morphological structure, affecting essential nanomaterial properties[39,40]

Ceramic nanoparticles: Ceramic nanoparticles, resistant to environmental stresses, form with a solid core through heat or a combination of heat and pressure, incorporating metallic or non-metallic elements[41,42]. Typically composed of inorganic compounds like silica or alumina, they may also include metals and metal oxides, yielding diverse nano molecules with varying shapes, sizes, and porosities. Engineered to evade the reticuloendothelial system, ceramic NPs undergo size and surface composition modifications[43]. Widely used in medical applications, ceramics such as calcium phosphates, alumina, silica iron oxides, carbonates, and titanium dioxide have been found[44].

Ceramics also play an important role in various applications in photocatalysis, dye photodegradation, imaging, and catalysis[45]. Researchers aim to develop advanced ceramics with minimal cytotoxicity and enhanced biocompatibility, addressing challenges through innovative strategies that integrate ceramic nanoparticles with biocompatible materials, considering characteristics like shape, size, and physicochemical attributes[46].

Lipid-based nanoparticles: Lipid-based nanoparticles (LBNPs), typically 10-100 nm in diameter, consist of a lipid core surrounded by lipophilic molecules, finding applications in oncology and biomedicine[47]. Liposomes, a key type of LBNP, use a phospholipid bilayer for enhanced drug solubility and stability, accommodating both hydrophobic and hydrophilic molecules. Incorporating cholesterol improves stability, decreases fluidity, and enhances permeability for hydrophobic drugs in liposomal formulations[48]. Solid lipid nanoparticles, sized between 50-1000 nm, and composed of physiological lipids in a solid state, offer a compelling alternative for drug delivery, featuring a matrix of mono-, di-, or triglycerides, fatty acids, and complex glyceride mixtures, with stability ensured by surfactants or polymers[49].

Semiconductor nanoparticles: Semiconductor nanoparticles, possessing hybrid characteristics of metals and nonmetals, have garnered attention for their versatility in diverse applications[50,51]. Their crucial broad bandgap, adjustable by researchers, makes them valuable in photocatalysis, photo optics, and electronic devices[52]. Additionally, their nano-scale dimensions provide benefits such as increased surface area-to-volume ratio, enhanced quantum confinement effects, and improved catalytic activity, contributing to exceptional performance in various applications[53].

Quantum dots

Quantum dots (QDs), semiconductor nanoparticles with size- and composition-dependent optoelectronic properties (1.5 to 10.0 nm), play a significant role in the electronic and biomedical industries[8]. Their success is attributed to superior features like photostability, size-dependent optical properties, high extinction coefficient, brightness, and a large Stokes shift, overcoming limitations of organic dyes. QDs, due to their ultrasmall size, are well-suited for imaging and biosensing applications. They facilitate the development of multimodal/multifunctional probes with increased surface area for optical trackability *in vitro* and in vivo, designed to detect pH, metal ions, DNA, and enzyme activity, and deliver various therapeutics[8].

Carbon-based nanoparticles

Carbon-based nanoparticles encompass five main materials: carbon nanotubes, graphene, fullerenes, carbon nanofiber, and carbon black, each with unique structural configurations and diverse applications in nanotechnology.

Graphene: Graphene, a two-dimensional carbon allotrope, is a single layer of carbon atoms arranged in a hexagonal lattice with exceptional properties, such as elasticity, mechanical strength, and unparalleled thermal and electrical conductivity. Synthesized in the laboratory, it forms a 1nm-wide honeycomb lattice, exhibiting semiconductor properties without an effective mass and zero band gap. Graphene demonstrates an ambipolar electric field effect, with a breaking strength of 42 Nm^{-1} and a Young's modulus of approximately 1.0, making it the strongest material ever tested. These attributes position graphene as a promising material for electrical and photonic devices, sensing platforms, and clean energy applications[5].

Fullerene: Fullerenes, a molecular form of carbon allotrope, consists of Cn clusters (n > 20) arranged on a spherical surface with carbon atoms at pentagon and hexagon vertices[54]. The extensively studied C60 fullerene, composed of 60 carbon atoms, is highly symmetric and spherical, with a 0.7 nm diameter and sp2 hybridized carbon atoms. Exhibiting exceptional symmetry and stability, fullerenes have 20 tripled axes, 12 fivefold axes, and 30 twofold axes[54]. These unique properties position fullerenes as promising nanoparticles widely utilized in biomedical sciences, acting as inhibitors for human immunodeficiency virus, contrast agents for magnetic resonance imaging, and sensitizers for photodynamic therapy[5,55].

Carbon nanotubes: CNTs, unique in carbon-based nanomaterials, possess versatile characteristics like length, diameter, chirality, and layer number, showcasing exceptional properties and widespread applications. Composed of graphite, CNTs, typically with at least two layers and an outer diameter ranging from 3 nm to 30 nm, are divided into two categories: single-walled nanotubes (SWCNTs) and multi-walled nanotubes (MWCNTs). SWCNTs, with a diameter of around 1 nm, exhibit high electrical conductivity, mechanical strength, and thermal conductivity due to their nearly one-dimensional structure, indicated by a length-to-diameter ratio of approximately 1000[56]. MWCNTs, robust cylindrical structures with a minimum diameter of 100 nm, demonstrate resilience and diverse structures rooted in graphene sheets, with an interlayer distance resembling that in graphite, about 3.3 Å. The initial proposal for gram-scale synthesis of double-walled carbon nanotubes in 2003 involved chemical vapor deposition, selectively reducing oxide solid solutions in methane and hydrogen[10,56-58]. Applications of CNTs include bicables, AFM tips, hydrogen storage, electrochemical electrodes, nanocomposites, field emission displays, and diverse electrical devices[59].

Composite nanoparticles

Composite nanoparticles are produced *via* the integration of two or more different components. The components bear different properties at the nanoscale level. This integration of diverse components eliminates the limitation of individual components which enables researchers to produce nanomaterials with specific properties and uses. These NPs exhibit unique physical and chemical properties and each component has strong mutual coupling effects on the other. The chemical properties of composite nanoparticles depend on their composition and structure. The mutual coupling effect between the components of composite NPs can lead to changes in the chemical properties of composite NPs[12]. Composite NPs are used in a variety of applications including electronics, optoelectronics, and biomedical sciences[60].

Composite nanoparticles can be classified into three main categories based on their structural features:

Simple hybrid NPs: These types of composite NPs formed by combining two or more components without a specific structural hierarchy. They exhibit unique properties due to the combination of different materials[61].

Core or shell structured composite NPs: These NPs are made up of two different regions: an inner core region and an outer shell. These two regions of NPa are composed of two or more different materials. The core and shell structure influences the properties of the nanoparticles, such as electromagnetic wave attenuation capacity, *etc*[62].

Multifunctional quantum NPs: These NPs have multiple functionalities, such as magnetooptical, and electrochemical properties. The specific structure of Multifunctional Quantum Composite NP is used in applications like biosensing, bioassays, catalysis, and separations[12].

EXPOSURE ROUTES OF NANOPARTICLES

A myriad number of nanoparticles are manufactured from diverse materials to serve a multitude of purposes, it is crucial to ensure the unswerving safety of these particles within the human body. To understand the degree and mechanism of nanoparticle-induced toxicity, it is essential to understand their route of exposure, toxicological profile, and fate in the human body. The route of exposure also acts as a crucial factor in deciding the potential toxicity of NPs. The potential routes of NP exposure are as follows:

Oral exposure

Oral Exposure of NPs occurs following intake of food, drinks, or additives and supplements containing NPs, swallowing of inhaled NPs, or oral administration of nanomedicines or nano-formulations. These particles are then passed through the following organs esophagus, stomach, small intestine, and large intestine, and are readily absorbed in the stomach epithelial cells[13,14,63].

However, the absorption rate of NPs depends on multiple factors such as shape and size, concentration, pH of the medium, *etc.* The size and charge of the NPs also influence the absorption rate; positively charged NPs were captured through negatively charged mucus, whereas, negatively charged nano-molecules easily entered the mucus layer. Particle size also plays a crucial role because larger NPs required more for ingestion as well as digestion[64,65]. It has been observed that NPs lower than 100 nm diameter, are directly taken up by endocytosis through regular epithelial cells of the small intestine[66,67]. Absorption can also occur through epithelial cells of Peyer's patches in the gut-associated lymphoid tissue. Some other research studies proposed that oral intake of NPs could be absorbed in the gastrointestinal tract, from where the particles can transmigrate to the liver and spleen *via* lymph nodes.

Inhalation

Nanoparticles have been observed to exert their effect on human health, primarily *via* dermal contact or inhalation. The NPs inhaled during production or usage, get deposited all over the respiratory tract and the smaller particles penetrate the lungs where they accumulate in the alveolar regions. The larger NPs with diameters ranging from 5-30 μm usually reside in the nasopharyngeal region and the smaller particles, with diameters ranging from 1-5 μm tend to deposit in the tracheobronchial region. The smallest NPs (0.1–1 μm) deposited over the alveolar region[68,69]. The particles smaller than 10mm are primarily absorbed inside the lung and may undergo translocation to various parts of the body including the kidney. Insoluble particles accumulated in the lung have the potential to trigger diverse local toxicological reactions. The smaller NPs easily translocated compared to the bigger ones, and after reaching the lung they can remain there for years and can make their way into the circulatory or lymphatic system and subsequently disseminate into other organs like the liver, spleen, and kidneys[15].

The elimination procedure of NPs is very complex and lengthy and depends on its physicochemical properties. The larger particles which are deposited at the extra-thoracic and intrathoracic bifurcation, have been trapped in the mucus layer and transported through the mucociliary escalator into the pharyngeal region. These mucus-laden NPs are then swallowed and enter into the gastrointestinal tract for further processing. The smaller particles in bronchioles and alveoli undergo mucus-associated transport and are then phagocytosed by alveolar macrophage. However, if these strategies are unable to reduce the toxicity, the lung defense system becomes stronger and eventually causes lung tissue damage[11,26,70].

Dermal exposure

Skin, the largest organ and primary protective barrier of the human body acts as the easiest route of NP entrance. The skin is divided into three layers: epidermis, dermis, and hypodermis While the epidermis effectively prevents the entry of micrometer-sized particles, but less effective as a barrier for particles in the nanoscale range. Dermal exposure to nanoparticles is unavoidable with the use of various cosmetics and medications. Several experimental investigations examined the feasibility of nanoparticle penetration through the skin barrier and reported that NPs are unable to traverse the skin whereas, contrasting findings from other studies, specifically those focused on metal NPs such as iron NPs, reported that they can successfully penetrate through hair follicles and ultimately reached to the basal and spinous layers [71,72] The epidermal entry of NPs is influenced by a variety of factors such as exposure medium, medium pH, temperature, *etc*[13,14,63].

The existing evidence suggests that NPs with a diameter of about 4 nm can permeate intact skin whereas, when the size grows up to 45 nm, NPs can only permeate *via* impaired or injured skin[73]. Beneath the dermal layer rich with blood vessels, macrophages, lymph vessels, dendritic cells, and nerve endings. Consequently, particles absorbed beneath distinct layers of the skin undergo swift transport within diverse circulatory systems[1].

NPS MEDIATED HEPATOTOXICITY

Silica nanoparticles

A series of investigations revealed that the administration of silica nanoparticles with smaller diameters (30 nm) exhibited more liver injury or lethality compared to larger ones (1000 nm)[2,74,75]. Suggesting an inverse correlation between the silica nanoparticle size and hepatotoxicity. Also in combinatorial toxicity assessment, SP30 (30 nm), the smallest NPs was found to synergize the other known chemical liver toxins (carbon tetrachloride, paraquat, cisplatin) in causing hepatic damage[75]. In another study, increased biodistribution with reduced urinary excretion was observed for lower aspect ratio of mesoporous silicon nanoparticles[76]. In an *in vitro* study when four amorphous SiNPs with different surface areas were applied on HepG2 cells, a clear perturbation in cholesterol biosynthesis was observed. Increased cholesterol biosynthesis was found to be directly proportional to the increased surface area, which might have an impact on steroidogenesis and bile formation[19]. In a metabolomic study, the same group demonstrated amorphous SiNPs mediated depletion of glutathione, NADPH oxidase mediated reactive oxygen species (ROS) production, and alterations in antioxidant profile indicating perturbation of glutathione metabolism and glutathione pool in hepatocytes[77].

In a dose and time-dependent manner, mesoporous SiNPs (MSN) caused cytotoxicity in L-02 cells. In NLRP3 knockout mice and caspase-1 knockout mice model, MSN-promoted inflammation and hepatotoxicity were found to be abolished compared to the normal mice, suggesting mSiNPs mediated ROS overproduction followed by activated NOD-like receptor protein 3 (NLRP3) inflammasome, resulting into pyroptosis through caspase-1 activation[78]. Rat exposed to silica NPs compared to control exhibited altered liver biochemical parameters such as elevated levels of low-density lipoproteins (LDL), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanina aminotransferasa (ALT) along with procalcitonin, iron, phosphorus, and potassium concentration. Histological modifications include Hydropic degeneration, Karyopyknosis, Sinusoidal dilatation, Hyperplasia of Kupffer cells, and infiltration of inflammatory cells with lowered liver index. Also negatively affects the expression of phase I and phase II drug metabolizing and drug transporter genes (*slc2a1, cyp4a12, ephx2, nat2*)[79].

Kupffer cells are well-known resident macrophages of the liver, contributing to the maintenance of liver normal physiological activity and homeostasis. Excessive accumulation of ROS and simultaneous release of bioactive mediators (H_2O_2, NO, and TNFα) indicates SiO_2NPs mediated activation and hyperplasia of KCs. BRL cells exhibited reduced viability, and structural alterations along with elevated levels of marker enzymes [lactate dehydrogenase (LDH), AST] when co-cultured (contactless) with SiNPs activated KCs, clearly suggesting that KCs activated by SiO_2NPs can cause liver injury *via* the release of H_2O_2, NO, and TNFα. In addition to that, infiltration of inflammatory cells and subsequent increase of TNFα, monocyte, lymphocytes, and neutrophils in the liver can be correlated with SiNPs activated KCs mediated inflammation in the liver[80].

Analysis of ^{1}H nuclear magnetic resonance (^{1}H NMR) results, unveiled lipid metabolism disorder in rats receiving intratracheal instillation of SiNPs causing hepatotoxicity in a dose-dependent manner. Biochemical analysis showed a significant increase in ALT, AST, triglyceride (TG), and LDL-C levels but a decrease in HDL-C levels in the treated group. Ten metabolic pathways were affected due to treatment, including the metabolism of amino acids (glutamate, cysteine, aspartate), purines, and glucose-alanine cycle that resulted in the production of 11 different metabolites compared to control[81].

Autophagy-mediated liver toxicity involves autophagic lysosomal reformation (ALR) an event where anomalous autophagy fails to terminate, which results in a persistent accumulation of enlarged autolysosomes. Mouse hepatocytes on exposure to SiO_2NPs prevent conversion of PI(4)P to PI(4,5)P2 on enlarged autolysosomal membrane due to loss of PIP5K1B, also clathrin fails to be recruited, leading to suppression of ALR and resulted into enlarged autolysosomes[82]. The molecular mechanism behind SiNp-induced autophagosome synthesis, accumulation, and autophagic dysfunction was worked out on L-02 cells. When treated with different concentrations of SiNPs, readily get internalized and induce ROS production, which in turn causes ER stress and UPR. Upregulated expressions of ATF4 and DDIT3 indicate involvement of EIF2AK3 and ATF6 pathway but not ERN1-XBP1 pathway. ATF4 and DDIT3 then transcriptionally upregulate expressions of LC3B and ATG12 (autophagic genes) that result in autophagosome formation[83]. In HepG2 cells accumulation of amorphous SiNPs in mitochondria leads to excessive ROS generation that in turn triggers autophagy and autophagic cell death in hepatocytes *via* the phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase/mammalian target of rapamycin (mTOR) pathway[84].

Overexpression of p53, bax, and caspase-3 in contrast to bcl-2 downregulation along with ROS generation in HepG2 cells insulted with SiNPs suggests activation of cell cycle check point genes and apoptotic pathway in accordance to cytotoxicity due to oxidative stress. Restoration of cell viability with an altered apoptotic marker profile was observed in the same cell when co-treated with vitamin C, a ROS scavenger[85]. Amorphous SiNPs exposure to human cells (HL-7702) and rat cells (BRL-3A) showed elevated expression of p53, Bax, cleaved caspase-3, and negative expression of Bcl-2 and caspase-3 levels, with increased ROS generation and decreased GSH level indicating oxidative stress-mediated cytotoxicity that leads to apoptotic activation *via* p53/casp-3/Bax/Bcl-2 pathway. Human liver cells exhibited more sensitivity than rat liver cells[86].

Compared to normal mice, SiNPs exhibited more severe effects in the liver of metabolic syndrome mice though improved insulin resistance. It has been established that SiNP exposure can accelerate liver damage in metabolic syndrome mice following deposition to mitochondria which results in mitochondrial injury and overproduction of ROS. That aggravated liver fibrosis (higher collagen deposition), hepatic ballooning, DNA damage (genotoxicity), and infiltration of inflammatory cells[87]. A recent study reveals SiNPs induced hepatotoxicity *via* perturbating mitochondrial quality control (MQC) process, promoting excessive mitochondrial fission (DRP1, FIS1, and MFN2 were up-regulated under SiNPs exposure, but MFN1 was down-regulated), mitophagy disorder (PINK/Parkin signaling, up-regulated PINK1 and *p*-Parkin, as well as an enhanced conversion of LC3B-I to LC3B-II) and downregulating mitochondrial biogenesis (inhibited mitochondrial biogenesis *via* PGC1α-NRF1-TFAM signaling, decline PGC1α, NRF1 and TFAM), leading to mitochondrial dysfunction followed by hepatocyte damage and liver biotoxicity[88]. From the above findings, it can be speculated that mitochondrial injury & instability in hepatocytes due to SiNP exposure resulted in liver oxidative stress.

Recent *in vitro* as well as *in vivo* investigation results indicate silicon NP insult can trigger LDH, ALT, and AST in serum concentration owing to hepatic damage. Compromised antioxidant enzyme profile [catalase (CAT), SOD, and GPx] with elevated levels of oxidative stress markers [NO, malondialdehyde (MDA), PCO, and H_2O_2] and MDA levels are engaged in hepatic ROS production[89]. Altered hepatic metabolism is observed in both free fatty acid - treated L-O2 cells and ApoE$^{-/-}$ mice model receiving SiNps treatment. Increased fatty acid biosynthesis, lipid deposition, liver total cholesterol/TG index along with decreased β-oxidation and lipid efflux resulting into perturbated lipid metabolism can be corroborated with the induction of oxidative stress-related liver injuries, may help the acceleration of liver diseases like metabolic associated fatty liver disease[90]. More over-upregulated expressions of pro-apoptotic genes (Bax, p53, Caspase-9/3) and downregulated anti-apoptotic genes Bcl-2 along with histopathological alterations of the liver such as sinusoidal dilatation, Kupffer cell hyperplasia, infiltration of inflammatory cells strongly indicates SiNPs induced hepatic toxicity *via* ROS-activated caspase signaling pathway, leading to induction of apoptosis in the liver[89]. Through integrative proteomic and metabolomic analyses, Zhu *et al*[91] identified key proteins (RPL3, HSP90AA1, SOD, PGK1, GOT1, PNP) indicative of abnormal protein synthesis, oxidative stress, and metabolic dysfunction in SiNP-induced hepatotoxicity. Metabolomic data revealed disruptions in vital metabolites [glucose, alanine, GSH, CTP, adenosine triphosphate (ATP)]. Bioinformatic analysis highlighted disturbances in glucose and amino acid metabolism, suggesting potential exacerbation of oxidative stress and liver injury. Key proteins associated with SiNP-induced hepatotoxicity include SOD, TKT, PGM1, GOT1, PNP, and NME2[91]. This study underscores the power of integrative omics analyses for nanoparticle toxicity assessments. Follow Table 1 for a comprehensive account.

Metal oxides nanoparticles

Consult Tables 2-10 for a comprehensive account of different metal oxide-induced hepatotoxicity.

Nickel oxides nanoparticles: The findings of several stress assays, liver function tests, and histopathology analyses make it abundantly evident that rats given NiO-NPs experience nitrative stress and oxidative stress-related liver damage[92, 93]. Chang *et al*[16] demonstrated that the liver cells of rats injected with NiO underwent ER stress and that this brought about the induction of apoptosis *via* many routes, including the PERK/eIF-2α, IRE-1α/XBP-1S, and caspase-12/-9/-3 pathways. A different investigation using a comparable experimental design found that the nuclear factor kappa B (NF-κB) signaling pathway is associated with hepatotoxicity[94].

In the HepG2 cells model, NiONPs caused cytotoxicity through ROS production and Bax/Bcl-2 pathway-mediated apoptotic induction. Also treated cells exhibited micronuclei formation, chromatin condensation, and DNA damage suggesting NiONPs mediated genotoxicity[95]. NiO was additionally found to induce hypoxic stress in the same human liver cells in a concentration-dependent manner, as evidenced by the activation of hallmark candidate genes, hypoxia-inducible transcription factor-1α (HIF-1α), and miR-210 microRNA and decreased levels of ribosome biogenesis. Nitric oxide (NO) levels that were too high caused Ca++ influx, which in turn led to mitochondrial instability and oxidative stress, further encouraging lysosomal degradation in connection with autophagic processes. Subsequently led to the development of apoptosis *via* the p53 and MAPKAPK-2 signaling pathways[96]. Rat liver and HepG2 cells under Nano-NiO exposure resulted in hepatic fibrosis. Upregulation of transforming growth factor 1 beta (TGF-β1), Smad2, Smad3, alpha-smooth muscle actin (α-SMA), matrix metalloproteinase 9 (MMP9), tissue inhibitors of metalloproteinase1 but simultaneously downregulation of E-cadherin and Smad7 in both models can be corroborated with hepatic fibrosis *via* activation of TGF-β1/Smad pathway, epithelial-mesenchymal transition (EMT), reformation and deposition of extracellular matrix[97]. A recent study reported NiNps-mediated hepatic injury following hepatic inflammatory response, ER stress, abnormal lipid metabolism that leads to hepatocyte apoptosis[98]. NiNPs exposure (15-45 mg/kg) in rats induced dose-dependent liver dysfunction, histological injuries, and oxidative stress. Elevated NF-kβ, nitrative stress markers, and inflammatory and apoptotic mediators were observed. The study highlights Ni NPs-induced hepatotoxicity, crucial for health risk assessment[99].

Tungsten trioxide nanoparticles: WO3 nanorods of varying lengths have been shown to cause hepatotoxicity in mice when given intraperitoneally. This effect is evident in the form of hepatocytic lesions, which include cellular edema, nuclear pyknosis in most hepatocytes, cytoplasmic vacuolation, and hydropic degeneration in hepatocytes surrounding the central vein. Additionally, liver function is impaired, as evidenced by elevated levels of serum ALT and AST, which are caused by oxidative stress (increased intracellular ROS, significant reduction in GSH and SOD activity), as well as an inflammatory response [increased NF-κB, tumor necrosis factor alpha (TNF-α), IFN-γ, and interleukin (IL)-4]. Shorter nanorods showed greater toxicity than longer nanorods in terms of severity. Adversity of WO3 nanorod was decreased by melatonin administration[17].

Table 1 Effects and molecular mechanisms underlying SiO_2NPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & Mechanism	Ref.
SiO_2NPs	15 nm (TEM)	HepG2 cell	1-200 ug/mL for 72hrs.	Bcl-2, GSH, Cell viability (decreased); p53, Bax, caspase-3, ROS production, LPO (increased)	[85]
				Oxidative stress & Apoptosis	
SiO_2NPs	15 nm (TEM)	Kupffer cells from Sprague Dawley rats; Sprague Dawley rats	50, 100, 200, 400, and 800 μg/mL for 24 h. 50 mg/kg single (i.v.)	ROS, AST, LDH, TNFα, H_2O_2, NO (increased); Kupffer cells (activation); Infiltration of inflammatory cells	[80]
				Activated Kupffer cells-mediated inflammation in liver toxicity	
SiO_2NPs	30, 50, 70, 300, 1000 nm (TEM)	BALB/c male mice	10-40 mg/kg (i.v.)	ALT, AST (increased)	[75]
				Acute liver injury	
Amorphous SiO_2 NPs	62.26nm (DLS)	HepG2	25, 50, 75, 100 μg/mL, 24 h	ROS levels; Autophagy and autophagic cell death *via* PI3K/Akt/mTOR pathway	[84]
				Oxidative stress	
Amorphous SiO_2 NPs	aSiNP-189 (20nm), aSiNP-116 (50nm), aSiNP-26 (110nm), aSiNP-8 (250nm) (EM)	HepG2	10–200 μg/mL, 24 h	Cholesterol biosynthesis (increased); May affect steroidogenesis & bile formation	[19]
Amorphous SiO_2 NPs	19.8 ± 2.7 nm (TEM)	HL-7702 cells; BRL-3A cells	31.4–500 μg/mL, 72 h	p53, Bax, cleaved caspase-3 (increased); GSH levels, caspase-3, Bcl-2 (decrease); Activation of p53/casp-3/Bax/Bcl-2 pathway; Human cells are more sensitive than rat cell	[86]
				Oxidative stress & apoptosis	
SiO_2NPs	30 nm (TEM)	Mouse hepatocytes	500 μg/mL, 24 h	ALT, AST (increased); ALR (blockage); Enlarged autolysosomes	[82]
				Inflammation	
Amorphous SiO_2 NPs	202.3 (DLS)	HepG2; ICR mice	50 mg/kg b.w. for 24 h (oral)	GSH, NADPH oxidase depletion; ROS (increased); Altered GSH metabolism	[77]
				Oxidative stress	
SiO_2NPs	10 nm (BET)	Albino Wistar rats	2 mg/kg daily 20, 35 or 50 injections (i.p.)	ALP, AST, ALT, LDH, procalcitonin, iron, phosphorus, potassium (increased); Phase I and II drug metabolizing and transporting enzymes (downregulation); Hydropic degeneration, karyopicnosis, Sinusoidal dialation, Kupffer cell hyperplasia, lowered liver index, infiltration of inflammatory cells	[79]
				Oxidative stress & Inflammation	
SiO_2NPs	15.4 ± 1.8 nm (TEM)	Kunming mice (normal & metabolic syndrome model)	10 mg/kg b.w. daily 30 d (oral)	Liver fibrosis (collagen deposition); Hepatic ballooning; DNA damage (genotoxicity) ROS production, mitochondrial damage, infiltration of inflammatory cells	[87]
				Mitochondrial instability & inflammation	
Mesoporous SiO_2 NPs	109.2 (DLS)	L02 cells; BALB/c mice	5–120 μg/mL, 24 and 48 h; 50 mg/kg 3 times a week for 3 wk (i.v.)	ALT, AST, ROS (increased); NLRP3 inflammasome activation; Pyroptosis *via* caspase-1 activation	[78]
				Oxidative stress and inflammation	
SiO_2NPs	58 nm (TEM)	L-02 cells	6.25, 12.5, 25, 50, and 100 μg/mL) for 12 h and 24 h	ROS production; ER stress; Activation of EIF2AK3 and ATF6 pathway; Induction of autosome formation	[83]
				Oxidative stress	
SiO_2NPs	58.04 ± 7.41 (TEM)	L02 cells	12.5, 25, 50, 100 μg/mL, 24 h	Affect mitochondrial quality control (MQC) process, Mitochondrial fission (increased); Induced mitophagy *via* activated	[88]

				PINK/Parkin signaling pathway; Decreased mitochondrial biogenesis *via* PGC1α-NRF1-TFAM signaling pathway; Mitochondrial dysfuntion **Mitochondrial dysfunction & oxidative stress**	
SiO_2NPs	58.11 ± 7.30 nm (TEM)	Sprague dawley rats	1.8, 5.4, 16.2 mg/kg b.w. (i.t.)	ALT, AST, TG, LDL-C (increased) HDL-C (decreased); Impact on Purine, amino acids metabolism, glucose-alanine cycle **Metabolic disorder**	[81]
SiO_2NPs	15nm (XRD)	Wistar rat	25 and 100 mg/kg b.w. for 28 consecutive days (i.p.)	AST, ALT, LDH, NO, MDA, PCO, H_2O_2, Bax, p53, Caspase-9/3 (increased) CAT, SOD, GPx, Bcl2 (decreased) **Oxidative stress & apoptosis**	[89]
SiO_2NPs	59.98nm (TEM)	Free Fatty Acid treated – L-02 cells; ApoE-/- mice	1.5, 3, 6 mg/kg b.w once per week for 12 times (i.t.)	LDH, AST, ALT, MDA (increased); GSH/GSSG (decreased); Fatty acid synthesis (increased); β-oxidation(decreased); Disturbed amino acid & lipid metabolism; Lipid accumulation leads to ER stress; Downregulated Nrf2 signaling **Oxidative stress, altered lipid metabolism**	[90]

Akt: Protein kinase B; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ATF6: Activating transcription factor 6; Bax: Bcl-2 associated X protein; Bcl2: B-cell lymphoma 2; CAT: catalase; DNA: Deoxy ribonucleic acid; EIF2AK3: Eukaryotic translation initiation factor 2-alpha kinase 3; ER: Endoplasmic reticulum; GPx: Glutathione peroxidase; GSH: Glutathione; GSSG: Glutathione disulfide.H_2O_2-hydrogen peroxide; HDL-C: High-density lipoprotein; LDH: Lactate dehydrogenase; LDL-C: Low-density lipoprotein; LPO: Lipid peroxidation; MDA: Malondialdehyde; mTOR: Mammalian target of rapamycin; NADPH: Reduced nicotinamide dinucleotide phosphate; NLRP3: NOD-like receptor protein 3; GSH: Glutathione; NO: Nitric oxide; NRF1/Nrf2: Nuclear factor erythroid 2-related factor1/2; p53-tumor suppressor protein p53; PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PI3K: Phosphatidylinositol 3-kinase; PINK: PTEN induced kinase, ROS: Reactive oxygen species, SOD: Superoxide dismutase; TFAM: Mitochondrial transcription factor A; TG: Triglyceride; TNFα: Tumor necrosis factor alpha.

Copper oxide nanoparticles: Transmission electron microscope investigation has confirmed the accumulation and distribution of CuONPs in rat liver tissue after oral administration. This would indicate that CuONPs can be easily absorbed through the intestinal wall and transported to the liver *via* blood. Serum levels of bilirubin that are high, heightened catalase and SOD activity, and altered glutathione metabolism enzyme profiles [glutathione reductase, GPx, and glutathione S-transferases (GST)], all strongly suggest that NPs exacerbated oxidative stress-related liver damage[100]. The primary marker of hepatic injury is an increase in vital enzymes such as serum ALT, and serum AST in the liver. CuONP-treated Wister rats have been shown to have histopathological changes, such as pyknotic, pleomorphic nuclei, binucleated hepatocytes with an increased population of apoptotic cells, with elevated levels of AST, ALT, and decreased levels of albumin in serum[18]. Mice receiving both chemically and biologically synthesized CuO-NPs (CNPs and BNPs), but mostly BNPs, showed distinct histopathological, biochemical, and apoptotic changes. Various types of histopathological alterations in hepatic tissues against their normal functioning range from hydropic degeneration and vacuolization to cell necrosis, loss of plasma membrane, more eosinophilic cytoplasm, karyorrhexis and complete loss of nucleus in few cells, activated Kupffer cells, lymphocytic infiltration around necrotized cells and congestion in sinusoids. Biochemical examination showed elevated levels of serum ALT and AST. Increased expression pattern of P^{53}, Casp-3 immunoreactivity suggested induction of apoptosis due to CuO toxicity in liver cells[101]. A comparable study has reported additional architectural abnormalities, such as ER swelling with lower count, increased intracellular space, fat accumulation, and cellular shrinkage related to the distribution of Nano-CuO in the liver. These discrepancies have been shown to affect hepatocyte growth, metabolism, and viability in both *in vitro* and *in vivo* investigations. JNK, PERK, C/EBP homologous protein (CHOP), ATF4, eIF2α, IRE1, Calpain, GRP78, ATF6, Bax, Caspase-3, and Caspase-12 have all been shown to have upregulated expressions in treatment group while Bcl-2 expression level gets diminished, is consistent with ROS-mediated oxidative stress-induced activation of the ER-stress pathway, that triggered apoptosis in liver tissue cells[102]. The liver of adult rats treated with CNPs (chemically synthesized CuO NPs) showed dose-dependent genotoxicity (DNA tailing), an enhanced oxidative stress response (lipid peroxidation), and histopathological changes (dilation and congestion of sinusoids) in contrast to GNPs (green synthesized CuO NPs)[103]. Mild to severe deleterious alterations in hepatic tissues including disorganized hepatic rays, dilated sinusoids with congestion, hepatocytic necrosis, glycogen breakdown, hemosiderosis, steatosis, hyperplasia of the bile duct and fibrous tissue proliferation, anti-inflammatory cell infiltration with caspase 3 immunoreactivity were also observed against the administration of nano-Cuo in dose-dependent manner[104].

Zinc oxide nanoparticles: In a study, Pasupuleti *et al*[20] reported that when SD rats were orally given nano-sized and micro-sized ZnO (5-2000 mg/kg), compared to micro-sized zinc oxide, nano-size zinc oxide exhibited an inverse dose-

Table 2 Effects and molecular mechanisms underlying NiONPs & NiNPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
NiO NPs	44 nm (TEM)	HepG2 cells	2-100 μg/mL for 24 h	Cell viability (reduced); ROS (increased); Micronuclei induction, chromatin condensation and DNA damage; bax and caspase-3 (upregulated); bcl-2 (downregulated) **Oxidative stress, apoptosis**	[95]
NiO NPs	20 nm (TEM)	Wistar rat	0.015, 0.06 or 0.24 mg/kg b.w. twice a week for 6 wk (i.t.)	NO, TNOS, iNOS, ·OH, LPO, HO1 (increased); CAT, GSHPx, T-SOD and TAOC, MT1 (decreased) **Oxidative & nitrative stress**	[93]
NiO NPs	20 nm (SEM)	Wistar rat	0.015, 0.06, and 0.24 mg/kg b.w. twice a week for 6 wk (i.t.)	GRP78, CHOP (increased); Activation of PERK/eIF-2α, IRE-1α/XBP-1S, and caspase-12/-9/-3 pathways **ER stress, apoptosis**	[16]
NiO NPs	20 nm (SEM)	Wistar rat	0.015, 0.06, and 0.24 mg/kg b.w. twice a week for 6 wk (i.t.)	ALT, AST, ALP, GGT, IL-1β and IL-6, TNF-α, NIK, IKK-α, NF-κB (increased); IL-4, IL-10, IκB(α) (decreased); Activation of NF-kB signalling pathway **Inflammation**	[94]
NiO NPs	13.16 ± 2.98 nm (TEM)	Wistar rat	125, 250 and 500 mg/kg single dose (oral)	ALP, LDH, ALT, AST, LPO (upregulation); GSH, SOD (downregulation) **Oxidative stress**	[92]
NiO NPs	21.6 ± 3.6 nm (TEM)	HepG2	5, 10, 25, 50 and 100 μg/mL, 24 h	HIF-1α, miR210, p53, Caspase-3, 8 and 9, NO, MMP (increased); Phagosome formation by lysosomal pathway **Hypoxia & oxidative stress, apoptosis**	[96]
NiO NPs	44 nm (TEM)	Wistar rat; HepG2	0.015, 0.06, and 0.24 mg/kg twice a week for 9 wk (i.t.); 25-200 μg/mL	TGF-β1, Smad2, Smad3, α-SMA, MMP9, TIMP1, EMT (upregulation); E-cadherin, Smad7 (downregulation); activation of TGF-β1/Smad pathway **Hepatic fibrosis, ECM deposition**	[97]
NiNPs	55.8 ± 14.0 nm (TEM)	C57/BL6 mice	10, 20 and 40 mg/kg/d for 7 and 28 d	ALT, AST, Ire1α, Perk and Atf6, TG increased; Lipid metabolism dysfunction; Inflammation **ER stress, apoptosis**	[98]

OH: Hydroxyl radical; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Atf6: Activating transcription factor 6; CAT: Catalase; CHOP: C/EBP Homologous Protein; ECM: Extra cellular matrix; eIF2α: Eukaryotic initiation factor 2 α; EMT: Epithelial mesenchymal transition; GGT: Gamma-glutamyl transpeptidase; GRP78: Glucose regulated protein 78; GSH: Glutathione; GSHPx: Glutathione peroxidase; HIF-1α: Hypoxia inducible factor 1; HO1: Heme oxygenase 1; IKK-α: IκB kinase alpha; IL-1β: Interleukin 1 β; IL-6: Interleukin 6; iNOS: Inducible nitric oxide synthase; IRE-1α: Inositol-requiring enzyme type 1α; IκB(α): Inhibitor kappa B alpha; LDH: Lactate dehydrogenase; LPO: Lipid peroxidation; miR210: miRNA210; MMP9: Matrix metallopeptidase 9; MMP: Mitochondrial membrane potential; MT1: Metallothionein 1; NF-κB: Nuclear factor kappa beta; NIK: NF-κB-inducible kinase; NO: Nitric oxide; p53-tumor protein p53; PERK: Protein kinase RNA like ER kinase; Smad2: Suppressor of mothers against decapentaplegic2; SOD: Superoxide dismutase; TAOC: Total antioxidative capacity; TG: Triglyceride; TGF-β1: Transforming growth factor 1 beta; TIMP1: TIMP metallopeptidase inhibitor 1; TNF-α: Tumor necrosis factor α; TNOS: Total nitric oxide synthase; TSOD: Total superoxide dismutase; XBP-1S: X box binding protein-1S; α-SMA: Alpha smooth muscle actin.

dependent increase in AST and ALT, that means nano-sized ZnO have shown higher toxicity at lower doses. Suggesting liver tissue assault and degeneration. Contrary to this result, Yang *et al*[105] demonstrated dose-dependent nanotoxicity of ZnO in mice models. A significant decrease in antioxidant (GSH) level causes an imbalance between oxidants and antioxidants, resulting in oxidative stress in the liver. Elevated expressions of transcription factor (xbp-1), ER chaperons (grp78, grp94, pdi-3), and phosphorylation of PERK and eIF2α in association to ER swelling and damage in hepatocytes strongly indicate ER stress. Upregulated expressions of proapoptotic genes (bax, chop), initiator caspase (Casp-9,12), effector caspase (casp-3), and subsequent diminished expression of bcl2, phosphorylation, and activation of JNK and CHOP/GADD153 strongly suggested ER stress-mediated opening of apoptotic pathways in liver tissue treated with nano-ZnO. Exposure to ZnONPs produces histological and histochemical modifications in liver tissues that may affect

Table 3 Effects and molecular mechanisms underlying WO_3NPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
WO_3 NPs	60-70 nm, length WO_3nanorods shorter (125−200 nm) and longer (0.8−2 μm)	BALB/c mice	2.5/5/10/20 mg/kg/d of shorter WO3 nanorods; 2.5/5/10/20 mg/kg/d longer WO3 nanorods for 14 d (i.p.)	ALT, AST; NF-κB, TNF-α, IFN-γ, IL-4 (increased); GSH, SOD (decreased) **Oxidative stress, inflammation**	[17]

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GSH: Glutathione; IFN-γ: Interferon gamma; IL-4: Interleukin 4; NF-κB: Nuclear factor kappa B; SOD: Superoxide dismutase; TNF-α: Tumor necrosis factor α.

Table 4 Effects and molecular mechanisms underlying CuONPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
CuO-NPs	33 nm (XRD)	Wister rats	300 mg/kg b.w. per day for 7 d (i.g.)	ALT, AST (increased)	[18]
CuO- NPs	40 nm (TEM)	Mature rats (Rattus norvegicus var. albinos)	0.5, 5, and 50 mg/kg b.w./d for 14 d (oral)	CAT, GPx, GR (increased) GST (decreased)	[100]
CuO-NPs	BNPs 4.14-12.82 nm CNPs 4.06-26.82 nm (XRD)	Mature mice	500 mg/kg b.w. single dose (oral)	ALT, AST, P^{53}, Caspase - 3 (increased); Hepatic necrosis	[101]
Nano-CuO	20-40 μm (TEM)	BRL-3A cells; Wister rat	5, 10, 20 μg/mL; 10 μg/g b.w. for 60 d (i.n.)	ALT, AST, T-BIL, D-BIL, I-BIL (increased) ALP (decreased); SOD (decreased); MDA, iNOS, GSH-PX (increased); MCP-1, IL-1, IL-1β, TNF-α, IL-6 (increased); JNK, PERK, CHOP, ATF4, eIF2α, IRE1, Calpain, GRP78, ATF6, Bax, Caspase-3, Caspase-12 (upregulated) **Oxidative stress induced ER stresspathway activation**	[102]
CuO-NPs	GNPs & CNPs	Sprague dawley rat	50 & 100 mg/kg b.w. twice a week starting before mating (oral)	CAT, GSH, GPx (decreased)	[103]
CuO-NPs	< 50 nm (TEM)	Wistar rat	5 mg, 10 mg, 25 mg/kg b.w. per day for 9 d (i.p.)	Mild to severe Liver tissue damage including necrosis of hepatocyte, anti-inflammatory cell infiltration	[104]

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ATF4/6: Activating transcription factor4/6; Bax: Bcl-2 associated X protein; CAT: Catalase; CHOP: C/EBP Homologous Protein; D-BIL: Direct bilirubin; eIF2α: Eukaryotic initiation factor 2 α; BNP: Biologically synthesized nanoparticle; CNP: Chemically synthesized nanoparticle; GNP: Green nanoparticle; ER: Endoplasmic reticulum; GPx: Glutathione peroxidase; GR: Glutathione reductase; grp78: Glucose regulated protein 78; GSH: Glutathione; GSH-PX: Glutathione peroxidase; GST: Glutathione-S-transferase; I-BIL: Indirect bilirubin; IL-1: Interleukin 1; IL-1β: Interleukin 1 β, IL-6: Interleukin 6; iNOS: Inducible nitric oxide synthase; IRE-1: Inositol-requiring enzyme type 1; JNK: Jun N-terminal kinase; MCP-1: Monocyte chemoattractant protein 1; MDA: Malondialdehyde; P^{53}: Tumor protein p53; PERK: Protein kinase RNA like ER kinase; SOD: Superoxide dismutase; T-BIL: Total bilirubin; TNF-α: Tumor necrosis factor α.

normal functioning. Degenerative liver cells exhibited nuclear changes (nuclear membrane irregularity, binucleation, nuclear vesiculation, anisokaryosis, and karyolysis), cytoplasmic changes (cytoplasmic vacuolation with parietal cytoplasmic swelling), and glycogen depletion followed by necrosis under ZnO insult. Inflammatory signs were sinusoidal dilation following Kupffer cell activation and enlargement, infiltration of inflammatory cells at lobular and portal triad[106]. ZnO-NPs-induced inflammatory liver injury *via* the production of inflammatory mediators (NO, TNF-, IL-6, C reactive protein, immunoglobulin G) has been documented[107]. Human liver cell HepG2 in response to short exposure to ZnO exhibited oxidative stress-mediated cytotoxic effects leading to LDH leakage, DNA damage, reduction in MMP, and increment in the ratio of proapoptotic/antiapoptotic proteins that lead to activation of mitochondrial apoptotic pathway. In addition to that ZnO was found to induce the phosphorylation of JNK, P38, and $P53^{ser15}$ without any significant changes in their expression level[108]. Above mentioned hepatic histopathological and immunohistochemical alterations along with oxidative stress are found to be promoted *via* modulation of JNK/p38MAPK and the STAT-3 signaling pathways[109]. A separate investigation in HepG2 cells revealed that ZnONPs override the toxic effects of ZnO (zinc oxide), exhibiting more hepatocyte inactivation, oxidative stress, mitochondrial damage, elevated

Table 5 Effects and molecular mechanisms underlying ZnONPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
ZnO NPs	Micro size; Nano size 63 nm (SEM)	Sprague Dawley rat	5, 50, 300, 100, 2000 mg/kg b.w for 14 d (oral)	AST, ALT (increased)	[20]
ZnO NPs	35 nm (TEM)	Wistar albino rats	2 mg/kg b.w. for 21; Days (i.p.)	Histopathological alterations; Kupffer cell activation	[106]
				Inflammation	
ZnO NPs	50 nm (TEM)	Wistar albino rats	600 mg/kg/b.w and 1 g/kg/b.w for 5 d	ALT, NO, TNF-α, IL-6, CRP, IgG (increased)	[107]
				Inflammation	
ZnO NPs	80 nm (TEM)	C57BL/6 mice	200 mg/kg/d (low dose) and 400 mg/kg/d (high dose) for 90 d (oral)	ALT, AST (increased); grp78, grp94, pdi-3, xbp-1(increased ER stress related proteins); Increased phosphorylation of PERK & eIF2α; caspase-3, 9, 12 (apoptosis); phosphorylation of JNK, and CHOP/GADD153; upregulation of Chop, Bax	[105]
				ERstress mediated activation of apoptotic pathway	
ZnO NPs	30 nm (TEM)	HepG2 cell	14–20 μg/mL for 12 h	AST, ALT, Bax (increased) Bcl2 (decreased) LDH leakage; JNK, P^{38} activation	[108]
				Apoptosis	
ZnO NPs	Less than 15 nm (TEM)	Sprague dawley albino rats	100, 200, 300 mg/kg b.w. per day for 14 d (oral)	ALT, AST, ALP (increased); Bax, caspase-3 (increased); Bcl2 (decreased); Modulation of JNK/p38MAPK & STAT-3 signalling pathways	[109]
				Apoptosis	
ZnO NPs	20-50 nm (TEM)	HepG2 cells; sprague dawley rat	20 μg/mL for 24 h; 25 mg/kg b.w. for 7 d (i.p.)	Cell inactivation; Intracellular calcium overload; Mitochondrial damage	[110]
				Oxidative stress	

ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, Bax: Bcl-2 associated X protein, Bcl2: B-cell lymphoma 2, CHOP: C/EBP Homologous Protein, CRP: C reactive protein, IgG: Immunoglobulin G, eIF2α: Eukaryotic initiation factor 2 α, GADD153: Growth arrest and DNA damage 153, Grp 78/94: Glucose regulated protein 78/94, IgG: Immunoglobulin G, IL-6: interleukin 6, JNK: Jun N-terminal kinase, LDH: Lactate dehydrogenase, MAPK: Mitogen activated protein kinase, NO: Nitric oxide, p38: Protein kinase, pdi-3: Protein disulfide isomerase -3, PERK: Protein kinase RNA like ER kinase, STAT-3: Signal transducer and activator of transcription 3, TNF-α: Tumor necrosis factor α, IL-6: Interleukin 6; xbp-1: X box binding protein-1.

intracellular calcium load along with weaker antioxidant level, and severe histopathological distortions. The expression pattern of differentially expressed genes and their transcripts are more for ZnONPs[110]. A recent study confirms the cytotoxic and genotoxic potentiality of ZnONPs in HepG2 cells in 2D and 3D culture after 24 h of exposure[111]. In dogs overused ZnONPs enhanced zinc accumulation in the liver with elevated serum liver indexes along with ROS generation and altered mitochondrial function. Strikingly ZnONPs attenuated apoptosis *via* the cytochrome c pathway instead, it induced autophagy through activating the mTOR/ATG5 pathway. Also involved in the disruption of the intestinal microbiome and 81 liver metabolites[112]. ZnO NPs induce crosstalk between protective autophagy and pyroptosis in hepatocytes. TFEB-mediated regulation influences ZnO NP-induced pyroptosis, with TFEB knockout exacerbating and overexpression alleviating it. TRAF-6 is identified in TFEB-mediated global regulation[113]. TFEB-regulated autophagy and lysosome prevent ZnO NPs-induced hepatocyte pyroptosis, providing insights for risk assessment and therapeutic strategies[113]. ZnO NPs also widely used in various applications, induce oxidative stress, leading to NLRP3-ASC-Caspase-1 complex assembly and pyroptosis in rat liver and HepG2 cells[114]. Inhibiting oxidative stress protects against ZnONPs-induced pyroptosis in hepatocytes, revealing a novel mechanism and potential clinical treatment strategies[114].

Titanium dioxide nanoparticles: Several major effects and molecular mechanisms underlying hepatotoxicity due to TiO_2 NP exposure have been reported in both *in vitro* and *in vivo* studies. Titanium dioxide exists in different commercially available forms. The natural one is an agglomerated, rod-shaped rutile form and the other is a glomerated metastable form, the anatase. Chen *et al*[115], in a study proved that both forms can significantly activate inflammatory signaling pathways like mitogen-activated protein kinase (MAPK) and NF-κB in HepG2 cells with reduced cell viability and ultrastructural alterations, though rutile form has more cytotoxic effect. In 80 CD-1 (ICR) mice, intragastric administration of TiO_2NPs resulted in increased expressions of Toll-like receptors (TLR2 &TLR4) and inflammation-related genes (IKK1, IKK2, NF-kB, NF-kBP52, NF-kBP65, TNF-α, NIK) with decreased expressions of IkB and Il-2 indicating TLRs/NIK/IkB kinase/NF-kB/TNF-α signaling pathway mediated inflammation in liver. At higher doses significant changes in liver coefficient, biochemical parameters (ALT, AST, ALP, LDH, pseudocholinesterase, leucine acid peptide) along with

Table 6 Effects and molecular mechanisms underlying TiO_2NPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
TiO_2NPs (Anatase)	7 nm (XRD)	80 CD-1 (ICR) mice	5, 10, 50 mg/kg b.w. every other day for 60 d (i.g.)	SOD, CAT, GSH-Px, MT, HSP70, GST (downregulation); CYP1A (upregulation)	[117]
				Oxidative stress, apoptosis	
TiO_2NPs (Anatase)	5 nm (XRD)	CD-1 (ICR) mice	5, 10, 50, 100, 150 mg/kg b.w. daily for 14 d (abdominal injection)	Accumulated in liver DNA; Inserted in DNA base pairs; Binds to DNA nucleotides; Alter DNA secondary structure; Liver DNA cleavage at higher dose	[119]
				Genotoxicity	
TiO_2NPs	< 25 nm anatase; < 100 nm rutile (SEM)	HepG2 cell	1, 10, 100 and 250 mg/mL incubated for 4, 24, 48 h	p21, mdm2, p53, gadd45α (increased expression); DNA strand break; DNA damage; ROS production	[120]
				Genotoxicity	
TiO_2NPs (Anatase)	5 nm (XRD)	80 CD-1 (ICR) mice	5, 10, 50 mg/kg b.w. for 60 d (i.g.)	TLR2, TLR4, IKK1, IKK2, NF-kB, NF-kBP52, NF-kBP65, TNF-α, NIK (upregulation); IkB, IL-2 (downregulation); ALT, AST, ALP, LDH, PCh, LAP (upregulation)	[116]
				Inflammation, apoptosis	
TiO_2NPs (Anatase & rutile)	Anatase –561.63 ± 26.26 nm; Rutile – 206.22 ± 2.18 nm (TEM)	HepG2 cell	5-320 μg/mL for 24 h	ERK1/2, p38 (increased phosphorylation); TNFα (upregulated); A20 (downregulated); Activation of MAPK & NF-kB pathway	[115]
				Inflammation	
TiO_2NPs; Rutile anatase; P25 (anatase: rutile = 75:25)	Rutile – 50 nm; Anatase – 50 nm; P25 – 21 nm (TEM)	Primary hepatocytes of Sprague Dawley rats	50 μg/mL, 72 h	ROS (upregulated); Urea, albumin, MnSOD, MMP, Mfn 1, Opa 1 (downregulated)	[122]
				Perturbation of mitochondrial dynamics, oxidative stress	
TiO_2NPs; Rutile	12-18 nm (TEM)	BRL 3A cells; sprague dawley rats	0.1-100 μg/mL for 6 h; 0.5-50 mg/kg BW intraperitoneal injection 24 h	Rapid G0/G1 to S transition, G2/M arrest; ALT, AST, ALP, LDH (upregulated)	[123]
				Hepatocytes with oxidative stress show more cytotoxicity	
TiO_2NPs; Anatase	10 (TEM)	B6C3F1 mice	50 mg/kg b.w. daily for 3 d (i.p.)	DNA strand break nucleotide oxidization; MT1H, MT1E (upregulation); Differential gene expression(increased)	[121]
				Oxidative stress, Genotoxicity, metabolic imbalance	
TiO_2NPs; Anatase	19 (XRD)	Wistar rat	100 mg/kg daily for 60 d (oral)	ALT, AST, ALP, LPO (increased); GSH, SOD, GPx, CAT (decreased); vacuolization, Sinusoidal dilation, inflammatory cells infiltration	[124]
				Oxidative stress	
TiO_2NPs; Anatase	10 nm (TEM)	Albino rats	100 mg/kg daily	ALT, AST, ALP, Bax, LPO (increased); GPx, SOD, GSH, Bcl-2, (decreased); hepatic apoptosis; Sinusoidal dilation, infiltration inflammatory cells, steatosis, hepatocellular necrosis	[125]
			60 d (oral)	**Oxidative stress**	
TiO_2NPs; anatase: Rutile (80: 20)	20 nm (TEM)	Wistar rat	300 mg/kg daily for 2 wk (oral)	ALT, AST, ALP, LDH, TNFα, NF-Kβ, TOS, LPO (upregulated); SOD, CAT, GPx, TAC (downregulated)	[118]
				Inflammation, Oxidative stress	
TiO_2NPs; Anatase	29 ± 9 nm (SEM)	Sprague dawley rats	2, 10, 50 mg/kg b.w. daily	LPO, GPx, SOD, GSSG, IL-1α, IL-4 and	[126,127]

			for 90 d (oral)	TNFα (increased); GSH (decreased); Mitochondrial swelling increased gut microbiota altered glycerophospholipid, Phosphatidylcholines metabolism; Hepatotoxicity indirectly through gut liver axis **Oxidative stress, inflammation**

A20: Alpha-induced protein-3; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Bax: Bcl-2 associated X protein; Bcl2: B-cell lymphoma 2; CAT: Catalase; CYP1A: Cytochrome p450 1A; DNA: Deoxy ribonucleic acid; ERK1/2: Extracellular signal-regulated protein kinases 1 and 2; gadd45α: Growth arrest and DNA damage 45 alpha; GPx/GSH-Px: Glutathione peroxidase; GSH: Glutathione; GSSG: Glutathione disulfide; GST: Glutathione S transferase; HSP70: Heat shock protein 70; IkB: Inhibitor kappa B; IKK1,2: IκB kinase; IL-1α: Interleukin 1 alpha; IL-2,4: Interleukin-2,4; LAP: Leucine acid peptide; LDH: Lactate dehydrogenase; LPO: Lipid peroxidation; MAPK: Mitogen activated protein kinase; mdm2: Murine double minute 2; Mfn 1: Mitofusin 1; MMP: Mitochondrial membrane potential; MnSOD: Manganese superoxide dismutase; MT: Metallothionein; MTIE: Metallothionein 1E; MTIH: Metallothionein 1H; NF-kB: Nuclear factor kappa beta; NIK: NF-κB-inducible kinase; Opa 1: Optic atrophy 1; p21: Cyclin-dependent kinase inhibitor 1; p38: Puncture38; p53: Tumor suppressor protein p53; PCh: Pseudocholinesterase; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TLR2/4: Toll like receptor 2/4; TNF-α: Tumor necrosis factor α; TOS: Total oxidant status.

Table 7 Effects and molecular mechanisms underlying MgONPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
MgO	-	3D Human Liver organoids male Sprauge Dawley rat	100 µg/mL incubated for 48 h. 40 mg/kg daily for 4 wk (oral)	ATP synthesis (decreased); ROS & Super oxide production (increased); ALT, AST (increased) **Oxidative stress**	[21]

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ATP: Adenosine triphosphate; ROS: Reactive oxygen species.

Table 8 Effects and molecular mechanisms underlying Al_2O_3NPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
Al_2O_3	< 50 nm	Developing chicken embryo, HepG2 cell culture model	10, 20, 40 µg/egg *via* injection from 8th to 12th day of incubation on an alternate day basis, 05, 10, 20 µg/mL for 12 h	ROS & Super oxide production (increased); ALP, ALT, AST activity (increased); HO-1, NQO-1 level (increased); Cell viability (decreased); SOD, CAT, GPx, TBARS, TNF-α, Caspase-3 activity (decreased) **Oxidative stress and cytotoxicity**	[128]

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HO-1: Heme oxygenase-1; NQO-1: NAD(P)H quinone oxidoreductase 1; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; TBARS: Thiobarbituric acid reactive substances; TNF-α: Tumor necrosis factor α; ROS: Reactive oxygen species.

Table 9 Effects and molecular mechanisms underlying Cr_2O_3NPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
Cr_2O_3-NPs	22.50 + 1.76 nm (TEM)	Wistar rats	50 mg/100 g bwt (LD), 200 mg/100 g bwt (HD); single dose for 1, 7, 14 d (oral)	ALT, AST, ALP, γGT, total bilirubin (increased)	[23]

ALP: Alanine phosphatase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, HD: High dose, LD: Low dose, γGT: Gamma glutamyltransferas.

mitochondrial swelling, apoptotic body formation, chromatin condensation, inflammatory cell infiltration suggests liver tissue injury caused by inflammation that in turn trigger activation of apoptosis[116]. The same group showed that TiO_2 NPs insult leads to ROS accumulation, over-expression of cytochrome p450 1A, and suppressed expression of stress-related genes (SOD, CAT, GSH-Px, MT, HSP70, GST), and NPs detoxifying/metabolizing genes[117]. Other investigation result shows that TiO_2NP ingestion at higher doses for longer periods leads to Kupffer cells hypertrophy, hydropic degeneration and vacuolization in hepatocytes, necrosis around the central vein followed by edema, infiltration of inflammatory cells along reduced antioxidant enzymes. Elevated levels of liver enzymes, higher lipid peroxidation, and upregulated expressions of inflammatory mediators (TNFα and NF-Kβ) suggest hepatic damage due to oxidative stress

Table 10 Effects and molecular mechanisms underlying iron oxide NPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
Na-oleate coated Fe_3O_4	8 ± 3 nm (TEM)	Wistar rat	0.0364, 0.364, & 3.64 mg/kg b.w. for 1 d, 1, 2, 4 wks (i.v.)	Temporary change in mitochondrial respiration; GPx, GST (increased); Lipidosis, mild necrosis; Enlarged sinusoid space **Oxidative stress**	[133]
Polyethylene glycol – 8000 coated Fe_3O_4	8.82 ± 0.70 nm (TEM)	Wistar rat	10 mg/kg b.w. single dose, once in a week, twice in a week for 30 d (i.v.)	ALT, AST, ALP (slightly increased); AST, LPO, SOD, GPx, Neutrophil count (increased); No significant tissue damage	[135]
Fe_3O_4	20 nm (TEM)	Wistar rat	40 mg/kg b.w. for 14 d (i.t.)	Congestion of sinusoid; Hepatocytic ballooning; Mononuclear cell infiltration; Tissue damage **Inflammation**	[132]
Fe_3O_4	41.3 ± 5.9 nm for USPIO, 112.6 ± 38.4 nm for SPIO (DLS)	L-02 cells	2.5, 5, 10, and 20 μg/mL) for 12 h	Cell survivility (decreased); Elevated expression of Genes related to acute phase inflammation, ER stress. HSP70, IL-6, PERK, ATF4, ER Ca^{++} (increased); USPIO show higher toxicity than SPIO **ER stress, inflammation**	[136]
Fe_3O_4	10 nm (TEM)	Hepatocytes of Lewis rat in sandwich culture model	100, 200, 400 μg/mL, single dose & cumulative dose; 24 h to 7 d	Cell survivility (decreased); ROS (increased); Albumin & urea synthsis (decreased) **Oxidative stress**	[134]
Fe_3O_4	29.6 ± 12.2 nm (TEM)	Albino wistar rat	30, 300, 1000 mg/kg b.w. for 28 d (nano & bulk) (oral)	GSH, CAT (decreased); SOD, GR, GST, LPO (increased); GPx (unchanged); Congested central vein in higher dose **Oxidative stress**	[130]
Fe_2O_3	30 nm (TEM)	Wistar rat	100, 200 mg/kg single dose (oral)	ALT (increased) iron deposition in hepatocyte & Kupffer cells Inflammation	[131]
Fe_2O_3	30 nm (TEM)	L-02 cells; BALB/C mice	2.5, 7.5, and 12.5 lg/mL) for 1, 3, 6 h; 20 mg/kg body weight for 24 h. (i.v.)	Cox2 (overexpression); COX-2 interaction with IP3R-GRP75-VDAC1 complex; Ca++ transfer increased; Bax, Cleaved Casp-3 (increased); Bcl2 (decreased) **Apoptosis**	[137]

ALP: Alanine phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Atf4: Activating transcription factor 4; Bax: Bcl-2 associated X protein; Bcl2: B-cell lymphoma 2; Ca++: Calcium ion; CAT: Catalase; COX-2: Cyclooxygenase-2; ER: Endoplasmic reticulum; GPx: Glutathione peroxidase; GR: Glutathione reductase; GRP75: Glucose regulated protein 75; GSH: Glutathione; GST: Glutathione S-transferase; HD: High dose; HSP-70: Heat shock protein 70; IL-6: Interleukin-6; IP3R: Inositol 1,4,5 triphosphate receptor; LD: Low dose; LPO: Lipid peroxidation; PERK: Protein kinase RNA like ER kinase; ROS: Reactive oxygen species; SOD: Super oxide dismutase; SPIO: Superparamagnetic iron oxide; USPIO: Ultra-small superparamagnetic iron oxide; VDAC1: Voltage-dependent anion channel 1; γGT: Gamma glutamyl transferase.

and inflammation[118].

Different spectral analyses and gel electrophoresis results of *in vivo* experiments unveil that liver DNA is a prime target of TiO_2NPs. In liver DNA, anatase form get accumulates either by inserting itself between base pairs or directly binding to 3 oxygen or nitrogen atoms [Ti-O(N)=1.87A] and 2 phosphorous atoms (Ti-P=2.38A) of nucleotide, affecting the configuration of DNA secondary structure. DNA laddering in gel slab at a higher dose of 150 mg/kg can be corroborated with liver DNA cleavage by NPs[119]. *In vitro* study with HepG2 cells also exhibited oxidative stress-induced DNA damage for both rutile and anatase forms. Elevated expression level of p53 and subsequent upregulated expression pattern of downstream DNA damage responsive genes (p21, mdm^2, gadd45α) confirms the TiO_2NPs mediated genotoxicity in hepatocytes[120]. Gene expression analysis and genotoxicity assessment demonstrated similar results, that TiO_2NPs promote oxidization of nucleotides which results in DNA strand break (DNA damage). Also disturbs the metabolic homeostasis of the liver through oxidative and stress-related impairment of glucose, lipid, and xenobiotic metabolism [121].

When primary hepatocytes were given exposure to rutile, anatase, and P25 (mixture of rutile & anatase) NPs, all three significantly exhibited hepatotoxicity. The Mitochondrial morphology and dynamics get compromised due to the downregulation of the fusion process, which leads to mitochondrial fragmentation in hepatocytes. Over production of ROS and subsequent loss of MnSOD enzyme activity and reduced MMP leads to oxidative stress that hampers the normal functionality of liver cells including biosynthesis of urea and albumin[122]. In a remarkable *in vitro* as well as *in vivo* experimentation Sha *et al*[123] click or tap here to enter text. Have proven that liver cells already in oxidative stress condition exhibit more susceptibility towards nano-TiO_2 mediated cytotoxicity. In contrast to G0/G1 phase arrest under only NM exposure, BRL-3A cells with prior oxidative stress conditions exhibited very fast G0/G1 phase to S transition, G2/M arrest with elevated cell death ratios. Increased expression levels of liver marker enzymes (ALT, AST, ALP, LDH) under the same experimental regime in an *in vivo* study indicated liver damage with prominent histopathological perturbation. Micro-TiO_2 didn't show such effects both in cells and rat liver. Again, in different studies orally administered thymol and tiron were seen to ameliorate TiO_2NPs mediated lipid peroxidation (LPO), oxidative stress, non-enzymatic and enzymatic alterations of antioxidant levels, augmentation of proapoptotic and downregulation of antiapoptotic genes along with biochemical and histopathological changes in liver tissue. Supporting hepatic injury by TiO_2NPs is mediated by oxidative stress and apoptosis[124,125].

In a dose-dependent manner TiO_2NPs treated rats show an increment in diversity and abundance of gut microbiota (*Firmicutes, Bacteroidetes, Tenericutes, Proteobacteria, etc*.) that has been found to produce a significant quantity of lipopolysaccharides and increased number of *Lactobacillus reuteri* but not *Romboutsia* in feces. On the contrary, it produces mitochondrial swelling, and an imbalance in oxidation/antioxidation status with the generation of altered metabolites (Glutamate, glutamine, and glutathione) in connection to energy-related metabolic disorders. Therefore, it can be predicted from the results that the indirect pathway of the gut-liver axis may play an important role, in connecting gut microbiota and liver metabolism. Subsequent investigation confirms that the gut microbiota under oxidative stress led to lipid metabolism disorders (glycerophospholipid and phosphatidylcholines) and caused liver toxicity *via* the gut-liver axis[126,127].

Mg-nano nanoparticles: In a recent experiment, researchers have tried to verify the hepatotoxic potentiality of Mg-nano in combination with valproate (anticonvulsant drug) and PTZ (pentylenetetrazole- used to induce convulsion mouse model) using 3D liver organoid and rat model. In the *in vitro* model the prepared suspension carrying Mg-nano decreased the production of ATP and increased ROS generation and super oxide production while *in vivo* result showed a significant increase of ALT, AST in serum but without any change in albumin or globulin concentration, suggesting Mg-nano as well as Valporate both can induce hepatotoxicity[21].

Aluminium oxide nanoparticles: Aluminum oxide nanoparticles (Al_2O_3-NPs) pose hepatotoxic effects on chicken embryos and cell cultures, inducing histological abnormalities, elevating tissue damage markers, causing oxidative stress, and impacting antioxidant enzymes[128]. Additionally, Al_2O_3-NPs affect red blood cells, liver metabolism, and stress response gene expression. The study reveals dose-dependent ROS generation, cytotoxic responses, and potentiating effects on TNF-α-induced apoptosis. Inhibition of p38 MAPK and JNK pathways modulates Al_2O_3-NPs-induced apoptosis in HepG2 cells, highlighting novel mechanisms and potential prevention strategies[128].

Chromium oxide nanoparticles: The investigated liver function biomarkers (ALT, AST, ALP, γgamma glutamyl transferase, total bilirubin levels) get elevated in a dose and exposure time-dependent fashion in rats after orally consuming Cr_2O_3-NPs. Routine histological examination clearly showed moderate to severe architectural damage including liver cell degeneration, Kupffer cell hyperplasia, parenchymal distortions, dilated central vein, and hemorrhage for both low and high doses, indicating the role of chromium oxide-NPs in liver toxicity[23].

Iron oxides nanoparticles: The bioavailability of nano iron oxide was found to be greater compared to bulk in different organs, including the liver[24,129]. Similarly, nano magnetite (Fe_3O_4) showed higher bioaccumulation, oxidative stress, and liver tissue damage than its bulk counterpart in another experiment also[130]. Orally administered nano maghemite (Fe_2O_3) was found deposited in hepatocytes and kupffer cells, resulting in very little perturbation of biochemical parameters with minimum effects on the liver[131]. Histopathological study revealed infiltration of mononuclear cells, ballooning, and hepatic damage with congestion in sinusoids but surprisingly with a decreased level of ALT during the investigation of concurrent effects of aerobic exercise and IONPs in liver enzymes of the treated subject[132]. In the rat model administration of coated Fe_3O_4 caused mild liver tissue injury with an altered antioxidant enzyme profile, suggesting oxidative stress-related response[133]. Similarly, increased ROS production and decreased cell viability with hampered albumin and urea synthesis in a dose-dependent manner was evident from another study with primary hepatocytes[134]. On dose interval treatment with PEG-8000 coated ultra-small superparamagnetic iron oxide nanoparticles, have shown temporary alterations in the liver biomarkers and hematological parameters, with lipid peroxidation[135]. In a separate experiment, USPIO was found to exhibit more toxic effects on liver tissue than SPIO. In USPIO treated L-02 cells, upregulated expressions of IL-1B, IL-6, IL-18, TNFSF12, TNFRSF12, SAA1, SAA2, JAK1, STAT5B, and CXCL14 genes with increased secretion of Il-6 and altered ER structure due to ER stress supports the occurrence of ER stress-mediated acute-phase inflammatory response that leads to cytotoxicity. Application of ER stress blocker or ATF4 siRNA attenuated the USPIOs effects supporting the involvement of PERK/ATF4 pathway[136]. MAMs [Mitochondria-associated endoplasmic reticulum (ER) membranes], a dynamic microdomain made up of proteins that maintain crosstalk between ER and mitochondria, play a crucial role in Ca^{++} ion and metabolite transfer between two organelles and cellular homeostasis. Both *in vitro* and *in vivo* results suggest SPIO-Nps (iron oxide) accumulation in hepatocytes triggers the overexpression followed by interaction of COX-2 with IP3R-GRP75-VDAC1 complex (inositol 1,4,5 triphosphate receptor, glucose-regulated protein 75, voltage-dependent anion channel 1), the fraction of MAMs that

facilitates Ca^{++} transfer. Thereby resulted in profuse Ca^{++} transfer from ER to mitochondria, producing Ca^{++} overload in mitochondria that sparks apoptosis in hepatocytes[137].

Orally administered nano-iron oxide, commonly used in food, disrupts the small intestinal barrier, leading to hepatic lipid metabolism disorders through the gut–liver axis. This disruption causes hepatic damage and iron deposition, impacting lipid homeostasis with decreased phosphatidylcholine and phosphatidylethanolamine and increased triglyceride levels. The study highlights the subchronic toxicity of nano-iron oxide and emphasizes the pivotal role of the gut-liver axis in its hepatotoxicity[138]. Fe_2O_3 nanoparticles (E172 food additive) exhibit no evident toxicity in body weight, histopathology, or oxidative stress in animal experiments. However, a sensitiveLC–MS/MS-based lipidomic study reveals significant alterations in hepatic glycerophospholipid metabolism, including decreased triacylglycerol and increased phosphatidylcholine. This study enhances understanding of the subacute effects of Fe_2O_3 NPs beyond conventional toxicology assessments[139].

Graphene oxide nanoparticles

Because of its special physico-chemical characteristics, graphene oxides are easily produced and tailored to order. They have a wide range of uses in the fields of electronics, nanomedicine, textiles, water purification, nanocomposite, and catalysis[140-143]. Several investigations unveiled the subacute toxicity caused by GO in different organs including the liver[144,145]. Patlolla *et al*[146] showed that in an SD rat model, GO-induced liver inflammation was associated with lower levels of cholesterol, HDL, and LDL. A separate study with a similar model revealed oxidative stress in accordance with the enhanced ROS production, increased activity of AST/GPT, ALT/GOT, alkaline phosphatase, and lipid hydro peroxide with structural alterations in hepatocytes. Varied degrees of histopathological modifications (sinusoidal abnormality, inflammation around portal and central vein, hepatocytic vacuolation) with an elevated level of serum enzyme markers and alterations in MDA, CAT contents concerning oxidative stress indicate GO-induced hepatotoxicity in Wistar rat[147] GO induced mild early apoptosis and inhibited phase-I drug-metabolism enzymes (CYP3A4, CYP2C9) in upcyte® hepatocytes[148]. Notably, CYP3A4 impairment coincided with an acute-phase response activation. The study highlights the potential health consequences of drug detoxification[148]. Follow Table 11 for a comprehensive account.

Carbon nanotubes nanoparticles

Carbon nanotubes are of two types, single-walled (SWCNTs) with one layer and multi-walled (MWCNTs) with multiple layers. When acid-oxidized MWCNTs (O-MWCNTs) and Tween-80-dispersed MWCNTs (T-MWCNTs) were administered intravenously to mice bodies both types showed inflammatory responses and oxidative stress-mediated liver toxicity. Compared to O-MWCNTs (with carboxyl group), T-MWCNTs (without carboxyl group) exhibited greater effects suggesting hepatotoxicity might be dependent on modification of carboxyl group. Whole genome-wise expression array revealed, upregulated expression of genes related to TNF-α, NF-κB signaling pathway, NK cell-mediated cytotoxicity, biosynthesis of cholesterol, metabolism by cytochrome P450, GPCRs (G protein-coupled receptors) were recorded for both the treatments[149].

NMR-based metabonomic study unveiled disruption of important metabolic pathways in rat model receiving SWCNTs. Decreased alanine but increased lactate concentration in plasma indicates impairment of amino acid metabolism. Similarly, decreased level of lipoproteins, and lipids together with the rise in choline, and phosphocholine in serum and liver extract support the disruption of membrane fluidity due to lipid peroxidation. All these strongly support nanotubes-induced hepatic injury through the modulation of energy, amino acid, and lipid metabolism[150].

Several investigations have revealed that MWCNT resulted in increased ROS production (H_2O_2), and LPO with a compromised antioxidant defense system (SOD, GPx, GSH, GST), suggesting oxidative stress-mediated hepatotoxicity[4,151,152]. In a series of experiments, Patlolla *et al*[153] and Patlolla *et al*[154] had shown that in a dose-dependent manner both carboxylated functionalized carbon nanotubes (SWCNT and MWCNT) exposure to mice resulted in ROS-mediated oxidative stress in association to increased liver biochemical markers and tissue damage.

Again, MWCNT exposure was found to stimulate pro-inflammatory cytokines (IL-6, IL-1B, COX-1, TNF-α), that serve as an inflammatory mediator to elicit inflammatory responses in the liver[4,151,152]. In an *in vivo* toxicity study, administration of both P- MWCNT (PEGylated) and NP- MWCNT (non-PEGylated) exhibited induction of hepatic inflammation through TNF-α and NF-κB signaling pathway without any oxidative damage to the liver tissue, though NP- MWCNT shows slightly higher toxicity[155]. Orally administered aqueous extract of *Cinnamomum burmannii* was reported to protect the liver against MWCNT assault by downregulating pro-inflammatory cytokine production and ameliorating the antioxidant system. Suggesting nanotubes triggered liver toxicity is due to oxidative stress and inflammation[4].

Histopathological examinations revealed that MWCNT insult produces clear ultrastructural perturbations including cellular swelling, hydropic degeneration, sinusoidal leukocytosis, sinusoidal space enlargement, vacuolar degeneration, inflammatory cell infiltration associated with focal hepatic and focal perivascular hepatic necrosis, spot necrosis, mitochondrial destruction, congested central vein, macrophage injury even blood coagulation[4,155,156].

Cd-MT (accumulated cadmium-metallothionein) mice when treated with oxidized MWCNTs have shown some striking results. Different doses of MWCNT exposure, alone promoted the release of free Cd^{++} from Cd-MT, a portion freely available in circulation for elimination while the other portion adsorbed by MWCNT, stayed together in the tissue. Also, co-exposure alleviated hepatotoxicity compared to single exposure[157]. But co-administration of a higher dose of MWCNTs with PbAc in NAFLD (non-alcoholic fatty liver disease) mice resulted in severe liver damage compared to lower combined or single dose (lower or higher) of MWCNTs or PbAc. Remarkable reduction in body weight, liver function, and augmentation of nonalcoholic steatohepatitis (steatosis, lobular inflammation) phenotype was noticed. MWCNTs alone or in combination were found to induce collagen deposition and lipidosis, which leads to hepatic fibrosis. Primary hepatocytes isolated from co-exposed NAFLD mice exhibited a higher rate of apoptosis followed by

Table 11 Effects and molecular mechanisms underlying GONPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
GO	100-500 nm (TEM)	Sprague dawley rats	2.5, 5, and 10 mg/kg/d for 7 d (i.v.)	Liver inflammation; Cholesterol, HDL, LDL (decreased)	[144]
GO	40 nm (TEM)	Sprague Dawley rats	10, 20 and 40 mg/Kg b.w. once for 5 d, (oral)	ROS, AST, ALT, LHP (increased)	[146]
GO	0.8-2 nm (TEM)	Wistar rats	0.4/2/10 mg/kg b.w.	AST, ALP, ALT, MDA (increased); CAT (decreased)	[147]

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CAT: Catalase; HDL: High density lipoprotein; LDL: Low density lipoprotein; LHP: Lipid hydro peroxide; MDA: Malondialdehyde; ROS: Reactive oxygen species.

oxidative stress and inflammation. A significant decrease in expression patterns of p-AMPKα and PPARγ at combined low doses but reverse expression pattern in the presence of AMP activated protein kinase (AMPK) activators suggests inhibition of AMPK/PPARγ pathway (adenosine 5'-monophosphate activated protein kinase/peroxisome proliferator-activated receptors γ) may be the reason behind hepatotoxicity[152]. Follow Table 12 for a comprehensive account.

Copper sulfide/cadmium sulfide nanoparticles

In a study using biomimetic synthesis and ion exchange strategy CuS/CdS nanocomposites were synthesized and tested for hepatotoxicity in liver cells and mice models. *In vitro,* study results unveiled that CuS/CdS nanocomposites cause oxidative stress-mediated apoptosis in liver cells which can be correlated with the perturbated intracellular antioxidant defense system in hepatocytes (SOD & GSH) and excessive accumulation of oxidative products (ROS, GSSG, MDA) that resulted into oxidative stress-mediated apoptosis in both hepatoma cells (BEL7402) and normal liver cells (L-02). Though the first one was more responsive than the latter one. Intravenous injection of nanocomposites to Balb/c mice has shown time-dependent accumulation of Cd^{2+} and Cu^{2+} in the liver, spleen, and kidney. Compared to Cu^{2+}, the liver and kidney retained a significant amount of Cd^{2+} which the physiological system was unable to remove[158]. Compared to CdS microparticles CdNPs exhibited more toxic effects in rat liver. Greater bioaccumulation of CdNPs leads to the overproduction of metallothionein and ligand formation that has increased its hydrophilicity, facilitating penetration through hepatocyte membrane and such interactions between membrane and NPs further facilitated ROS generation (H_2O_2, NO) and oxidative stress (lipid peroxidation), disrupting membrane integrity. Biochemical analysis showed increased ALT, AST, and ALP in serum. Ultrastructural study exhibited cytoplasmic degeneration, organellar proliferation (microsome, ER, peroxisome, mitochondria), and extensive parenchymal degeneration suggesting hepatotoxicity[159].

The hepatic bile salt export pump (BSEP) is crucial for secreting bile salts from hepatocytes to bile and the hepatic MRP2 transporter contributes to bile flow, detoxification, and chemoprotection maintaining a healthy liver. Lowered expression of BSEP mRNA and protein followed by diminished activity of BSEP was observed in the CuSNPs treated group while MRP2 function remain unaltered. Hepatocytes also showed spheroid injury with altered ROS and mitochondrial membrane potential[160]. In a separate experiment, different-sized (LNPs - 17.8 nm and SNPs -2.8 nm) copper sulfide nanoparticles ($Cu_{2-x}S$ NPs), biomineralized with Bovine Serum Albumin were administered in SD rats through tail vein to assess safety and liver toxicity. Both the particles were found to intervein important biochemical pathways including, lipid metabolism, cholesterol/bile acid metabolism, copper ion transport/metabolism, inflammatory and drug metabolism-cytochrome P450 pathway. SNPs are discharged through feces, 7 and 14 d after single administration causing manageable liver toxicity, so it could be a promising nano agent. On the contrary LNPs with more retention power in Kupffer cells, were found to be involved in prolonged and delayed liver toxicity[161]. Follow Table 13 for a comprehensive account.

Cobalt nanoparticle

The human fetal liver cell line L02 demonstrated dose- and time-dependent cytotoxicity following exposure to varying doses of Nano-Co for 12 or 24 h. It has been predicted that cobalt nanoparticles reach hepatocyte intracellular regions through both endocytosis-driven and endocytosis-free pathways. This led to the generation of ROS and mtROS (mitochondrial reactive oxygen species), which in turn caused oxidative stress damage. Availability of IL-1β and IL-18 in the extracellular space suggests mtROS-mediated activation of NLRP3 (NOD-like receptor protein 3) inflammosome response, resulting in the upregulation of caspase-1 p20, IL-1β, and IL-18. Thus Nano-Co induced modulation of ROS/NLRP3 pathway was found to be involved in hepatotoxicity[162]. Follow Table 14 for a comprehensive account.

Nanoclay particles

In mice, intra-veinous administration of nanoclay resulted in acute hepatotoxicity. Elevated level of ALT and AST in serum with routine histological study results indicates toxic effects for higher doses (10 or 20 mg/kg). When co-administered with chemical (carbon tetra chloride, paraquat) or drug (cisplatin) exhibited synergistic increment in liver biomarkers compared to their individual effects[163]. Follow Table 15 for a comprehensive account.

Table 12 Effects and molecular mechanisms underlying carbon nanotube induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
MWCNTs	O-MWCNT; T-MWCNT; Length 356 ± 185 nm	Kunming mice	10 and 60 mg/kg b.w. (Iv) sacrificed at 15 & 60 d	GSH, SOD (decreased at 15 days); AST, T-Bil (increased); Spotty necrosis, Infiltration of inflammatory cells in portal region, mitochondrial swelling and lysis; Cyp2B19 (upregulated); Cyp2C50, Gsta2 (downregulated) **Oxidative stress, Inflammation**	[149]
PEGylated; MWCNT	P- MWCNT; NP-MWCNT; Length of less than 1 μm; Diameter of 10-20 nm	Kunming mice	10 and 60 mg/kg b.w. (Iv) sacrificed at 15 & 60 d	Blackish discoloration of the liver (MWCNTs accumulation); AST, Bag4, Gab1 genes (increased); Infiltration inflammatory cells, cellular necrosis, focal necrosis; Mitochondrial swelling/lysis; NP- MWCNT shows more toxicity than P-MWCNT **Inflammation**	[155]
Carboxylated functionalized SWCNT	lengths of 15–20 μm; Diameter of 15–30 nm	Swiss webster mice	0.25, 0.5 & 0.75 mg/kg b.w. per day for 5 d (Ip)	ROS, LHP, ALT, AST, ALP, (increased); Histological alterations **Oxidative stress**	[153]
Carboxylated functionalized; MWCNTs	lengths of 15–20 μm; Diameter of 15–30 nm	Swiss webster mice	0.25, 0.5 & 0.75 mg/kg b.w. per day for 5 d (Ip)	ROS, LHP, ALT, AST, ALP, (increased); Histological alterations **Oxidative stress**	[154]
MWCNTs	Length 5-50 μm; Diameter 20-30 nm (SEM)	Swiss albino mice	10 and 60 mg/kg b.w. (oral) sacrificed at 7, 14, 21, 28 d	SOD, CAT activity (decreased); Macrophage injury, cellular swelling, unspecific inflammation, spot necrosis, blood coagulation. The sinusoid and hepaticvenule diameter increased by the high dose **Oxidative stress**	[156]
SWCNTs	Length several μm; Diameter 0.8-1.2 nm (TEM)	Wistar rat	7.5 (low), 15 (medium), and 22.5 (high) mg/kg b. w. Intratracheal instillation once for 15 d	ALB, ALP, TP, TC (decreased at high conc.); Focal necrosis, inflammatory cell infiltration, Cellular swelling at centrilobular part, membrane fluidity destruction, impaired amino acid & lipid metabolism **Metabolic disruption, Hepatotoxicity**	[150]
Oxidised MWCNTs	Length 1-2 μm; Diameter 10-30 nm (TEM)	Kunming mice (Cd-MT accumulated mice)	500 μg/mouse for 4 h	ALT, AST, TBil, BUN (increased); Released Cd^{++} from Cd-MT; Adsorb a part of free Cd^{++} **Coexpossure ameliorated hepatotoxicity**	[157]
Carboxylated MWCNTs	Length 12 μm; Diameter 11.5 nm (TEM)	Wistar rat	0.25, 0.50, 0.75 and 1.0 mg/kg b.w. for 5 consecutive days (Ip)	ALT, AST, ALP, GGT (increased); LPO, H_2O_2, CAT, GPx, activity (increased); SOD, GST (decreased); IL-6, IL-1β, COX-1, iNOS, TNF-α (increased); micronucleated polychromatic erythrocytes (MNPCE) **Oxidative stress, Inflammation**	[151]
MWCNTs	Polycrystalline; Length 600-700 nm; Size 650 nm	Albino rat	1 g/kg b. w. (oral) 4 wk	LPO, H_2O_2, TT, CATactivity (increased); SOD, GSH, GPx, GST (decreased); IL-6, IL-1β, COX-1, TNF-α (increased); hydropic degeneration focal hepatic & perivascular hepatic necrosis associated with inflammatory cells, infiltration, sinusoidal leukocytosis, vacuolar degeneration, congestion of central vein **Oxidative stress, Inflammation**	[4]
Carboxylated MWCNTs	diameter: 5–15 nm, length: 0.5-2 μm (TEM)	C57BL/6J mice (NAFLD)	MWCNT; LD-10 mg/kg b.w. HD-30 mg/kg b.w. PbAc LD-150 mg/kg b.w. HD-300 mg/kg	Death at high dose on 5th day. ALT, AST, ALP (decreased); Nonalcoholic steatohepatitis lobular inflammation, hepatic	[152]

	b.w. MWCNT+ PbAc, LD-10 mg/kg +150 mg/kg HD-30 mg/kg +300 mg/kg (Intragastrically) daily for 80 d	fibrosis, steatosis, apoptotic induction in primary hepatocytes of NAFLD mice; SOD, GST, GSH (decreased); H_2O_2, GPx, MDA, LPO (increased); Lipid peroxidation; IL-6, IL-1β and TNF-α (inflammatory cytokines) inhibiting AMPK/PPARγ pathway **Oxidative stress, Inflammation**	

ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AMPK: AMP activated protein kinase; AST: Aspartate aminotransferase; Bag4: BAG cochaperone 4; BUN: Blood urea nitrogen; CAT: Catalase; COX-1,2: Cyclooxygenase-1,2; Cyp2B19: Cytochrome P4502B19; Cyp2C50: Cytochrome P4502C50; Gab1: GRB2 associated binding protein 1; GGT: Gamma glutamyl transferase; GPx: Glutathione peroxidase; GSH: Glutathione; Gsta2: Glutathione S-transferase, alpha2; GST: Glutathione-S transferase; H_2O_2: Hydrogen peroxide; IL-1β: Interleukin-1beta; IL-6: Interleukin-6; iNOS: Inducible nitric oxide synthase; LHP: Lipid hydroperoxide; LPO: Lipid peroxidation; MDA: Malondialdehyde; NAFLD: Non-alcoholic fatty liver disease; O-MWCNT-acid: Oxidized multi-walled CNTs; PPARγ: Peroxisome proliferator-activated receptor-γ; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TBil: Total bilirubin; TC: Total cholesterol; T-MWCNT: Tween-80-dispersed multi-walled CNTs; TNF-α: Tumor necrosis factor alpha; TP: Total protein; TT: Total thiol.

Table 13 Effects and molecular mechanisms underlying CuS/CdS-NPs induced hepatonanotoxicit

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
CdS NPs	5-9 nm (TEM)	Wistar rat	10 mg/kg alternate days for 45 d	Hepatosomatic index (decreased); ALT, AST, ALP, LPO, H_2O_2, NO (increased); GSH (depletion); Cytoplasmic degeneration/coagulation, sinusoidal inflammation, parenchymal degeneratin, mitochondria, peroxisome, microsomes increased in number **Oxidative stress**	[159]
CuS/CdS	8.7 nm	hepatoma cells BEL7402 and L-02 normal liver cells; Balb/c mice	4 mg/kg, i,v injection	SOD, GSH (down regulation); ROS, GSSG, MDA (up regulation) **Oxidative stress**	[158]
$Cu_{2-x}S$	17.8 nm (LNPs); 2.8 nm (SNPs)	Sprague Dawley rats	5 mg/kg through tail vein single dose	ALT, AST, TBA, LDH (increased) ALB (decreased)	[161]

ALB: Albumin, ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, GSH: Glutathione, GSSG: Glutathione disulfid, H_2O_2: Hydrogen peroxide, LDH: Lactate dehydrogenase, LPO: Lipid peroxidation, MDA: Malondialdehyde, NO: Nitric oxide, ROS: Reactive oxygen species, SOD: Superoxide dismutase, TBA: Total bile acid.

Table 14 Effects and molecular mechanisms underlying cobalt NPs induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
Nano-Co	10-40 nm	Normal human liver L02 cells	2.5, 5, 7.5, 10, 20, and 40 μg/mL) for 12 h or 24 h	Modulation of ROS/NLRP3 pathway	[162]

NLRP3: NOD-like receptor protein 3; ROS: Reactive oxygen species.

Nanocellulose modified with oxalate ester

Structural alteration of nanocellulose (CNS) may increase its application but such modification can lead to toxicity. Short-term exposure of Wistar rat to chemically modified CNS (NCD), mainly higher dose showed an elevated level of ALT, and AST in serum, with increased myeloperoxidase (MPO) but decreased CAT, and glutathione peroxidase (GPx) activities, indicating disruption in ROS balance. Further over-expressions of iNOS and Bax in treated groups compared to control suggests oxidative stress-mediated inflammation and induction of apoptosis in hepatocytes[29].

Polystyrene nanoparticles

Polystyrene nanoparticles (PS NP) owe their origin to the degradation of microplastics. In aged -PS NPs (aPS) the oxygen-containing functional groups get increased on its surface. In a recent investigation, comparative toxicity of PS NPs and aPS NPs was done to evaluate their effects on the liver after short-term exposure. Metabolomic, biochemical, and histopathological results reveal that both types of NPs can affect glucose and lipid metabolism through modulating

Table 15 Effects and molecular mechanisms underlying nanoclay, NCD, polystyrene, chytosan induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
Nano-Clay	57.8 ± 12.3 nm & 648.3 ± 232.2 nm	BALB/C mice	1, 5, 10, 20 mg/kg b.w. (Iv) 24 h; Co-administered with Ccl4, paraquat, cisplatin	ALT, AST (increased)	[163]
NCD (modified nanocellulose with oxalate esters)	100 nm (SEM)	Wistar rat	50 & 100 mg/kg b.w. (oral) for 7 d	ALT, AST (increased); CAT, GPx activity (decreased); MPO activity (increased); iNOS, Bax (increased); dialated sinusoidal space, vacuolated hepatocytes, cellular infiltration **Oxidative stress**	[29]
Polystyrene	PS NPs 158.8 ± 1.3 nm; aPS NPs 117.0 ± 1.8 nm (SEM)	ICR mice	50 mg/kg/d (oral) for 7 d	Glucose, HDL-C, TG, TC (increased in blood); LDL-C (decreased in blood); Activation of PI3K/AKT/GLUT4 & SREBP-1/PPARγ/ATGL signaling pathways; TG decomposition; Lipid accumulation (increased); Nuclear pyknosis, blurred intercellular space, central hepatic vein congestion, hepatic ballooning; Compared to PS NPs, aPS NPs showed higher toxicity **Disruption of glycolipid metabolism**	[28]
Chitosan (CsNPs)	18 ± 1 nm (DLS)	BHAL cell	≥ 0.5% w/v for 4 h	Readily internalized; Disrupt membrane integrity; ALT leakage; CYP3A4 enzyme activity (increased); necrotic or autophagic cell death	[27]

ALT: Alanine aminotransferase, aPS: UV aging Polystyrene, AST: Aspartate aminotransferase, ATGL: Adipose triglyceride lipase, Bax: Bcl-2 associated X protein, CAT: Catalase, CYP3A4: Cytochrome P4503A4, GLUT4: Glucose transporter 4, GPx: Glutathione peroxidase, HDL-C: High-density lipoprotein, iNOS: Inducible nitric oxide synthase, LDL-C: Low-density lipoprotein, MPO: Myeloperoxidase, NLRP3: NOD-like receptor protein 3, p-AKT: Phosphoprotein kinase B, PI3K: Phosphatidylinositol 3-kinase, PPARγ: Peroxisome proliferator-activated receptor-γ, PS: Polystyrene, ROS: Reactive oxygen species, SREBP-1: Sterol regulatory element binding protein-1, TC: Total cholesterol, TG: Triglyceride.

PI3K/AKT/GLUT4 and SREBP-1/PPARγ/ATGL signaling pathways respectively. Increased glucose but decreased lipoprotein concentration in serum indicates NPs mediated glycolipid metabolism disruption that provokes the exposed mice to self-regulate various lipoprotein levels in serum. Pyknotic nucleus, congested central vein, unclear sinusoids. vacuolation, hepatocyte ballooning suggests polystyrene NPs mediated liver toxicity[28].

Chitosan nanoparticles

Chitosan molecules being considered biocompatible have been tested for liver toxicity. Compared to the chitosan molecule, CsNPs showed higher cellular uptake though having poor cell adhesiveness. Availability of more ALT in the extracellular space of BHAL cells after 4 h of exposure indicates loss of membrane integrity. In a concentration-dependent manner CYP3A4 activity was seen to increase suggesting activation of defence mechanism for clearance of CsNPs. Also, it caused significant damage to the nucleus and cytoplasm, indicating necrotic cell death of hepatocytes[27].

Hydroxyapatite nanoparticles

Hydroxyapatite NPs (HANP) showed antitumor activity in HepG2 cells within a range of 20-80nm particle size. Its cellular uptake and nuclear localization followed by efficacy was found to diminish with increasing particle size. Treated cells exhibited caspase-3, and caspase-9 activation with increased proapoptotic markers (Bax, Bid) and with a concomitant decrease in Bcl-2 and cytochrome c release from mitochondria to the cytoplasm, confirmed HAPN-mediated activation of mitochondrial-dependent apoptotic pathway[164]. A similar result was documented in another *in vitro* experiment, where incubation of buffalo rat liver (BRL) cells with 80 nm HANPs at 200 μg/mL, exhibited diminished cell viability, LDH leakage, induced apoptosis, and necrosis, and MAPK pathway-mediated cytotoxicity. *In vivo*, study results showed infiltration of inflammatory cells near the portal area, increased WBC count, ALT, AST, and TNF-α in serum of treated rats with increased levels of H_2O_2, MDA suggesting HANPs induced oxidative stress-related liver injury[165]. Follow Table 16 for a comprehensive account.

Quantum dots

Mice with both acute and chronic exposure to cadmium selenium (CdSe) QDs showed predominant liver accumulation. Enlarged central vein and disordered hepatic cords were observed for chronic exposure only. In contrast the *in vitro* study unveiled that, Hepa 1-6 cells (murine liver cells) became condensed and decreased in size while J774A.1 cell (macrophage -substitute for Kupffer cell) became condensed and round. Beta-mercaptoethanol (β-ME) pretreatment was found to attenuate the QDs-induced increase of MDA level, suggesting QDs-induced oxidative stress in the liver involves the production of free radicals with compromised ROS scavengers (GSH-Px) that have provoked cytotoxicity in hepatocytes and macrophages, potentiating impairment of cellular differentiation without causing any death[166]. Similarly, perturbated redox homeostasis in mice treated with Cd/Se/Te-based quantum dot 705 has been documented. Increased levels of copper, zinc, and selenium with trace elements and their corresponding transporters (ZIP8, ZIP14, and CTR-1),

Table 16 Effects and molecular mechanisms underlying hydroxyapatite nanoparticles induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
Hydroxyapatite nanoparticles	50 nm (XRD)	HepG2 cells; L-02 cells	100 μg/mL for 24, 48 h	Caspase-3, 9 (activated); Bax, Bid (upregulated); Bcl-2 (downregulated); Cytosolic appearance of cytochrome c **Apoptosis**	[164]
Hydroxyapatite nanoparticles	80 nm (TEM)	BRL cells; Sprague–Dawley rat	25, 50, 100, 200, 400 and 800 μg/mL for 1 h; 50 mg/kg (Iv) single dose, sacrificed at 48 h	Decreased cell viability; Increased LDH leakage; Induced apoptosis & necrosis; MAPK signaling pathway activation; WBC count, ALT, AST, TNF-α, H_2O_2, MDA (increased); Infiltration of inflammatory cells near portal area **Oxidative stress, inflammation, apoptosis, necrosis**	[165]

ALT: Alkaline phosphatase; AST: Aspartate aminotransferase; Bax: Bcl-2 associated X protein; Bcl2: B-cell lymphoma 2; Bid: BH3 interacting-domain death agonist; H_2O_2: Hydrogen peroxide; LDH: Lactate dehydrogenase; MAPK: Mitogen activated protein kinase; MDA: Malondialdehyde; TNF-α: Tumor necrosis factor alpha.

over-expressed oxidative stress markers (heme oxygenase-1 expression, 8-oxo-7,8-dihydro-2¢-deoxyguanosine) along with reduced SOD, GPx activity, GSH/GSSG ratio indicates oxidative stress. Also upregulated pro-inflammatory mediators (Il-6, TNF-α) and liver markers (ALT, AST) signify liver damage due to oxidative stress-mediated inflammatory response[167]. CdSe/ZnS QDs were also reported to induce oxidative stress, inflammation, pyroptosis, and liver dysfunction. Application of Z-YVAD-FMK (caspase-1inhibitor), 2-APB (Ca^{2+} channel blocker), BAPTA-AM (intracellular Ca^{2+} chelator), NAC (a total ROS scavenger), Mito-TEMPO (a mtROS scavenger) and further silencing NLRP3 was reported to alleviate QDs mediated pyroptosis of hepatocytes, confirming the underlying mechanisms includes intracellular Ca^{2+} mobilization that triggered mtROS generation and subsequent activation of NLRP3 inflammosome leading to caspase-1mediated pyroptosis. A similar result was in agreement when NLRP3 knocked out mice exposed to QDs[168]. On the contrary except QDs accumulation in mitochondria, lysosome, and lipid droplets no significant signs of liver damage were observed when Kunming mice were subjected to Mn-doped ZnS QDs and polyethylene glycol-coated QDs exposure[169]. Similarly except slight increment of liver markers (ALT, AST, ALP) in serum, no such remarkable liver tissue damage was recorded in mice exposed to cadmium-free inidium-based QDs[25]. Again, cadmium telluride (CdTe) QDs administration was found to elevate oxidative stress in AML 12 (murine hepatoma cells alpha mouse liver 12) and mice model, concomitant increased expression pattern of the tumor-suppressor gene (p53), proapoptotic gene (Bax) and decreased level of antiapoptotic marker (Bcl-2) suggests activation of mitochondria-mediated apoptotic pathway in hepatocytes. NF-E2-related factor 2 (Nrf2) deficiency was found to attenuate CdTe-QDs provoked injury and apoptosis suggesting the underlying mechanism involves modulation of the Nrf2 signaling pathway[170]. A series of investigations have proved that mitochondria are the prominent target of CdTe-QDs in hepatocytes. In different cell lines and mice models, it was found that interaction between CdTe-QDs and mitochondrial membrane resulted in mitochondrial enlargement, membrane potential disruption, opening of permeability transition pore, impaired oxidative phosphorylation *via* diminishing activity of electron transport chain enzymes, ROS accumulation, redox damage, ATP depletion and increased PGC-1α. Together all these indicate oxidative mediated stress-mediated release of cytochrome c and Bax to promote intrinsic and extrinsic pathways of apoptosis in CdTe-QDs exposed hepatocytes[9,171-173]. When normal and carcinoma liver cells were incubated with CdTe/CdS QDs for 24 h, both the cells showed similar lysosomal accumulation of QDs followed by abnormal activation of lysosomal enzymes that triggered lysosome-dependent ROS production and autophagy. Inhibition of lysosomal enzymes were also found to prevent ROS production and activation of autophagic flux and thereby rescued hepatocytes from cytotoxic effects of QDs[3] A recent *in vivo* investigation unveils the sub-acute low dose of CdTe QDs uptake leads to both activation of NF-KB pathway through overproduction of ROS that also indirectly regulates NLRP3 inflammasome assembly to trigger inflammatory cascades *via* inflammatory cytokines (IL-1β, TNF-α, IL-6) and activation of Kupffer cells to cause liver tissue injury. In *in vitro* study pretreatment of KUP5 cells with NAC (*N*-acetylcysteine – ROS scavenger) and DHMEQ (Dehydroxymethylepoxyquinomicin- NF-KB translocation inhibitor) before QDs, reversed the activation of Kupffer cells following down-regulation of NF-κB, caspase-1, and NLRP3[174]. A recent study highlights the varied impact of CDs (Carbon Quantum Dots) on liver cells (KUP5 and AML12 cells *in vitro*) and the importance of the TFEB-lysosome pathway in regulating autophagy and apoptosis induced by CDs on liver cells for a comprehensive toxicological safety evaluation[175]. Follow Table 17 for a comprehensive account.

Gold nanoparticles

Gold is generally unreactive in its natural state but becomes reactive in its ionic form. It can also exist as gold salts, allowing the synthesis of nanomaterials with properties like easy synthesis, high particle reactivity, and strong optical characteristics[176,177]. In recent days, gold nanoparticles (AuNPs) have gained considerable attention in various fields, especially in biomedical sciences due to their unique physicochemical properties[178]. Nevertheless, there are many concerns regarding their potential hepatotoxic effects that have raised questions about their safety use in such applications. Numerous inflammatory and cytotoxic responses have been observed with smaller-sized AuNPs in comparison to

Table 17 Effects and molecular mechanisms underlying quantum dots induced hepatonanotoxicity

NPs	Size	Tested model	Dose & route of administration	Effects & mechanism	Ref.
Cd/Se/Te QD705	12.3 ± 5.2 nm (TEM)	ICR mice	100 µL of 40 and 160 pmol (IV) sacrificed at 12 and 16 wk	ALT, AST (increased); GPx, HO-1, 8-oxo-dG (increased); Cu/Zn/Se (increased); SOD activity (decreased); GSH/GSSG; Unbalanced antioxidation systems; Trace metals, trace metal transporters; TNFα, IL-6 (increased) **Oxidative stress and inflammation**	[167]
CdSe QD	4 nm (TEM)	Kunming mice Hepa 1–6 cells	200 nMCdCl2, 20 nM & 200 nM QDs (acute) for 48 h (IP); 20 nMCdCl2, 5 nM & 10 nM QDs for 6 wk (chronic) (IP); 20 nM CdCl2, 5 nM, 10 nM and 20 nM QDs for 24 & 48 h	ROS, MDA (increased); GSH-Px (decreased); Enlarged central vein, disordered hepatic cords; Reduced cell size, condensation; Round and condensed macrophage **Oxidative stress**	[166]
Mn-doped ZnS QDs	3.8 ± 0.1 nm (TEM)	Kunming mice	1 & 5 mg/kg (QDs); 5 mg/kg (QDs PEG) (IV) for 7 da sacrificed on 8th & 28th day	QDs accumulated in mitichondia, lysosome, lipid droplets; No hepatic damage	[169]
CdTe QDs	2.2 nm (TEM)	AML 12; ICR mice	27.66, 41.49, 53.94, 70.12, 91.16 & 118.50 µg/mL for 24 & 48 h. 4.125, 8.25 and 16.5 mg/kg body weight (IV) once a week for 4 wk	LPO, MDA, SOD, CAT, P53, Bcl-2, Nrf2, HO-1 (increased); Bax (decreased); ATP concentration (decreased); Nrf2 signaling pathway activation **Oxidative stress, apoptosis**	[170]
CdTe QDs	7.3 ± 1.2 nm (TEM)	HepG2 cell	10 µg/mL containing 1 µg/mL of cadmium for 24 h	MMP disruption, mitochondrial swelling, increased intracellular ca2+ levels, impaired cellular respiration & decreased ATP synthesis; PGC-1α (increased) **Mitochondrial toxicity & dysfunction**	[171]
CdTe QDs	15.25 ± 0.34 nm (TEM)	BALB/c mice	0.4, 2, 5, 6, 7, and 10 mg/kg b.w (Iv) for 24 h; 5 mg/kg bw (Iv) 2 h, 24 h, 3 d, and 1 wk	Enlarged mitochondria with increment in number; Affects ETC complex & ATP synthesis energy metabolism impairment **Mitochondrial dysfunction**	[172]
CdSe/Zn-QD	7.1 nm (TEM)	L02 cells; C57BL/6 mice; NLRP3 knockout mice	5, 10, 20, 40, 80 nM, 24 and 48 h; 10 nmol/kg (IV) results at 2 wk	Dose-dependent decrease in cell viability pyroptosis; Caspase-1 activity(increased); NLRP3 inflammasome activation; mt ROS production (increased); Cytoplasmic Ca2+ (increased) levels ALT, AST, MPO, TNFα, IL-1β (increased); γ-GT (decreased) **Oxidative stress and inflammation**	[168]
Cd free indium - based QDs	4 nm (TEM)	Lister Hooded rats	12.5 & 50 mg/kg b.w. (Iv) for 24 h. 1 wk, 4 wk	ALT, AST, ALP (slightly increased); No hepatic damage	[25]
CdTe/CdS QDs	12 nm (TEM)	HL-7702; HepG2 cells	1- 32 nM for 48 h	Lysosomal internalization; Abnormal activation of lysosomal enzymes; ROS generation (increased); Autophagy **Apoptosis independent nanotoxicity**	[3]
CdTe QDs	15.25 ± 0.34 nm (TEM)	BALB/c mice	0.4, 2, 5, 6, 7, and 10 mg/kg b.w (Iv) for 24 h. 5 mg/kg b.w. (Iv)2 h, 24 h, 3 d (d), and 1 wk (w)	AST, ALT, T-bil (increased); Albumin (decreased); liver accumulation	[173]
CdTe QDs	15.25 ± 0.34 nm (TEM)	BALB/c mice	0.4, 2, 5, 6, 7, and 10 mg/kg b.w (Iv) for 24 h. 5 mg/kg b.w. (Iv) 2 h, 24 h, 3 d (d), and 1 wk (w)	tGSH, ATP (depletion) GST, CAT (decreased) SOD activity (increased); Hmox I, Ncf-1, Ncf-2 (upregulated expression); PGC-1α (increased) **Oxidative stress, apoptosis**	[9]
CdTe QDs	2.2-3.0 nm (TEM)	ICR mice; KUP5 cells	2.5 & 10 µM/kg·b.w. (Iv) single dose once per wekk for 14 d; 5, 50 & 500 NM	IL-1β, TNF-α, IL-6 (increased); Assembly of NLRP3 inflammasome; ROS productin (increased); Activation of NF-KB pathway; Kupffer cell activation **Oxidative stress, Inflammation**	[174]

8-oxo-dG: 8-oxo-7,8-dihydro-2¢-deoxyguanosine; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ATP: Adenosine triphosphate; Bax: Bcl-2 associated X protein; Bcl-2: B-cell lymphoma 2; CAT: Catalase; Cu-copper; ETC: Electron transport chain; GSH-Px: Glutathione peroxidase; GSSG: Glutathione disulfide; GST: Glutathione S-transferase; HO-1/Hmox I: Heme oxygenase 1; IL-1β: Interleukin 1 β; IL-6: Interleukin-6; LPO: Lipid peroxidation; MDA: Malondialdehyde; MMP: Mitochondrial membrane potential; MPO: Myeloperoxidase; Ncf-1,2: Neutrophil cytosolic factor 1,2; NF-κB: Nuclear factor kappa beta; NLRP3: NOD-like receptor protein 3; Nrf2: Nuclear factor erythroid 2-related factor 2; P53: Tumor suppressor protein p53; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS: Reactive oxygen species; Se: Selenium; SOD: Superoxide dismutase; T-Bil: Total bilirubin; tGSH: Total glutathione; TNFα: Tumor necrosis factor alpha; γ-GT: Gamma glutamyl transferase; Zn: Zinc.

contact with larger-sized AuNPs with the same mass concentration because of their highly reactive role with biological constituents, and have stressed the harmful effects produced by a large number of nanoparticles[179]. AuNPs activate hepatic macrophages and consequently stimulate the occurrence of immune hepatitis and liver dysfunction[180,181]. Serum ALT and AST levels, indicative of liver damage, remained within the normal range in NC (Normal Chow) diet-fed mice 24 h or 7 d after AuNP administration, suggesting AuNPs' non-toxicity under normal diet conditions[182]. Conversely, MCD (methionine and choline-deficient) diet-fed mice exhibited elevated ALT and AST levels post-AuNP administration, indicating hepatotoxicity. The experiment revealed that MCD diets induced hepatic TG accumulation through the inhibition of mitochondrial beta-oxidation and blocking hepatic export of very low-density lipoprotein, but AuNP-induced hepatotoxicity was attributed to increased inflammatory response and apoptosis, not accumulated TG contents[182]. Intravenously injected AuNPs rapidly accumulate in Kupffer cells in the liver, stimulating these cells and leading to increased monocyte function, upregulated cytokine secretion, and subsequent liver damage through enhanced necrosis, apoptosis, and abnormal ROS production[183]. The toxicity of AuNPs is associated with their capacity to stimulate inflammatory responses and accelerate stress-induced apoptosis, with smaller nanoparticle sizes contributing to toxicity[184]. AuNPs induce hepatocellular injury through ROS generation, promoting oxidative stress[185]. This oxidative stress, characterized by lipid peroxidation, protein damage, and DNA modifications, is exacerbated by inflammatory responses and pro-inflammatory cytokines. The correlation between nanoparticles and oxidative stress suggests fatty acid peroxidation as a probable cause for AuNP-triggered DNA destruction[186]. Khan *et al*[187] measured oxidative stress markers in rats exposed to AuNPs, revealing increased MDA levels specifically in the liver, indicating AuNPs' liver-specific oxidative stress. The mutagenic and carcinogenic nature of MDA, a product of fatty acid peroxidation, suggests its potential to combine with DNA, leading to DNA damage and potentially activating programmed cell death pathways[187]. Research has shown that AuNPs can enter hepatocytes through various mechanisms, including endocytosis and direct penetration of the cell membrane[188,189]. Once internalized, these may accumulate in specific subcellular compartments, such as the ER or mitochondria which leads to inducing organelle-specific toxicity. The disruption of cellular organelles can trigger a cascade of events leading to hepatocellular damage [190,191]. Cell migration, crucial for mammalian cell survival and differentiation and regulated by external signals, was significantly reduced by 70% in HeLa cells treated with MUAM-AuNPs, as demonstrated in a gap-filling assay by Lee *et al*[192]; this reduction was attributed to the loss of long F-actins aligned with the migration axis, impacting migration-related signaling pathways, disrupting extracellular matrix organization, and ultimately impeding cell migration[192-194]. Additionally, AuNPs induced differential gene expression in treated samples, involving both upregulated and downregulated genes associated with cellular metabolism, protein catabolism, cell cycle, and G1/S transition; notably, downregulation of genes related to the G1 phase and nucleic acid metabolism suggested inhibition of DNA synthesis. In a separate experiment, 1.4-nm triphenyl monosulfonate (TPPMS)-coated AuNPs caused necrotic cell death through elevated oxidative stress and loss of mitochondrial potential, while Tiopronin-coated AuNPs induced necrosis *via* increased ROS production and apoptosis due to mitochondrial dysfunction; citrate AuNPs also exhibited dose-dependent ROS production leading to apoptosis[195,196]. Moreover, Au clusters significantly increased ROS production by inhibiting TrxR1 activity, inducing apoptosis, and disrupting mitochondrial membrane polarization[197]. Finally, irradiation in the presence of AuNPs led to an interaction with the cell membrane protein disulfide isomerase, disrupting thiol balance, causing cellular redox imbalance, and ultimately inducing oxidative stress[196].

Silver nanoparticles

Silver nanoparticles (AgNP)-intoxication significantly disturbs normal liver function, elevates hepatic lipid peroxidation, increases liver DNA damage, and induces biochemical and histological alterations in rats[198]. The toxicity of AgNPs mainly originates from the degraded forms of AgNPs, the "particle-specific effect" or the triggered oxidation stress[199]. After cellular intake, these (AgNPs) would enter the acidic endo/Lysosomes (pH4.5–6.5) and undertake chemical transformation from particulate silver to elemental silver, Ag+, Ag-O- and Ag-S- species[200]. The Ag+ released from AgNPs dissolution is thought to bind intracellular sulfhydryl group (−SH)-containing molecules and leads to cytotoxicity, which is known as the "Trojan-horse" mechanism[199]. AgNPs also help in intracellular ROS production and cause cellular damage, *e.g.* genotoxicity, mitochondrial dysfunction, and cell membrane damage[201]. Ag ions have been reported to cause disturbance and destruction of mitochondrial function through interaction with thiol groups of inner mitochondrial membrane proteins and AgNPs decrease the activity of mitochondrial respiratory chain complexes and reduce antioxidant factors like glutathione, thioredoxin, superoxide dismutase, and N-acetylcysteine in liver cells[202, 203]. Xu *et al*[201] investigated two normal hepatic cell lines (NCTC1469 and L-02) and two hepatoma cell lines (Hepa1–6 and HepG2) to assess the cytotoxicity of AgNPs. They have shown AgNPs could certainly lead to intra-cellular oxidation stress and cytotoxicity through acting GST molecules and thus suppressing its enzyme activity, although GST expressions were not significantly affected. The research also highlighted the binding of High Molecular Weight proteins to Ag+ became saturated and more Low molecular weight molecules (*e.g.* metallothionein) were continually synthesized by cells

to neutralize AgNPs and Ag+ for detoxification. It indicates that the dissolution of internalized AgNPs resulted in the formation of Ag-protein complexes. As a consequence, the damage of protein molecules by AgNPs and Ag+ would destroy the intra-cellular homeostasis of the liver. Assar *et al*[204] pointed out that after 15 and 30 d of exposure to the maximum dose of AgNPs in rats, a drop in liver weight was observed to a striking rise in lipid peroxidation, leading to structural changes to lipid vacuoles. This finding also showed that a state of oxidative injury was provoked by silver nanoparticles in a dose-dependent way by the raised hepatic MDA (malondialdehyde) levels and the depletion of the antioxidant defensive mechanism by reducing the hepatic reduced glutathione (GSH) levels. The most severe hepatic damage was associated with increasing the AgNP-administered dose and expanding exposure time. Research findings from Matés[205], Srivastava *et al*[206], Ansar *et al*[207], and Piao *et al*[208] have detailed that continuous elevation of Ag+ concentration leads to continuous induction of hydroxyl radical, ultimately consumes more intracellular GSH, and disturbing the homeostasis of free radical scavenging. AgNPs raised MDA levels causing oxidative damage in rats[209]. Many studies support that the liver is the main target organ for AgNP action. The histological assessment of the liver indicated pathological changes that were dose and time-dependent and happened in the liver after 30 d of increasing concentrations of AgNP exposure. Sooklert *et al*[210] and Elje *et al*[111] showed that low levels of dissolved Ag were found in the Ag-NPs exposure shortly after exposure in the HepG2 human liver cells, and the amounts were lower than the measured EC50 for cytotoxicity of $AgNO_3$ and identified six genes from HepG2 Liver cells, with three showing significant up-regulation of FOS and JUN, and two demonstrating up-regulation of EGR1, CXCL8, HSPB1, and MT2A. Notably, high-dosage AgNP exposure increased fold changes in genes associated with cell proliferation (FOS, JUN, and EGR1)[210]. An increased intracellular level of ROS can also activate cell-death-regulating pathways, such as p53, AKT, and MAP kinase[185]. Microscopic images revealed nuclear membrane distortion, blebbed nuclei formation, and accumulation of autophagic vacuoles in AgNP-treated liver cells, along with increased mitochondria, cytoplasmic vacuoles containing silver nanoparticles, and swollen lipid droplets. In hepatocytes, CEBPA (CCAAT enhancer binding protein alpha) is highly expressed and plays a critical role in regulating many metabolic liver genes, while CEBPB (CCAAT enhancer binding protein beta) is up-regulated during liver regeneration and plays a crucial role in the development of liver or acute inflammatory response[211]. The proto-oncogenes FOS and JUN, which are known to play important roles in both cell survival and the signaling pathway involved in hepatotoxicity, were highly up-regulated in the presence of AgNPs. In addition to that, heat shock protein family members HSPB1, HSPA4L, and HSPH1 were also significantly up-regulated[212]. Xin *et al*[213], reported that AgNPs induced oxidative stress, and consequently increased expression of heat shock protein and heme oxygenase (HMOX1) in both liver and lung cells. Sooklert *et al*[210] identified 24 interesting candidate genes as possible targets of AgNP-induced hepatocellular toxicity. SOX15, a highly upregulated gene, acts as a transcription activator involved in embryonic development regulation and cell fate determination. TLL1, the most noticeable down-regulated gene, is necessary for various developmental events. AgNPs may exert cytotoxic effects through SOX15 upregulation or TLL1 downregulation in hepatic cells. Deregulated autophagy after AgNP treatment was also seen which may lead to increased cell death either independently or synergistically with apoptosis or necrosis[214]. Wen *et al*[215] and Recordati *et al*[216] observed increased hepatocellular necrosis and gall bladder hemorrhage in mice injected with AgNPs, particularly with 10nm AgNPs. AgNP administration induced exacerbated hepatic steatosis, heightened liver injury, and elevated risk of NAFLD development and progression[215,216]. The effects were attributed to hyperactivation of SREBP-1c-mediated de novo lipogenesis, pro-inflammatory cytokine activation, and increased oxidative stress and DNA methylation[216]. Kim *et al*[217] demonstrated that cAgNPs (citrate-coated and stabilized) caused significant changes in ALP and LDH levels, indicating liver tissue damage persisting up to 28 d after exposure and suggesting prolonged impairment of liver structure and functions following a single exposure. From Lee *et al*[218], it was reported that the deposited AgNPs in hepatocytes were found to be individual particles with a size smaller than 100 nm in diameter. AgNPs accumulated in hepatocytes' endosomes and lysosomes, with additional deposition in Kupffer cells (> 100 nm agglomerates)[218]. Kupffer cells played a role in inflammation observed with mild inflammatory cell infiltration in portal vein areas. Elevated ALT and AST levels indicated liver damage persisting up to one month after AgNP administration[218]. Maternal exposure to AgNPs *via* the intragastric route led to increased silver content in rat offspring livers, causing a significant reduction in body weight and dilated blood vessels. Liver damage, indicated by vacuolation and lipid peroxidation, was associated with elevated caspase-9 concentration, suggesting AgNPs induce apoptosis through the intrinsic pathway in offspring livers[219].

CONCLUSION

In summary, the extensive examination sheds light on the intricate landscape of hepatotoxicity induced by various nanoparticles (NPs), revealing distinct mechanisms and effects associated with different nanomaterials. Size-dependent hepatotoxicity is observed in SiNPs, with smaller particles causing more severe liver injury. The combined toxicity of SiNPs with other liver toxins highlights potential synergies in NP-induced liver damage. Oxidative stress, inflammation, apoptosis, and genotoxicity are induced by NiO-NPs, WO3 NPs, Nano-CuO, and other nanomaterials, illustrating the complexity of NP-mediated hepatotoxic effects (Figures 1-3).

Integrative omics analyses identify key proteins and disrupted metabolic pathways in SiNP-induced hepatotoxicity, underscoring the necessity for a multifaceted understanding of NP-induced liver damage. CNTs, including SWCNTs and MWCNTs varieties, contribute to hepatotoxicity through inflammatory responses and oxidative stress, with variations in toxicity observed among different types of CNTs.

Moreover, exposure to CuS/CdS-NPs, cobalt nanoparticles, nanoclay particles, nanocellulose, polystyrene nanoparticles, chitosan nanoparticles, hydroxyapatite nanoparticles, quantum dots, and gold nanoparticles elucidates

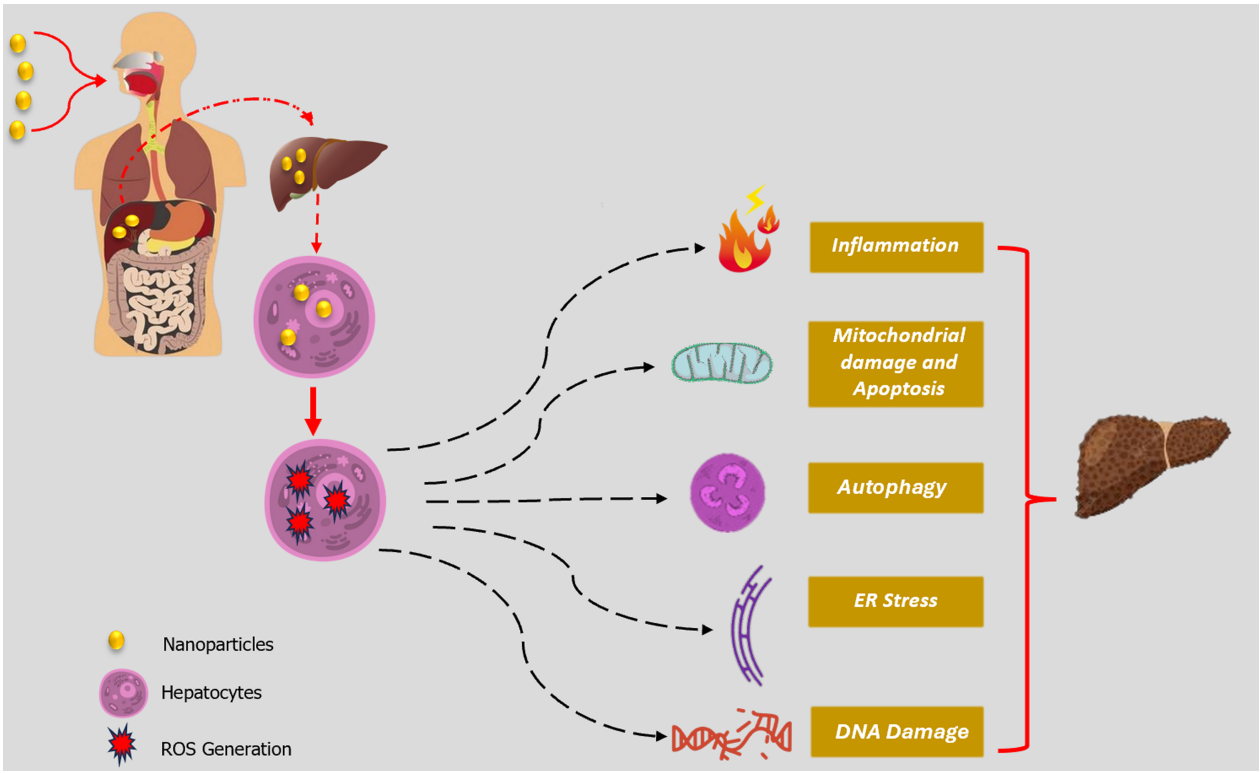

Figure 3 Different modalities of nanoparticles induced hepatotoxicity. ER: Endoplasmic reticulum; ROS: Reactive oxygen species.

diverse hepatotoxic effects, underscoring the importance of considering nanoparticle characteristics in toxicity assessments.

Despite these toxicities, it is noteworthy that nanoparticles play a pivotal role in diverse biomedical applications, showcasing their versatility and impact. In cancer therapy, catalytic strategies employing substances like hydrogen peroxide and glucose, alongside biocompatible nanomaterials, promise efficient treatment with minimal side effects[220]. Nanomaterials contribute significantly to the fight against coronavirus disease 2019, aiding in rapid diagnostics, vaccine development, and therapeutic interventions[221]. Transition metal-based nanoparticles, particularly those with anisotropic shapes, offer unique properties for biomedical applications, including drug delivery and imaging[222]. Precision nanoparticles (PNPs) emerge as discrete structures with precisely tailored heterogeneity, addressing challenges associated with uncontrolled nanoparticle variability[223]. PNPs significantly enhance the performance of nanoparticle-based vehicles in various biological processes, presenting a promising avenue for improved biomedical outcomes.

Therefore, the study concludes by emphasizing the urgent need for a comprehensive understanding of NP-induced hepatotoxicity to ensure the safe use of nanomaterials, suggesting further *in vivo* studies and exploration of potential protective strategies. Additionally, the proposal of herbal gold nanoparticles as a potential hepatoprotective agent opens avenues for future research and development in the field. Overall, the findings underscore the complexity and diversity of nanomaterial-induced hepatotoxicity, emphasizing the importance of continued research for safer nanomaterial applications in various contexts.

FOOTNOTES

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Author contributions: Das SK, Sen K, Ghosh B, Ghosh N and Sinha K performed the research and wrote the manuscript; Sinha K and Sil P conceptualized and designed the study; Das SK drew the figures; all authors have read and approved the final manuscript. Sil PC initiated and conceptualized the review, bringing extensive experience and expertise in the field of hepatotoxicity and nanomaterials. Sil PC oversaw the overall design of the review, provided critical insights into the interpretation of scientific literature, and ensured the accuracy and relevance of the content presented. Additionally, Sil PC played a pivotal role in synthesizing complex scientific concepts and findings, contributing significantly to the intellectual content and scholarly rigor of the manuscript. Sinha K was selected as co-corresponding author based on his demonstrated scientific acumen, research leadership, and ability to effectively communicate and collaborate with co-authors. Sinha K actively participated in the review process, conducting thorough literature reviews, analyzing data, and synthesizing key findings. Moreover, Sinha K played a crucial role in manuscript preparation, including drafting sections, revising content based on feedback, and ensuring the coherence and clarity of the final manuscript.

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ORIGINAL ARTICLE

Case Control Study

Expression and clinical significance of short-chain fatty acids in patients with intrahepatic cholestasis of pregnancy

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Abstract

BACKGROUND

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific liver condition that typically arises in the middle and late stages of pregnancy. Short-chain fatty acids (SCFAs), prominent metabolites of the gut microbiota, have significant connections with various pregnancy complications, and some SCFAs hold potential for treating such complications. However, the metabolic profile of SCFAs in patients with ICP remains unclear.

AIM

To investigate the metabolic profiles and differences in SCFAs present in the maternal and cord blood of patients with ICP and determine the clinical significance of these findings.

METHODS

Maternal serum and cord blood samples were collected from both patients with ICP (ICP group) and normal pregnant women (NP group). Targeted metabolomics was used to assess the SCFA levels in these samples.

RESULTS

Significant differences in maternal SCFAs were observed between the ICP and NP

groups. Most SCFAs exhibited a consistent declining trend in cord blood samples from the ICP group, mirroring the pattern seen in maternal serum. Correlation analysis revealed a positive correlation between maternal serum SCFAs and cord blood SCFAs [r (Pearson) = 0.88, *P* = 7.93e-95]. In both maternal serum and cord blood, acetic and caproic acids were identified as key metabolites contributing to the differences in SCFAs between the two groups (variable importance for the projection > 1). Receiver operating characteristic analysis demonstrated that multiple SCFAs in maternal blood have excellent diagnostic capabilities for ICP, with caproic acid exhibiting the highest diagnostic efficacy (area under the curve = 0.97).

CONCLUSION

Compared with the NP group, significant alterations were observed in the SCFAs of maternal serum and cord blood in the ICP group, although they displayed distinct patterns of change. Furthermore, the SCFA levels in maternal serum and cord blood were significantly positively correlated. Notably, certain maternal serum SCFAs, specifically caproic and acetic acids, demonstrated excellent diagnostic efficiency for ICP.

Key Words: Intrahepatic cholestasis of pregnancy; Short-chain fatty acids; Maternal serum; Cord blood; Caproic acid

Core Tip: Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific liver condition that typically arises in the middle and late stages of pregnancy. Short-chain fatty acids (SCFAs), prominent metabolites of the gut microbiota, have significant connections with various pregnancy complications. This work assesses the SCFA levels in maternal serum and cord blood samples which are collected from both patients with ICP and normal pregnant women by using targeted metabolomics, then the correlation between maternal and cord blood SCFAs are explored. At the same time, the clinical diagnostic potential of key differential SCFAs are assessed.

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INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific liver condition that typically arises in the middle and late stages of pregnancy. ICP is characterized by increased bile acid levels in maternal blood and persistent skin itching [1]. Although the clinical symptoms in patients with ICP often subside quickly after delivery, the perinatal mortality rate for fetuses and newborns can be as high as 5%. Additionally, ICP tends to recur in up to 70% of subsequent pregnancies [2]. While the exact pathogenesis of ICP remains incompletely understood, it is believed to be influenced by genetic, hormonal, and environmental factors[2].

Recent research has shed light on the role of gut microbiota and their metabolites in the progression of ICP[3]. Short-chain fatty acids (SCFAs), prominent metabolites of the gut microbiota, play a pivotal role in preserving host metabolism, maintaining intestinal barrier function, fostering immune tolerance, and regulating autoimmune activity[4]. SCFAs have significant connections with various pregnancy complications[5], and some SCFAs hold potential for treating such complications[6]. However, the metabolic profile of SCFAs in patients with ICP remains unclear. Hence, we herein used targeted metabolomics technology to analyze SCFAs in the maternal and cord blood of both patients with ICP and normal pregnant (NP) women. The primary objectives were to investigate the changes in SCFAs in patients with ICP and their offspring, examine the correlation between maternal and umbilical blood SCFAs, and assess their clinical significance and diagnostic value. Ultimately, the study aims to identify novel biomarkers for diagnosing and treating ICP by focusing on gut microbiota metabolites.

MATERIALS AND METHODS

Study population

In this study, we selected 34 patients with ICP (ICP group) who delivered at our hospital between October 2020 and March 2022 to comprise the study group. Additionally, we included 30 NP women from the same period as controls (NP group), based on predefined inclusion and exclusion criteria. The inclusion criteria encompassed meeting the diagnostic standards for ICP as established by the Chinese Medical Association[7] and having reached a gestational age of ≥ 28 wk with no other pregnancy-related complications. Furthermore, participants needed to volunteer for the study and provide informed consent.

The exclusion criteria specified that participants should not have a history of major diseases affecting organs or systems, including cardiovascular, cerebrovascular, pulmonary, hepatic, renal, or endocrine conditions. They should also be free from severe internal or external complications apart from ICP and should not have taken antibiotics, probiotics, or prebiotics within one month before the sample collection. Additionally, they should not have experienced diarrhea or other gastrointestinal symptoms. The study received approval from the Ethics Committee, and all participants signed written informed consent forms. All methods used in this study adhered to the principles of the Helsinki Declaration.

Collection of clinical data and biological samples

The demographic and clinical information for both groups of participants, encompassing data such as age, pre-pregnancy body mass index, and pregnancy-related weight gain, was meticulously collected. Fasting venous blood samples were obtained from the subjects before delivery, and umbilical cord blood samples were collected during the delivery process. Following collection, the samples were promptly stored at 4 °C and allowed to stand for a duration of 4 h. Subsequently, the samples underwent centrifugation, and the resulting serum was extracted and stored at −80 °C.

Furthermore, clinical indicators, including but not limited to hemoglobin, total bile acid, and D-dimer levels; fetal biparietal diameter; abdominal circumference; and fetal birth weight, were extracted from the medical record system. These clinical indicators encompassed blood parameters before delivery, fetal ultrasound data, and the outcome of the pregnancies. The ultrasound metrics for the fetuses were derived from the last obstetric ultrasound conducted by the subjects within one week before the delivery.

Detection and analysis of SCFAs

The maternal and cord blood samples were meticulously collected and subjected to a process involving a mixture of 50% H_2SO_4 and an extraction solution. This extraction solution comprised an internal standard, 2-methyl pentanoic acid (25 mg/L), and methyl tert-butyl ether. The procedure involved a sequence of steps, including vortexing, oscillation, low-temperature ultrasound, and centrifugation, culminating in allowing the mixture to stand before extracting the supernatant. Subsequently, the supernatant underwent detection of SCFAs, specifically acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid, valeric acid, and caproic acid, utilizing a gas chromatography-mass spectrometer (SHIMADZU GC2030-QP2020 NX, J&W Scientific, Folsom, CA, United States).

To assess intergroup differences in SCFAs between maternal and cord blood samples, principal component analysis and orthogonal projections to latent structures discriminant analysis were used. Important differential metabolites were identified based on variable importance for the projection (VIP) values. Cluster and correlation analyses of SCFAs were conducted using R software (v.4.2.2), utilizing the pheatmap package, cor() function, and cor.test() function. Furthermore, potential biomarkers were evaluated and screened using the receiver operating characteristic (ROC) curve.

For pathway analysis, the seven types of SCFAs were integrated into the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, leading to the identification of 24 pathways (Supplementary Table 1). Finally, the KEGG pathway enrichment results were visualized using the website https://www.bioinformatics.com.cn.

Statistical analysis

Statistical analysis was conducted using SPSS (v.26.0) and GraphPad Prism (v.8.0.2) software. The Kolmogorov-Smirnov test was used to inspect the normality and homogeneity of variance of all the data. For quantitative data that adhered to a normal distribution, group-wise statistical differences were assessed using Student's *t*-tests. The data was presented as mean ± SD. In cases where the quantitative data did not follow a normal distribution, the Wilcoxon rank sum test was used. Values were presented as median and interquartile range (IQR) for data that were not normally distributed for continuous variables. Categorical data was expressed as percentages (%), and the statistical distinctions between groups were examined using the chi-square test or Fisher's exact test. To explore the correlation between SCFAs and clinical indicators, the Spearman correlation coefficient was used. Additionally, the relationship between maternal serum and cord blood SCFAs was analyzed using linear regression and the Pearson correlation coefficient. The resultant plots were generated utilizing R software (v.4.2.2), particularly with the ggplot2 (v.3.4.2) package. Statistical significance was defined as $P < 0.05$.

RESULTS

Clinical data of subjects

In this study, a total of 64 subjects were included, 34 with ICP and 30 NP women. The baseline data and clinical indicators for all participants were presented in Supplementary Tables 2-5, with Table 1 highlighting the indicators that exhibited statistical differences. The analysis revealed no statistically significant differences ($P > 0.05$) between the two groups in terms of age, years of education, and the number of pregnancies and births. However, the ICP group exhibited lower uterine height, and abdominal circumference during pregnancy than the NP group ($P < 0.05$). Also, Compared with the NP group, the ICP group showed a lower trend in weight gain during pregnancy ($P > 0.05$). Additionally, the hemoglobin level of the ICP group was significantly lower than that of the NP group ($P < 0.05$), while the serum alanine aminotransferase, total bile acid, and glycyrrhetinic acid levels were significantly higher in the ICP group ($P < 0.001$).

Pre-delivery ultrasound examinations indicated that the ICP group had smaller biparietal diameter, fetal head circumference, abdominal circumference, and femoral length than the NP group ($P < 0.05$). Furthermore, perinatal outcomes revealed that the ICP group had an earlier gestational delivery, a higher rate of cesarean section, and lower birth weights

Table 1 Maternal and fetal clinical characteristics of patients with intrahepatic cholestasis of pregnancy and normal pregnant group, mean ± SD

Index		NP (*n* = 30)	ICP (*n* = 34)	*P* value
Baseline information	Maternal fundal height (cm)	34.60 ± 2.43	32.79 ± 3.19	0.014
	Maternal abdominal circumference (cm)	102.17 ± 5.89	97.47 ± 8.73	0.016
Blood routine	Hemoglobin (g/L)	4.20 ± 0.60	3.79 ± 0.44	0.003
	Hematocrit	127.33 ± 12.14	117.41 ± 12.32	0.002
	Leukocyte (× 10^9/L)	10.70 ± 3.23	8.10 ± 2.34	< 0.001
	Percentage of neutrophils (%)	77.96 ± 6.69	72.50 ± 8.86	0.008
Liver function	Direct bilirubin (μmol/L)	1.45 ± 0.73	2.37 ± 2.09	0.018
	Glutamic-pyruvic transaminase (U/L)	9.40 ± 3.19	38.33 ± 48.76	< 0.001
	Total bile acid (μmol/L)	2.65 ± 1.35	22.64 ± 18.11	< 0.001
	Cholyglycine (mg/L)	1.38 ± 0.18	6.58 ± 8.92	< 0.001
Fetal ultrasound	Biparietal diameter (mm)	93.40 ± 3.56	91.49 ± 4.09	0.032
	Fetal head circumference (mm)	334.40 ± 11.03	327.86 ± 12.40	0.029
	Fetal abdominal circumference (mm)	337.20 ± 19.36	326.57 ± 18.67	0.028
	Fetal femur length (mm)	71.47 ± 3.47	68.89 ± 3.87	0.007
Pregnancy outcome	Gestational week of delivery (wk)	39.39 ± 0.84	37.82 ± 1.64	< 0.001
	Vaginal delivery [*n* (%)]	23(76.67)	11(32.35)	< 0.001
	Neonatal birth weight (g)	3476.00 ± 436.12	3063.14 ± 386.62	< 0.001

ICP: Intrahepatic cholestasis of pregnancy; NP: Normal pregnant.

for newborns than the NP group ($P < 0.001$).

Characteristics of altered maternal serum SCFAs metabolic spectrum in the ICP group

The metabolic profile of maternal serum SCFAs in the ICP and NP groups exhibited significant differences, as indicated by the results of targeted metabolomics quantitative analysis (Figure 1A and B). In the serum of the ICP group, all SCFAs, except for isobutyric acid, demonstrated a decreasing trend (Figure 1C-E). Notably, acetic acid and caproic acid were identified as key metabolites responsible for the differences in serum SCFAs between the two groups, each with a VIP value greater than 1 (Figure 1C). Quantitative analysis further confirmed that acetic acid represented the most abundant SCFA in both the ICP and NP groups. Furthermore, isobutyric acid displayed a notable difference in serum content between the two groups, with significantly higher levels in the ICP group than the NP group, while being present at very low levels in the NP group (Figure 1E).

Characteristics of SCFAs metabolic spectrum changes in cord blood of the ICP group

The metabolic profile of SCFAs in cord blood was investigated in both ICP and NP groups. Targeted metabolomics analysis revealed that there were no significant differences in the metabolic profiles of SCFAs between the two groups (Figure 2A and B). However, the VIP diagram indicated that acetic acid and caproic acid were key metabolites contributing to the differences in SCFAs between the two groups, each with a VIP value greater than 1 (Figure 2C). Most SCFAs exhibited a decreasing trend in the ICP group compared with the NP group (Figure 2D and E). Among the seven SCFAs detected, the acetic acid level was the highest in cord blood, and its level was significantly lower in the ICP group than in the NP group ($P < 0.01$, Figure 2E). Additionally, the isobutyric acid levels in cord blood showed an opposite trend to that in maternal serum, with a significant decrease observed in the ICP group compared with that in the NP group ($P < 0.01$, Figure 2E).

Cord blood SCFAs' correlation with SCFAs found in maternal serum

Further analysis was conducted to explore the correlation between SCFAs in maternal blood and SCFAs in cord blood. The stacked bar chart depicting the percentage of SCFAs in the blood samples of the subjects (Figure 3A) highlighted that the isobutyric acid and caproic acid levels in the total SCFAs of maternal serum were exceptionally low but significantly increased in cord blood SCFAs. Notably, the acetic acid levels were the highest in both maternal serum and cord blood. However, the acetic acid levels were notably lower in cord blood than in maternal serum (75.18% *vs* 84.99%).

The scatter plot of the linear regression (Figure 3B-D) indicated a robust positive correlation between the total SCFAs in cord blood of the ICP and NP groups and the SCFAs in maternal serum ($P < 0.001$). These findings suggest a close

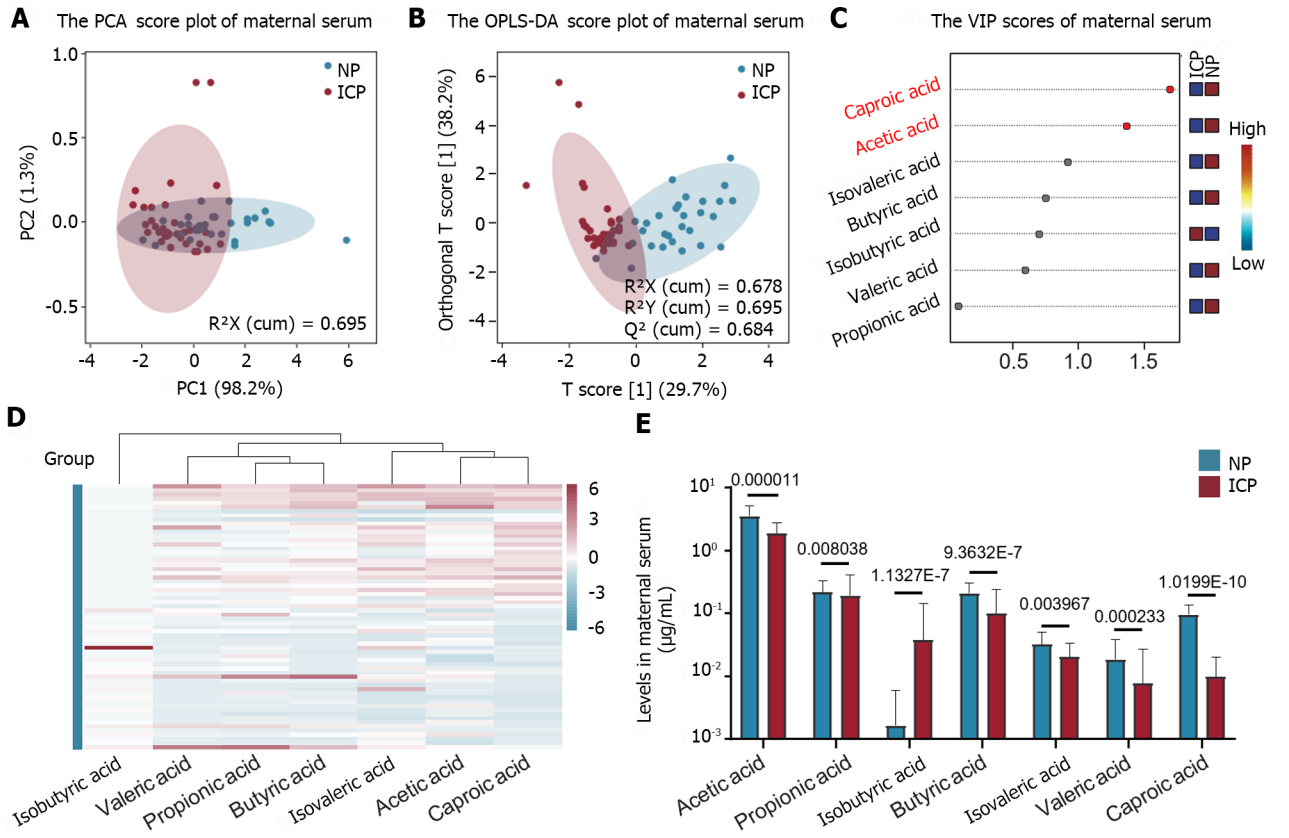

Figure 1 Characteristics of altered maternal serum short-chain fatty acids metabolic spectrum in intrahepatic cholestasis of pregnancy patients. A: Principal Component Analysis score plot: intrahepatic cholestasis of pregnancy (ICP) patients displayed a clear distinction from the normal pregnant women; B: Orthogonal Projections to Latent Structures Discriminant Analysis score plot: ICP patients showed a clear distinction from the normal pregnant women; C: Variable importance for the projection diagram: The differences in the maternal serum short-chain fatty acids (SCFAs) between the two groups of subjects can be attributed to the significant impact of acetic acid and caproic acid as crucial metabolites; D: Heat map: All SCFAs in the serum of ICP patients, except for isobutyric acid, demonstrated a decreasing trend; E: Quantitative analysis bar chart: Acetic acid showed the highest concentration in maternal serum. The content of isobutyric acid displayed the most significant difference between the two groups, thereby exhibiting extremely low levels in the normal pregnant women. NP: n = 30; ICP: n = 34. PCA: Principal Component Analysis; SCFAs: Short-chain fatty acids; OPLS-DA: Orthogonal Projections to Latent Structures Discriminant Analysis; VIP: Variable Importance for the Projection.

correlation between SCFAs in cord blood and maternal serum. However, among all subjects, only acetic acid and caproic acid exhibited a significant positive correlation for an individual SCFA (P < 0.05, Table 2).

Clinical significance and functional analysis of differential SCFAs

To further explore the correlation between SCFAs and clinical indicators, SCFAs in maternal serum and cord blood from the two subject groups with statistically different clinical indicators were analyzed (Spearman analysis, Figure 4A). The results revealed that the correlation between acetic acid and caproic acid in maternal serum and cord blood exhibited a consistent trend with clinical indicators. For instance, both of these SCFAs displayed a negative correlation with total bile acid levels (P < 0.05), and caproic acid demonstrated a significant positive correlation with neonatal birth weight (P < 0.001). However, isobutyric acid showed a significant positive correlation with total bile acids in maternal serum but exhibited an opposite trend in cord blood (P < 0.05). ROC curve analysis showed that multiple SCFAs in maternal serum possessed excellent diagnostic capabilities for ICP (area under the curve > 0.8), with caproic acid demonstrating the highest diagnostic efficacy (area under the curve = 0.97, Figure 4B). To gain insights into the function of SCFAs, a KEGG pathway enrichment analysis was conducted. It was observed that SCFAs participate in 24 pathways, primarily involving metabolic and biological pathways, such as carbohydrate metabolism, energy metabolism, the digestive system, and the nervous system (Supplementary Table 5, Figure 4C). Among these pathways, the digestion and absorption of proteins in the digestive system were predominantly influenced by SCFAs. Acetic acid was involved in most pathway metabolisms, isobutyric acid participated in four of them, while caproic acid was not linked to the mentioned pathways. For detailed information, please refer to Supplementary Table 5, which may provide valuable insights into the metabolism and digestive status of the ICP group.

Table 2 Pearson's correlation between maternal serum short-chain fatty acids and cord blood short-chain fatty acids

	ALL		NP		ICP	
	r (Pearson)	*P* value	r (Pearson)	*P* value	r (Pearson)	*P* value
Acetic acid	0.33	0.03	0.03	0.90	-0.24	0.45
Propionic acid	9.42E-04	1.00	0.02	0.93	-0.12	0.70
Isobutyric acid	-0.21	0.19	0.21	0.35	-0.17	0.61
Butyric acid	0.11	0.48	0.05	0.82	0.35	0.26
Isovaleric acid	0.05	0.76	-0.12	0.59	0.04	0.89
Valeric acid	-0.17	0.29	-0.23	0.30	-0.03	0.92
Caproic acid	0.38	0.01	0.22	0.33	0.18	0.58

ALL: Intrahepatic cholestasis of pregnancy and normal pregnant; ICP: Intrahepatic cholestasis of pregnancy; NP: Normal pregnant.

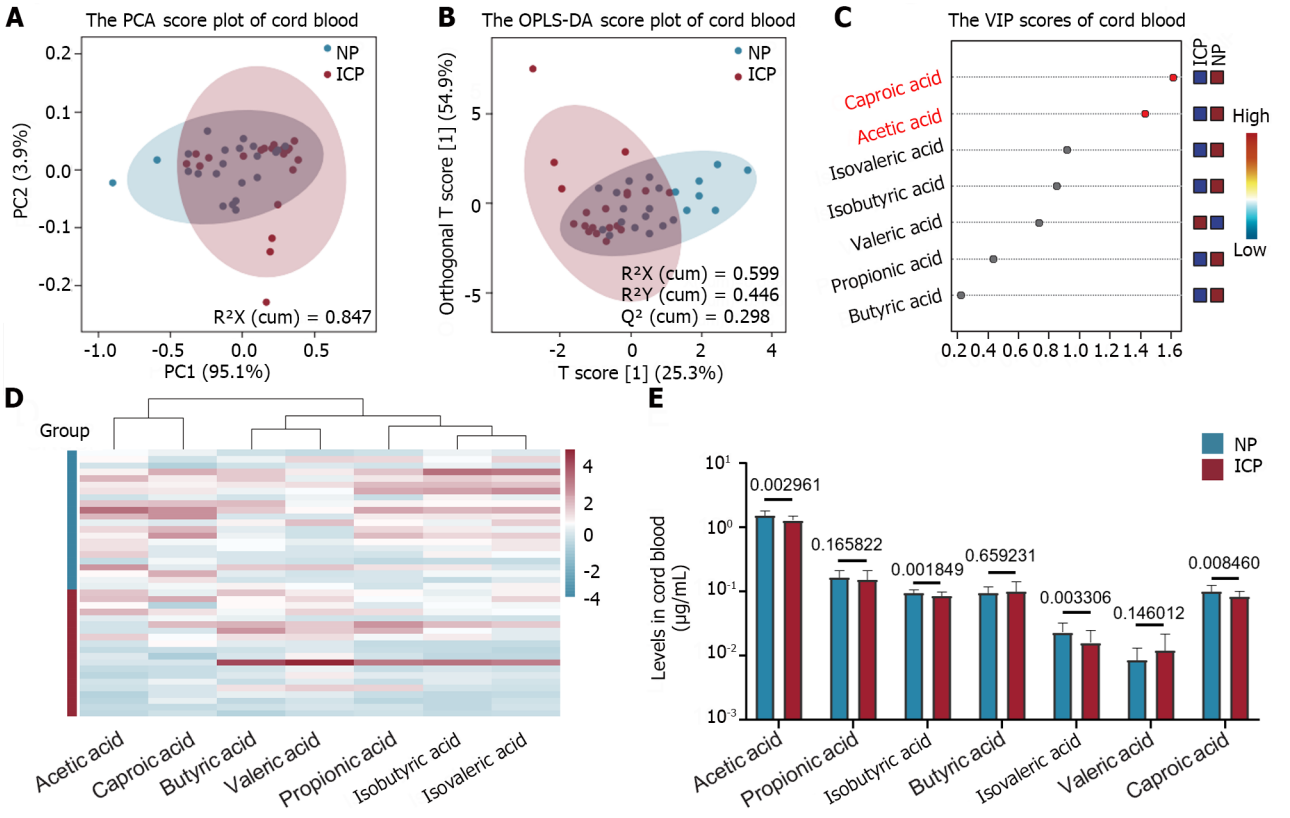

Figure 2 Characteristics of short-chain fatty acids metabolic spectrum changes in the cord blood of intrahepatic cholestasis of pregnancy patients. A: Principal Component Analysis score chart: intrahepatic cholestasis of pregnancy (ICP) patients experience inadequate separation from the normal pregnant women; B: Orthogonal Projections to Latent Structures Discriminant Analysis score chart: A partial overlap was observed between patients with ICP and normal pregnant women; C: Variable importance for the projection diagram: Acetic acid and caproic acid were identified as important metabolites that induced differences in short-chain fatty acids (SCFAs) in the cord blood between the two groups; D: Heat map: Except for valeric acid, other SCFAs displayed a decreasing trend in the cord blood of ICP patients; E: Quantitative analysis bar chart: Acetic acid demonstrated the highest content in the cord blood. The isobutyric acid content was lower in ICP patients. NP: n = 22; ICP: n = 20. PCA: Principal Component Analysis; SCFAs: Short-chain fatty acids; OPLS-DA: Orthogonal Projections to Latent Structures Discriminant Analysis; VIP: Variable Importance for the Projection.

DISCUSSION

SCFAs are the byproducts of dietary fiber fermentation in the gut microbiota under anaerobic conditions. Different gut microbiota can produce varying SCFAs, and the top three SCFAs in the human body are acetic acid, propionic acid, and butyric acid[8]. Prior studies have indicated that, during pregnancy, the total SCFAs in maternal peripheral blood circulation decrease significantly compared with those in non-pregnancy, with reductions in acetic acid and propionic acid levels, while butyric acid levels increase[9]. As pregnancy progresses, the level of butyric acid in peripheral blood

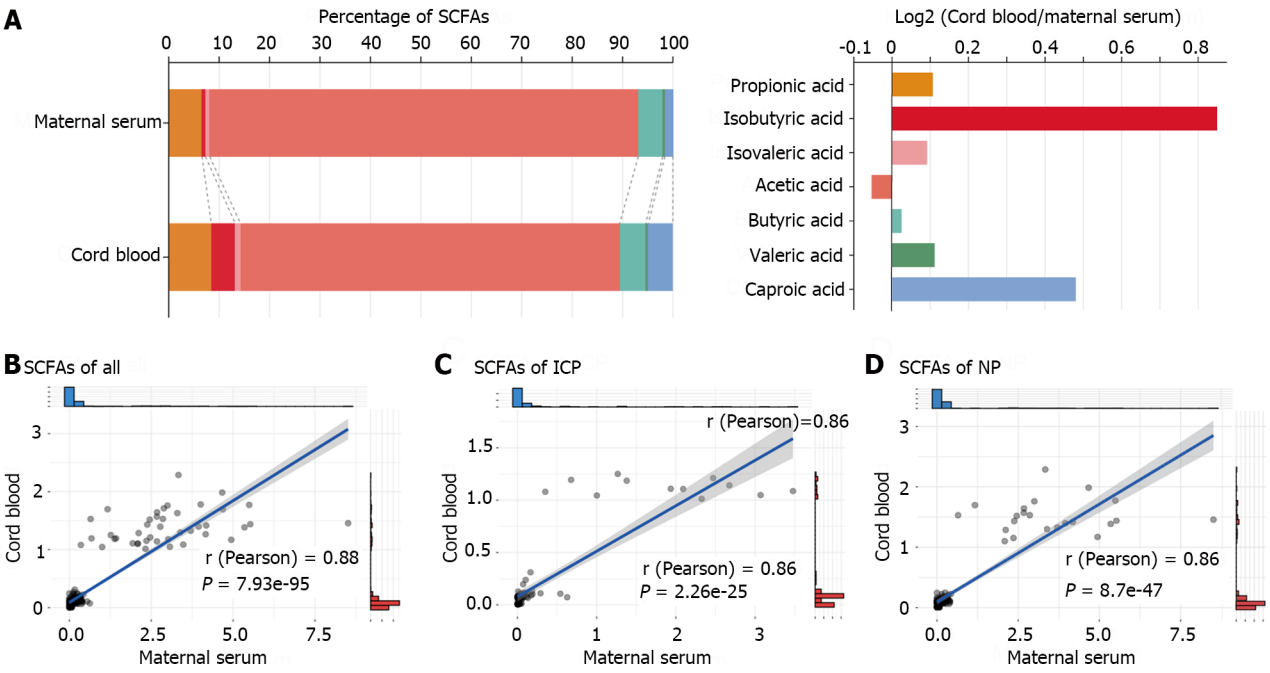

Figure 3 Cord blood short-chain fatty acids' correlation with short-chain fatty acids found in maternal serum. A: Stacked bar chart of the percentage of short-chain fatty acids (SCFAs) in maternal serum and cord blood: The proportion of isobutyric acid and caproic acid in maternal serum's total SCFAs was extremely low, while, in the cord blood SCFAs, it was significantly higher; B: Linear regression scatter plots of SCFAs in the two groups of subjects: A significant positive correlation existed between the total SCFAs in maternal serum and cord blood in both the subject groups; C: Linear regression scatter plot of SCFAs in intrahepatic cholestasis of pregnancy patients: There was a significant positive correlation between total SCFAs in the maternal serum and cord blood; D: Linear regression scatter plot of SCFAs in normal pregnant group: A strong positive correlation between the total SCFAs found in the maternal serum and cord blood. SCFAs: Short-chain fatty acids.

circulation increases, while the acetic acid, propionic acid, and isobutyric acid levels remain relatively stable[10]. SCFAs are associated with various pregnancy complications and can impact fetal growth and development in the womb[11-13]. A recent report in Science Advances found that SCFAs produced by the maternal microbiome play a role in supporting normal placental development, and their absence can limit placental growth and damage placental vascularization[14]. However, the alterations in SCFAs in the maternal blood of patients with ICP and their influence on fetal health remain unclear.

This study uncovered that both ICP and NP groups exhibited significantly higher acetic acid, propionic acid, and butyric acid levels in maternal serum SCFAs than other SCFAs. In umbilical cord blood SCFAs, acetic and propionic acids were the most abundant, with similar butyric acid, isobutyric acid, and caproic acid levels, while the isovaleric acid and valeric acid levels were the lowest. These changes may be linked to placental transport functions. Correlation analysis revealed that both acetic acid and caproic acid in maternal and cord blood were negatively correlated with total bile acids in peripheral blood. ROC analysis indicated that multiple SCFAs in maternal blood exhibited good diagnostic capabilities for ICP, with caproic acid demonstrating the highest diagnostic efficacy. KEGG pathway analysis suggested that acetic acid is involved in most pathway metabolisms, which may be related to the metabolism in patients with ICP.

Acetic acid is the most abundant SCFA in the human body and is produced by species in the Bacteroidetes phylum, one of the most abundant microbial groups in the intestine[13]. Acetic acid can promote the recruitment of immune cells in the intestine, thereby regulating intestinal inflammation[15]. Moreover, acetic acid has an inhibitory effect on liver adipogenesis, reducing lipid aggregation in adipose tissue[16]. Research has shown that when acetic acid is added only to the drinking water of pregnant mice during pregnancy, their offspring are immune to induced allergic airway disease [13], suggesting that acetic acid can traverse the placenta and have an impact on the fetus. This study discovered that compared with the NP group, acetic acid in the maternal serum and cord blood of the ICP group exhibited a significant decline and was significantly negatively correlated with total bile acids in maternal circulation. These results suggest that acetic acid plays a protective role in the progression of ICP, although the precise mechanisms in intrahepatic cholestasis and ICP require further investigation.

Caproic acid, as a putrefactive SCFA, results from the fermentation of amino acids or proteins that are not digested or absorbed in the small intestine, leading to the production of protein breakdown products[17]. Animal experiments have demonstrated that supplementation of exogenous caproic acid can increase phospholipid metabolism in the mother and enhance progesterone synthesis in the ovaries, potentially improving the embryonic survival rate in early pregnancy[18]. Interestingly, obese pregnant women have been observed to have lower caproic acid levels in their feces than normal-weight pregnant women[19]. In the context of this study, both maternal serum and cord blood caproic acid levels displayed a decreasing trend in patients with ICP, and maternal serum caproic acid levels exhibited a positive correlation with weight gain during pregnancy, which might be related to metabolic adaptation. Previous research has indicated that caproic acid can inhibit NF-κB transactivation and possess anti-inflammatory effects[20]. However, further exploration is

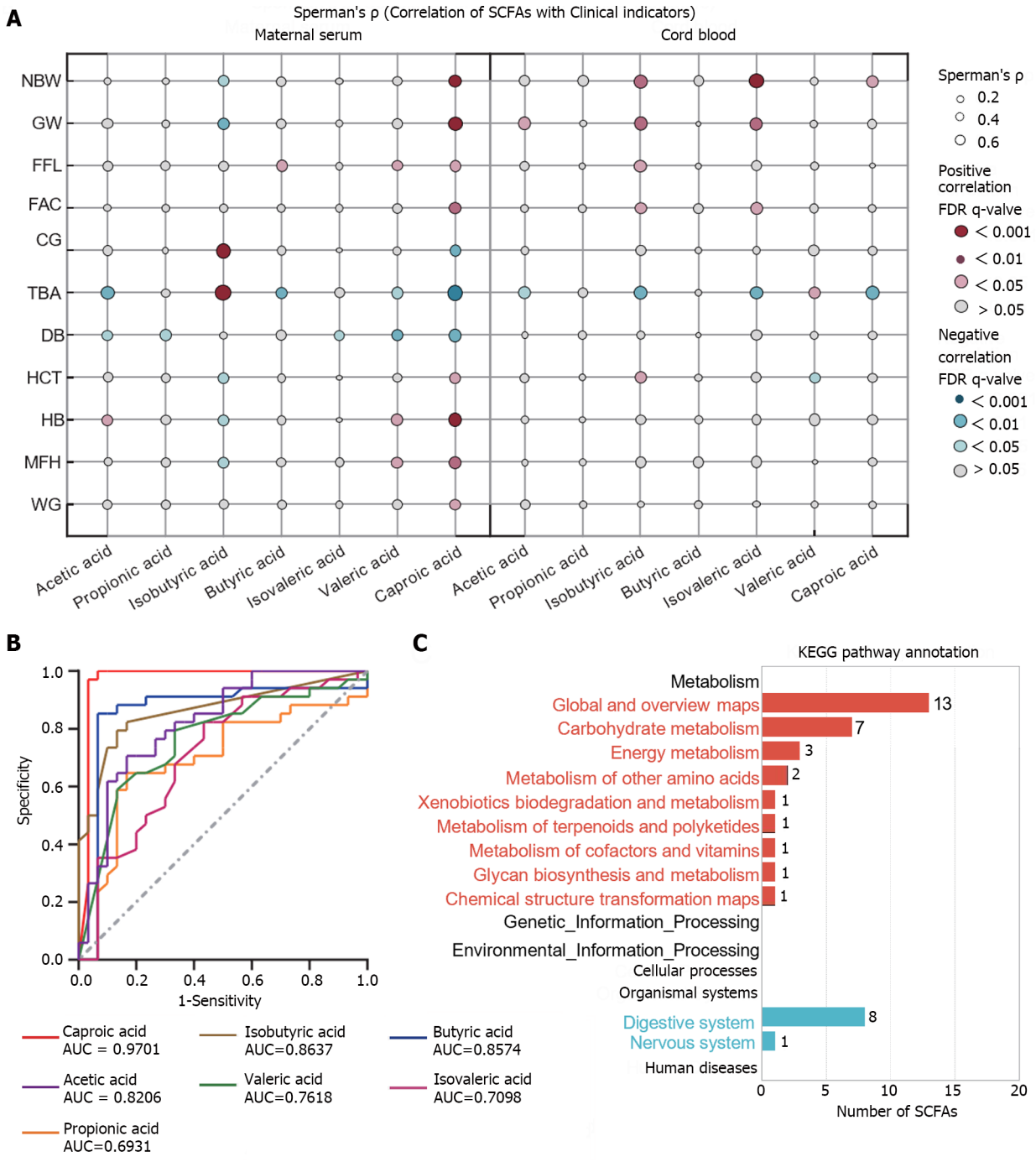

Figure 4 Clinical significance and functional analysis of differential short-chain fatty acids. A: Bubble chart depicting the correlation between short-chain fatty acids (SCFAs) and clinical indicators: Whether in maternal serum or cord blood, the correlation between acetic acid and caproic acid as well as clinical indicators showed the same trend. The size of the bubble represents the correlation levels. The color of the bubble represents the FDR levels. Red: Positive correlation; Blue: Negative correlation; B: The receiver operating characteristic (ROC) curve of maternal serum SCFAs for individual diagnosis of intrahepatic cholestasis of pregnancy: the diagnostic accuracy of caproic acid was 97.01%; C: Summary of the secondary classification of Kyoto Encyclopedia of Genes and Genomes pathway enrichment results: mainly involving metabolic pathways and biological system pathways, including carbohydrate metabolism, capacity metabolism, digestive system, and nervous system. For maternal blood, NP: n = 30; ICP: n = 34. For cord blood, NP: n = 22; ICP n = 20. NBW: Neonatal birth weight; GW: Gestational week of delivery; FFL: Fetal femur length; FAC: Fetal abdominal circumference; CG: Cholyglycine; TBA: Total bile acid; DB: Direct bilirubin; HCT: Hematocrit; HB: Hemoglobin; MFH: Maternal fundal height; WG: Weight gain during pregnancy.

necessary to determine whether it exerts anti-inflammatory and protective effects on patients with ICP and their fetuses.

In contrast to other SCFAs, this study identified a significant increase in isobutyric acid in the maternal serum of patients with ICP. Your previous research has also noted that the level of isobutyric acid in the peripheral blood circulation of pregnant women with pregnancy complications, such as pre-eclampsia and gestational diabetes, significantly increased compared with the NP group, aligning with the findings of this study[5,21]. This suggests that isobutyric acid plays a pivotal role in promoting the progression of pregnancy complications. Serino[22] has commented that SCFAs produced by gut microbiota are linked to host health. While it is generally believed that SCFAs have a more

significant positive impact on host metabolism than harm, in specific situations, excessive production of SCFAs can have detrimental effects on the host, making it challenging to definitively classify SCFAs as beneficial or harmful to the host. The results of this study imply that elevated isobutyric acid levels in maternal blood circulation not only lead to pathological changes in the mother but also impact the growth and development of the fetus in the uterus.

CONCLUSION

This study conducted an analysis of SCFAs in both maternal serum and umbilical cord blood from the ICP and NP groups, elucidating the trends of SCFA changes in the two sample types. It was observed that a significant positive correlation exists between maternal serum SCFAs and umbilical cord blood SCFAs, distinguishing it from most existing studies that concentrate on fecal or human serum samples. The comprehensive analysis of changes in SCFAs in patients with ICP provides valuable insights. However, this study has certain limitations. Firstly, the sample size was relatively small, and samples were collected from a single hospital within the same period, which may affect the robustness of the results. Expanding the clinical sample size and collecting samples from other regions in synchrony would enhance the reliability of the data. Secondly, SCFAs, being closely related to factors such as place of residence, lifestyle, diet, and medication, were not fully accounted for in the subjects' information, potentially introducing bias into the results. Thirdly, the effects and mechanisms of SCFAs, especially acetic acid, caproic acid, and isobutyric acid, on patients with ICP and their fetuses have not been comprehensively explored. Further *in vivo* and *in vitro* experiments are required to elucidate their mechanisms of action, providing a solid theoretical foundation for SCFAs as diagnostic and therapeutic targets for ICP.

ARTICLE HIGHLIGHTS

Research background

Intrahepatic cholestasis of pregnancy (ICP) is a liver condition specific to pregnancy. Short-chain fatty acids (SCFAs), important metabolites produced by the gut microbiota, are significantly linked to several pregnancy complications.

Research motivation

However, the metabolic profile of SCFAs in patients with ICP is still uncertain.

Research objectives

The study aimed to examine the correlation between maternal and umbilical blood SCFAs and investigate the changes in SCFAs in patients with ICP and their offspring. Additionally, the research sought to assess the clinical significance and diagnostic value of these SCFAs. Ultimately, the study aimed to identify novel biomarkers for diagnosing and treating ICP by focusing on gut microbiota metabolites.

Research methods

Therefore, in this study, we utilized targeted metabolomics technology to analyze SCFAs in the maternal and cord blood of patients with ICP and normal pregnant (NP) women.

Research results

The study revealed that maternal serum SCFAs in both the ICP and NP groups showed significantly higher levels of acetic acid, propionic acid, and butyric acid compared to other SCFAs. In umbilical cord blood, acetic and propionic acids were found to be the most abundant, with similar levels of butyric acid, isobutyric acid, and caproic acid, while isovaleric acid and valeric acid levels were the lowest. Furthermore, the correlation analysis indicated a negative correlation between both acetic acid and caproic acid in maternal and cord blood, and total bile acids in peripheral blood.

Research conclusions

Significant alterations were observed in the SCFAs of maternal serum and cord blood in the ICP group, compared with the NP group. It is notable that the SCFA levels in maternal serum and cord blood were significantly positively correlated in the ICP group. Additionally, certain maternal serum SCFAs, specifically caproic and acetic acids, exhibited excellent diagnostic efficiency for ICP.

Research perspectives

Additional *in vivo* and *in vitro* experiments are needed to clarify the mechanisms of action of SCFAs, establishing a strong theoretical basis for their use as diagnostic and therapeutic targets for ICP.

FOOTNOTES

Author contributions: Ren SJ, Zhou YP and Xuan RR designed the research; Ren SJ, Feng JT, Xiang T and Liao CL performed the research; Feng JT, Xiang T and Liao CL analyzed the data; Ren SJ, Zhou YP and Xuan RR wrote the paper; all authors participated in primary and final drafting; all authors have read and approve the final manuscript; all authors significantly contributed to the study.

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Institutional review board statement: The protocol for this research project has been approved by a suitably constituted Ethics Committee of the institution and it conforms to the provisions of the Declaration of Helsinki. Committee of The First Affiliated Hospital of ningbo university, Approval No. KY20220912. All informed consent was obtained from the subjects.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All authors have no conflicts of interest to declare.

Data sharing statement: The data that support the findings of this study are available from the corresponding author at fyxuanrongrong@nbu.edu.cn upon reasonable request.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Klebsiella pneumoniae infections after liver transplantation: Drug resistance and distribution of pathogens, risk factors, and influence on outcomes

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Abstract

BACKGROUND

Liver transplantation (LT) is the only curative treatment for end-stage liver disease. However, LT recipients are susceptible to infection, which is the leading cause of early mortality after LT. *Klebsiella pneumoniae* infections (KPIs) in the bloodstream are common in LT recipients. We hypothesized that KPIs and carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections may affect the outcomes of LT recipients.

AIM

To assess KPI incidence, timing, distribution, drug resistance, and risk factors following LT and its association with outcomes.

METHODS
This retrospective study included 406 patients undergoing LT at The Third Xiangya Hospital of Central South University, a tertiary hospital, from January 2015 to January 2023. We investigated the risk factors for KPIs and assessed the impact of KPIs and CRKP infections on the prognosis of LT recipients using logistic regression analysis.

RESULTS
KPI incidence was 7.9% (n = 32), with lung/thoracic cavity the most frequent site of infection; the median time from LT to KPI onset was 7.5 d. Of 44 *Klebsiella pneumoniae* isolates, 43 (97.7%) and 34 (77.3%) were susceptible to polymyxin B or ceftazidime/avibactam and tigecycline, respectively; > 70% were resistant to piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, meropenem, and levofloxacin. Female sex [odds ratio (OR) = 2.827, 95% confidence interval (CI): 1.256-6.364; P = 0.012], pre-LT diabetes (OR = 2.794, 95%CI: 1.070-7.294; P = 0.036), day 1 post-LT alanine aminotransferase (ALT) levels ≥ 1500 U/L (OR = 3.645, 95%CI: 1.671-7.950; P = 0.001), and post-LT urethral catheter duration over 4 d (OR = 2.266, 95%CI: 1.016-5.054; P = 0.046) were risk factors for KPI. CRKP infections, but not KPIs, were risk factors for 6-month all-cause mortality post-LT.

CONCLUSION
KPIs occur frequently and rapidly after LT. Risk factors include female sex, pre-LT diabetes, increased post-LT ALT levels, and urethral catheter duration. CRKP infections, and not KPIs, affect mortality.

Key Words: Liver transplantation; *Klebsiella pneumoniae* infections; Carbapenem-resistant *Klebsiella pneumoniae*; Risk factors; Outcomes

Core Tip: Despite advances in liver transplantation (LT) technology, *Klebsiella pneumoniae* infections (KPIs) remain challenging to treat. Timely prevention of KPIs is therefore critical. Many risk factors play crucial roles in the occurrence of KPIs after LT and in determining recipient prognosis. We examined the role of KPIs in the prognosis of LT recipients and the risk factors for KPIs after LT. By analyzing the distribution of KPIs and drug resistance, we demonstrated that risk factors are associated with surgical operative variables. Identifying these risk factors provides a basis for preventing KPIs, which, in turn, may improve the prognosis of LT recipients.

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INTRODUCTION

Liver transplantation (LT) is the only curative treatment for end-stage liver disease[1]. However, the lifelong use of immunosuppressant drugs makes LT recipients susceptible to infection, which is the most common cause of early mortality after LT[2]. In recent years, studies have demonstrated that infections in LT recipients are more likely to be caused by gram-negative than gram-positive pathogens[3]. The gram-negative bacterium *Klebsiella pneumoniae* (*K. pneumoniae*) is a common cause of infection, with reports indicating that 6.9%-14.2% of LT recipients experienced bloodstream infections caused by this pathogen[4,5].

The major concern regarding *K. pneumoniae* infections (KPIs) is the incidence of carbapenem-resistant *K. pneumoniae* (CRKP), which ranges from 2.5% to 35%; CRKP-associated mortality is as high as 35%-83% among LT recipients[5-12]. Therapeutic options for these infections are limited.

Although some studies have demonstrated the effects of CRKP infection on the prognosis of solid organ transplant (SOT) recipients, the impact of KPIs or CRKP infections in LT recipients remains unclear[5,13,14]. The present study examined the drug resistance and distribution of *K. pneumoniae* isolates and the effect of KPIs, particularly CRKP infections, on outcomes after LT. The findings of this study should provide clues for preventing KPIs and improving the outcomes of LT recipients with KPIs.

MATERIALS AND METHODS

Study design and patient samples

We conducted a single-center retrospective study including all adult patients who underwent LT at The Third Xiangya Hospital of Central South University from January 1, 2015, to January 31, 2023. Four patients with donor-derived KPIs and two patients aged under 18 years were excluded from the analysis, along with two patients who died within 48 h of transplantation due to massive intraoperative blood loss or primary graft nonfunction. Finally, 405 patients who received donations after brain death and 1 patient who received a donation after circulatory death were included in the analysis. All LT recipients underwent modified piggyback LT. Induction immunosuppression consisted of corticosteroids with or without basiliximab, and maintenance immunosuppression involved a corticosteroid taper and tacrolimus/cyclosporin A with or without mycophenolate mofetil or enteric-coated mycophenolate sodium. Standard perioperative antibacterial prophylaxis consisted of third-generation cephalosporins or carbapenems administered for 3-5 d. Teicoplanin, caspofungin, and other antibiotics were prescribed according to the infection status and identified pathogens. Antithymocyte globulin was prescribed when acute rejection episodes were not resolved by glucocorticoid therapy or when glucocorticoids were unsuitable for preventing acute rejection. This study was approved by the Ethics Committee of The Third Xiangya Hospital (approval number: 24029) and conducted in accordance with the principles outlined in the Declaration of Helsinki.

Clinical data collection

All patients were routinely followed-up in the outpatient department post-LT. The clinical data of LT recipients aged ≥ 18 years were extracted from inpatient and outpatient electronic medical records, including demographic information and infection characteristics. The follow-up periods were 3 months for microbiological data and 6 months for mortality. We also analyzed the prevalence of KPIs and CRKP infections and lengths of intensive care unit (ICU) and hospital stays after LT. Analysis was performed to identify risk factors for KPIs, 6-month all-cause mortality, and ICU stays of at least 7 d after LT.

Definitions

Infections were defined using the standards of the Centers for Disease Control and Prevention/National Healthcare Safety Network[13]. Infection was confirmed based on a positive culture together with clinical signs of an active infection, including chills, fever, hypotension, or imaging findings from computed tomography or chest radiography. The source of infection was confirmed by a positive culture accompanied by clinical manifestations[13]. CRKP was defined as an insusceptibility to at least one carbapenem, with a minimum inhibitory concentration of ≥ 4 μg/mL for imipenem or meropenem (Clinical and Laboratory Standards Institute, 2017). Reoperations included both retransplantation and post-LT laparotomy. Acute rejection was determined by biopsy.

Microbiological studies

Patient samples, including blood, sputum, bronchoalveolar lavage fluid, urine, ascites, bile, organ preservation solution, and catheter drainage fluid, were collected for clinical bacterial culture. Sputum samples were obtained from the trachea or were induced. Blood, urine, sputum, and abdominal drainage fluid were subject to routine bacterial culture once a day for 5-7 d after LT. Samples were collected for culture when an infection was suspected within the 3 months following LT. Blood samples were cultured and analyzed using a BD9240 automatic blood culture instrument (BD, Franklin Lakes, NJ, United States).The identification and susceptibility tests for culture-positive cases were conducted according to standard bacteriological procedures using a Bruker mass spectrometer and VITEK® 2 system (bioMérieux, Marcyl'Étoile, France). The minimum inhibitory concentration as measured by agar dilution was used to assess the antimicrobial susceptibility of the bacteria. When analyzing drug resistance, all intermediates were classified as resistant.

Statistical analysis

Statistical analysis was performed using SPSS software version 26.0 (IBM Corporation, Armonk, NY, United States). Categorical variables are expressed as frequencies and percentages. Continuous variables with and without normal distributions are expressed as means ± SD and medians and interquartile ranges, respectively. Chi-squared tests or Fisher's exact tests were used to compare categorical variables. Binary logistic regression based on forward stepwise regression was used to identify risk factors using odds ratios (OR) and 95% confidence intervals (CI). Risk factors with P-values < 0.01 after univariate analysis were included in the multivariate analysis. Two-tailed P-values < 0.05 were considered statistically significant.

RESULTS

General patient characteristics and prognosis

The 406 LT recipients included in the analysis had a mean age of 47.3 ± 10.6 years with a median Model for End-Stage Liver Disease (MELD) score of 23.0; 17.7% of patients were female. Liver failure occurred as a result of hepatitis virus-related cirrhosis/necrosis/tumor (n = 304), alcoholic liver disease (n = 31), mixed cirrhosis (n = 19), autoimmune hepatitis (n = 15), primary biliary cirrhosis (n = 11), cryptogenic cirrhosis (n = 9), Budd-Chiari syndrome (n = 5), hepatolenticular degeneration (n = 3), failure of previous LT (n = 3), drug-induced liver injury (n = 2), polycystic liver (n = 2), and familial

hereditary amyloidosis (n = 2). Prior to LT, patients had a median creatinine level of 0.8 mg/dL, albumin level of 34.5 g/L, white blood cell count of 5.2 × 10^9/L, lymphocyte count of 0.8 × 10^9/L, and platelet count of 72.0 × 10^9/L. Two months before LT, 160 (39.4%) patients experienced infections, with 140 (34.5%) experiencing pulmonary infections and 13 (3.2%) experiencing multiple-site infections, all of which involved the lungs. The median surgical time, blood loss, and number of red blood cell (RBC) transfusions were 378.5 min, 3000.0 mL, and 12.0 units, respectively. In the 3 months following LT, 32 (7.9%) patients were infected with 44 strains of *K. pneumoniae*; 21 (65.6%) patients were infected with CRKP. The median time from transplantation to KPI onset was 7.5 d. After LT, 18 (4.4%) and 395 (97.3%) patients were treated with anti-thymocyte immunoglobulin and tacrolimus, respectively. The median alanine aminotransferase (ALT) and albumin levels on day 1 and the median creatinine level on day 3 after LT were 694.5 U/L, 37.2 g/L, and 0.9 mg/dL, respectively. Overall, 94 patients required mechanical ventilation, 19 required renal replacement therapy, and 67 experienced acute rejection after LT. Moreover, 17 (4.2%) patients underwent reoperation. The median postoperative ICU and hospital stays were 6.0 and 26.0 d, respectively. The 6-month mortality rate was 7.9% (n = 32). Rates of KPI and CRKP infection were significantly higher in patients who died (both 18.8%; n = 6/32) than in those who survived (7.0%; n = 26/374 and 4.0%; n = 15/374, respectively). The baseline demographic, clinical, and laboratory characteristics are summarized in Table 1.

Distribution and drug resistance of K. pneumoniae

The most common site of KPI was the lung/thoracic cavity (n = 15), followed by the bloodstream (n = 12) and abdominal/ biliary tract (n = 12) (Table 2).

The KPIs were resistant to the following antibiotics, from the highest to lowest rate: Piperacillin/tazobactam, levofloxacin, aztreonam, meropenem, cefepime, ceftazidime, cefoperazone/sulbactam, amikacin, trimethoprim/sulfamethoxazole, tigecycline, ceftazidime/avibactam, and polymixin B. Among the 44 *K. pneumoniae* isolates, 1 (2.3%) was resistant to ceftazidime/avibactam, 1 (2.3%) was resistant to polymixin B, and 10 (22.7%) were resistant to tigecycline (Table 3).

Analysis of the risk factors for KPIs after LT

Univariate logistic regression analysis of patients with and without KPIs identified female sex (P = 0.002), duration of surgery ≥ 450 min (P = 0.033), ALT level ≥ 1500 U/L 1 d after LT (P < 0.001), duration of post-LT urethral catheterization over 4 d (P = 0.009), and post-LT mechanical ventilation (P = 0.015) as risk factors for post-LT KPIs. A MELD score ≥ 22 at LT (P = 0.066), pre-LT diabetes (P = 0.067), infection in the 2 months prior to LT (P = 0.098), and anti-thymocyte globulin use (P = 0.063) showed a trend toward a higher incidence of KPIs but did not reach significance.

Multivariate analysis identified female sex (OR = 2.827, 95%CI: 1.256-6.364; P = 0.012), pre-LT diabetes (OR = 2.794, 95%CI: 1.070-7.294; P = 0.036), ALT level ≥ 1500 U/L 1 d after LT (OR = 3.645, 95%CI: 1.671-7.950; P = 0.001), and post-LT urethral catheter duration over 4 d (OR = 2.266, 95%CI: 1.016-5.054; P = 0.046) as independent risk factors for the development of post-LT KPIs. All data from the univariate and multivariate analyses are presented in Table 4.

Prognosis of patients with KPI or CRKP infection after LT

Pearson's chi-squared test was used to assess the effects of KPIs on the prognosis of LT recipients. Notably, patients with KPIs were more likely to have ICU stays of at least 7 d after LT than those without (56.3% *vs* 35.3%; P = 0.018). Patients with KPIs also had higher 6-month all-cause mortality than those without KPIs (17.6% *vs* 5.0%; P = 0.017). In contrast, patients with KPIs were not more likely to have post-LT hospitalization stays ≥ 21 d (P = 0.592) than those without (Table 5).

Univariate and multivariate analyses were performed to determine whether KPIs were independent risk factors for 6-month all-cause mortality. The multivariate analysis showed that KPIs were not a risk factor for 6-month all-cause mortality after LT. However, CRKP infections (OR = 5.330, 95%CI: 1.534-18.524; P = 0.008), female sex (OR = 2.829, 95%CI: 1.098-7.288; P = 0.031), intraoperative RBC transfusions ≥ 12 units (OR = 3.466, 95%CI: 1.259-9.543; P = 0.016), day 3 post-LT creatinine levels ≥ 2 mg/dL (OR = 9.724, 95%CI: 4.077-23.194; P < 0.001), and post-LT mechanical ventilation (OR = 4.118, 95%CI: 1.790-9.476; P = 0.001) were identified as risk factors for 6-month all-cause mortality after LT (Table 6).

Multivariate logistic regression analysis of factors related to prolonged ICU stays identified MELD scores ≥ 22 at LT (OR = 1.695, 95%CI: 1.086-2.645; P = 0.020), intraoperative blood loss ≥ 3000 mL (OR = 1.790, 95%CI: 1.139-2.813; P = 0.012), ALT levels ≥ 1500 U/L 1 d after LT (OR = 1.915, 95%CI: 1.123-3.265; P = 0.017), post-LT renal replacement therapy (OR = 4.058, 95%CI: 1.327-12.409; P = 0.014) and post-LT mechanical ventilation (OR = 3.402, 95%CI: 2.052-5.639; P < 0.001), but not KPIs or CRKP infections, as independent risk factors for post-LT ICU stays of at least 7 d (Table 7).

DISCUSSION

LT recipients are susceptible to opportunistic infections and antibiotic-resistant bacterial transmission due to malnutrition, complex surgical procedures, and immunosuppressive drugs[1]. *K. pneumoniae* is the most common gram-negative pathogen isolated from patients with LT[1]. In our study, the rates of KPI and CRKP infection were 7.9% and 5.2%, respectively, which were lower than the rates of 18.4% and 8.0%, respectively, reported by Liu *et al*[1] and Kalpoe *et al*[6].

K. pneumoniae most commonly infects the bloodstream and urinary tract post-LT[6,15]. Pneumonia, tertiary peritonitis, and surgical site infections have been reported as complications of KPIs in LT recipients[8,15]. The present study found that the lung/thoracic cavity was the most frequent site of infection, followed by the bloodstream, abdominal/biliary tract, urinary tract, perianal region, and liver.

Table 1 Demographic, laboratory, and clinical variables of 406 liver transplantation recipients

Characteristics	Value
Recipient age (yr), mean ± SD	47.3 ± 10.6
Recipient gender, no. of female (%)	72 (17.7)
Recipient BMI, median (IQR), kg/m^2	22.8 (20.8-25.1)
Hospital stay prior to LT, median (IQR), days	10.0 (1.0-22.3)
MELD score at LT, median (IQR)	23.0 (15.0-30.0)
Infection within 2 months prior to LT, *n* (%)	160 (39.4)
Pulmonary infection	140 (34.5)
Abdominal/biliary infection	6 (1.5)
Urinary tract infection	1 (0.2)
Multiple site infection[1]	13 (3.2)
Pre-LT use of broad-spectrum antibiotics	166 (40.9)
Underlying liver diseases, *n* (%)	406 (100)
Viral cirrhosis/necrosis/tumor	304 (74.9)
Alcoholic cirrhosis	31 (7.6)
Autoimmune hepatitis	15 (3.7)
Primary biliary cirrhosis	11 (2.7)
Mixed cirrhosis	19 (4.7)
Others[2]	26 (6.4)
Pre-LT type 2 diabetes, *n* (%)	48 (11.8)
Pre-LT creatinine, median (IQR), mg/dL	0.8 (0.7-1.0)
Pre-LT WBC count, median (IQR), × 10^9/L	5.2 (3.4-8.1)
Pre-LT lymphocyte count, median (IQR), × 10^9/L	0.8 (0.5-1.2)
Pre-LT platelet count, median (IQR), × 10^9/L	72 (43.8-106.5)
Pre-LT albumin level, median (IQR), g/L	34.5 (30.9-38.1)
Donor age (yr), mean ± SD	42.1 ± 13.0
Steatosis ≥ 30%, *n* (%)	42 (10.3)
Cold ischemia time, mean ± SD	6.2 ± 1.5
Duration of surgery, median (IQR), min	378.5 (333.0-425.0)
Intraoperative bleeding, median (IQR), mL	3000.0 (2000.0-5000.0)
Intraoperative RBC transfusion, median (IQR), units	12.0 (8.0-18.0)
Post-LT infections due to *Klebsiella pneumoniae*, *n* (%)	32 (7.9)
Post-LT infections due to CRKP, *n* (%)	21 (5.2)
Median interval between the onset of infections due to *Klebsiella pneumoniae* and LT, median (IQR), days	7.5 (2.0-17.8)
Post-LT immunosuppressant treatment, *n* (%)	406 (100)
Tacrolimus	395 (97.3)
Ciclosporin A	5 (1.2)
Mycophenolate mofetil/enteric-coated mycophenolate sodium	277 (68.2)
Sirolimus	5 (1.2)
Glucocorticoid	406 (100)
Basiliximab	214 (52.7)

Anti-thymocyte globulin	18 (4.4)
ALT on day 1 after LT, median (IQR), U/L	694.5 (383.0-1242.0)
Creatinine on day 3 after LT, median (IQR), mg/dL	0.9 (0.7-1.4)
Albumin level on day 1 after LT, median (IQR), g/L	37.2 (33.9-40.7)
Post-LT duration of urethral catheter, median (IQR), days	3.0 (2.0-5.0)
Post-LT mechanical ventilation, *n* (%)	94 (23.2)
Reoperation, *n* (%)	17 (4.2)
Acute rejection, *n* (%)	67 (16.5)
Post-LT renal replacement therapy, *n* (%)	19 (4.7)
ICU stay after LT, median (IQR), days	6.0 (5.0-7.0)
Hospitalization stay after LT, median (IQR), days	26.0 (21.0-30.0)
All-cause mortality within 6 months after LT, *n* (%)	32 (7.9)

[1]There were 9 cases of pulmonary and abdominal/bile duct infections, 1 case of pulmonary and urinary tract infections, 1 case of pulmonary and bloodstream infections, 1 case of pulmonary and intracranial infections, and 1 case each of pulmonary, abdominal and bloodstream infections.
[2]There were 9 cases of cryptogenic cirrhosis, 5 cases of Budd-Chiari syndrome, 3 cases each of hepatolenticular degeneration and transplant liver failure, 2 cases each of drug-induced liver injury, polycystic liver, and familial hereditary amyloidosis.
ALT: Alanine aminotransferase; BMI: Body mass index; ICU: Intensive care unit; Fis: Fungal infections; IQR: Interquartile range; LT: Liver transplantation; MELD: Model for End-Stage Liver Disease; RBC: Red blood cell.

Table 2 Infection sites of 44 episodes of infections caused by *Klebsiella pneumoniae*

Infection sites	Lung/thoracic cavity	Blood stream	Abdominal/biliary tract	Urinary tract	Perianal abscess	Liver abscess
Klebsiella pneumoniae (44)	15	12	12	3	1	1

Table 3 Rate of drug-resistance of 44 isolates of *Klebsiella pneumoniae* to 12 commonly used antibiotics, *n* (%)

Antimicrobial	n	Percentage
TZP	34	77.3
CAZ	31	70.5
CFS	30	68.2
FEP	31	70.5
ATM	31	70.5
MEM	31	70.5
AN	21	47.7
LVF	33	75.0
SXT	20	45.5
TIC	10	22.7
POL	1	2.3
CAZ/AVI	1	2.3

ATM: Aztreonam; TZP: Piperacillin/tazobactam; CFS: Cefoperazone/sulbactam; CAZ: Ceftazidime; FEP: Cefepime; AN: Amikacin; LVF: Levofloxacin; MEM: Meropenem; TIC: Tigecycline; SXT: Trimethoprim/sulfamethoxazole; POL: Polymixin B; CAZ/AVI: Ceftazidime/avibactam.

K. pneumoniae is a particularly concerning pathogen because it has limited antibiotic sensitivity and often develops multidrug resistance during treatment[16,17]. In our study, > 70% of the *K. pneumoniae* isolates were resistant to piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, meropenem, or levofloxacin. The prevalence of CRKP infections was 5.2% in LT recipients, which is slightly lower than the rate of 7.0% reported in a previous study on LT recipients in China[1]. The rate of *K. pneumoniae* resistance to carbapenems reached 70.5%, which is similar to the rate of 63.3% re-

Table 4 Univariate and multivariate logistic regression analysis of risk factors for infections due to *Klebsiella pneumoniae* within 3 months after liver transplantation, *n* (%)

Variables	With *K. pneumoniae* infections(32)	Without *K. pneumoniae* infections(374)	*P* value	OR (95%CI)
Total				
Univariate analysis				
Female sex	12 (37.5)	60 (16.0)	0.002	
Recipient age ≥ 55 yr	10 (31.3)	91 (24.3)	0.385	
Recipient BMI ≥ 25	9 (28.1)	97 (25.9)	0.787	
MELD score at LT ≥ 22	23 (71.9)	206 (55.1)	0.066	
Hospital stay prior to LT ≥ 7 d	23 (71.9)	216 (57.8)	0.119	
Viral cirrhosis/necrosis/tumor	21 (65.6)	283 (75.7)	0.209	
Alcoholic cirrhosis	3 (9.4)	28 (7.5)	0.969	
Pre-LT diabetes	7 (21.9)	41 (11.0)	0.067	
Pre-LT use of broad-spectrum antibiotics ≥ 3 d	16 (50.0)	150 (40.1)	0.275	
Pre-LT creatinine ≥ 2 mg/dL	1 (3.1)	28 (7.5)	0.574	
Infection within 2 months prior to LT	17 (53.7)	143 (38.2)	0.098	
Pre-LT WBC count ≥ 10 × 10^9/L	4 (12.5)	55 (14.7)	0.937	
Pre-LT lymphocyte count ≤ 0.5 × 10^9/L	6 (18.8)	92 (24.6)	0.458	
Pre-LT platelet count ≤ 50 × 10^9/L	12 (37.5)	123 (32.9)	0.595	
Pre-LT albumin level < 30 g/L	9 (28.1)	71 (19.0)	0.212	
Donor age ≥ 50 yr	13 (40.6)	121 (32.4)	0.340	
Steatosis ≥ 30%	2 (6.3)	40 (10.7)	0.624	
Cold ischemia time ≥ 360 min	15 (46.9)	189 (50.5)	0.691	
Duration of surgery ≥ 450 min	10 (31.3)	61 (16.3)	0.033	
Intraoperative bleeding ≥ 3000 mL	23 (71.9)	214 (57.2)	0.101	
Intraoperative RBC transfusion ≥ 12 U	20 (62.5)	201 (53.7)	0.340	
ALT on day 1 after LT ≥ 1500U/L	14 (43.8)	66 (17.6)	<0.001	
Creatinine on day 3 after LT ≥ 2 mg/dL	4 (12.5)	57 (15.2)	0.874	
Albumin level on day 1 after LT < 30 g/L	4 (12.5)	24 (6.4)	0.347	
Post-LT duration of urethral catheter ≥ 4 d	22 (68.8)	167 (44.7)	0.009	
Post-LT mechanical ventilation	13 (40.6)	81 (21.7)	0.015	
Reoperation	3 (9.4)	14 (3.7)	0.286	
Acute rejection	6 (18.8)	61 (16.3)	0.721	
Post-LT renal replacement therapy	3 (9.4)	16 (4.3)	0.382	
Glucocorticoidse ≥ 1500 mg	21 (65.6)	235 (62.8)	0.754	
Basiliximab use ≥ 40 mg	14 (43.8)	145 (38.8)	0.580	
Anti-thymocyte globulin use	4 (12.5)	14 (3.7)	0.063	
Multivariate analysis				
Female sex			0.012	2.827 (1.256-6.364)
Pre-LT diabetes			0.036	2.794 (1.070-7.294)

ALT on day 1 after LT ≥ 1500U/L	0.001	3.645 (1.671-7.950)
Post-LT duration of urethral catheter ≥ 4 d	0.046	2.266 (1.016-5.054)

ALT: Alanine aminotransferase; BSIs: Bloodstream infections; CI: Confidence intervals; LT: Liver transplantation; MELD: Model for End-Stage Liver Disease; OR: Odds ratios; RBC: Red blood cell; BMI: Body mass index; *K. pneumoniae*: *Klebsiella pneumoniae*.

Table 5 The postoperative outcome for patients with/without infections caused by *Klebsiella pneumoniae* following liver transplantation, *n* (%)

Variables	With infections caused by *K. pneumoniae*(32)	Without infections caused by *K. pneumoniae* (374)	X^2	*P* value
ICU stay after LT ≥ 7 d	18 (56.3)	132 (35.3)	5.557	0.018
Hospitalization stay after LT ≥ 21 d	26 (81.3)	302 (80.7)	0.288	0.592
All-cause mortality within 6 months after LT	6 (18.8)	32 (8.6)	5.651	0.017

ICU: Intensive care unit; LT: Liver transplantation; *K. pneumoniae*: *Klebsiella pneumoniae*

ported by Liu *et al*[1]. Previous retrospective studies recommend polymyxin E, amikacin, and tigecycline for SOT recipients with CRKP infections[18,19]. However, the existing options (polymyxins, aminoglycosides, tigecycline, and carbapenems) for carbapenem-resistant Enterobacteriaceae are limited by their low efficacy, resistance, suboptimal pharmacokinetics, and high toxicity rates[20,21]. Our results identified ceftazidime/avibactam and polymyxin B as the first choice for KPI treatment, with tigecycline the second choice. The CRKP infection rate in patients who died was significantly higher than that in patients who survived in our study, which is consistent with previous studies that identified CRKP infections as the most lethal among all gram-negative infections in SOT recipients[22,23].

Previous studies have demonstrated the following risk factors for CRKP infections in LT recipients: Colonization with CRKP, hepatocellular carcinoma, chronic kidney disease, preoperative infection, MELD score > 20, mechanical ventilation, exposure to cephalosporine-carbapenem/piperacillin-tazobactam, renal replacement therapy, hepatitis C virus recurrence, length of ICU stay, and Roux-en-Y biliary choledochojejunostomy[1,8,11,15].

Our analysis demonstrated that pre-LT diabetes is independently associated with the development of post-LT KPIs. The underlying mechanism may involve diabetes-induced immunosuppression. A previous study established a relationship between the risk factors of necrotizing soft tissue *Klebsiella* infections and diabetes mellitus[24]. Singh *et al*[25] revealed that diabetes mellitus is an independent and significant predictor of bacteremia in LT recipients.

We also revealed a post-LT urethral catheter duration of > 4 d to be an independent risk factor for post-LT KPIs. A univariate analysis performed by Zhang *et al*[26] suggested an association between urinary catheterization and bacterial and fungal infections after LT; however, this association was lost following multivariate analysis.

We identified female sex as a risk factor for KPIs, consistent with the findings of a study by Abbott *et al*[27], which claimed that females are more likely to be hospitalized for septicemia following kidney transplantation. In contrast, Bert *et al*[28] found male sex to be significantly associated with bloodstream infections post-LT. The most likely cause of the increased risk of KPIs in female LT recipients is their greater vulnerability to urinary tract infections. However, only 3 of the 44 *K. pneumoniae* strains in our study involved urinary tract infections. The reason for this is unclear, and therefore confirmation that the prolonged use of urethral catheters and female sex are independent risk factors for post-LT KPIs is required in further larger-sample studies.

Elevated post-LT ALT levels were also found to be an independent risk factor for post-LT KPIs. To the best of our knowledge, this is the first study to identify this risk factor, which resulted in a 3.6-fold increased risk of post-LT KPIs [28]. Higher ALT levels early after LT indicate severe intraoperative blood loss or hypotension or poor graft quality, all of which render LT recipients more susceptible to infection.

The present study revealed that KPIs have no impact on ICU or hospital stays or 6-month all-cause mortality rates. However, 6-month all-cause mortality is impacted by CRKP infections, in addition to female sex, intraoperative RBC transfusion, day 3 post-LT creatinine level, and post-LT mechanical ventilation. These results are consistent with those of a previous study that identified mechanical ventilation and CRKP infections as risk factors for three-month mortality after LT[1]. Previous studies have also shown that CRKP infections are independently associated with mortality rates in SOT recipients, which range from 40% to 75%[1,23,29,30].

Limitations of the study

This study has several limitations. First, the retrospective single-center design implies an inherent selection bias and represents only the regional prevalence of KPIs and CRKP infections in LT recipients. Second, many studies have stated that colonization with *K. pneumoniae*, particularly CRKP, prior to LT may be important for the risk of post-LT KPIs and CRKP infections. Unfortunately, surveillance for *K. pneumoniae* is not routinely performed at our center.

Table 6 Univariate and multivariate Logistic regression analysis of risk factors for 6-month all-cause mortality after liver transplantation, *n* (%)

Variables	Death(32)	Survival(374)	*P* value	OR (95%CI)
Total				
Univariate analysis				
Female sex	10 (31.3)	62 (16.6)	0.037	
Recipient age ≥ 55 yr	14 (43.8)	87 (23.3)	0.010	
Recipient BMI ≥ 25	4 (12.5)	102 (27.3)	0.068	
MELD score at LT ≥ 22	24 (75.0)	205 (54.8)	0.027	
Hospital stay prior to LT ≥ 7 d	24 (75.0)	215 (57.5)	0.053	
Viral cirrhosis/necrosis/tumor	25 (78.1)	279 (74.6)	0.659	
Alcoholic cirrhosis	1 (3.1)	30 (8.0)	0.513	
Pre-LT diabetes	4 (12.5)	44 (11.8)	1.000	
Pre-LT creatinine ≥ 2 mg/dL	6 (18.8)	23 (6.1)	0.008	
Infection within 2 months prior to LT	19 (59.4)	141 (37.7)	0.016	
Pre-LT WBC count ≥ 10×10^9/L	7 (21.9)	52 (13.9)	0.219	
Pre-LT lymphocyte count ≤ 0.5×10^9/L	12 (37.5)	86 (23.0)	0.066	
Pre-LT platelet count ≤ 50×10^9/L	8 (25.0)	127 (34.0)	0.302	
Pre-LT albumin level < 30g/L	6 (18.8)	74 (19.8)	0.888	
Donor age ≥ 50 yr	7 (21.9)	127 (34.0)	0.163	
Steatosis ≥ 30%	3 (9.4)	39 (10.4)	1.000	
Cold ischemia time ≥ 360 min	20 (62.5)	199 (53.2)	0.248	
Duration of surgery ≥ 450 min	8 (25.0)	63 (16.8)	0.244	
Intraoperative bleeding ≥ 3000 mL	26 (81.3)	211 (56.4)	0.006	
Intraoperative RBC transfusion ≥ 12 U	25 (78.1)	196 (52.4)	0.005	
ALT on day 1 after LT ≥ 1500 U/L	8 (25.0)	72 (19.3)	0.433	
Creatinine on day 3 after LT ≥ 2 mg/dL	18 (56.3)	43 (11.5)	< 0.001	
Albumin level on day 1 after LT < 30 g/L	6 (18.8)	25 (6.7)	0.564	
Post-LT infections due to *Klebsiella pneumoniae*	6 (18.8)	26 (7.0)	0.017	
Post-LT infections due to CRKP	6 (18.8)	15 (4.0)	< 0.001	
Post-LT mechanical ventilation	19 (59.4)	75 (20.1)	< 0.001	
Reoperation	3 (9.4)	14 (3.7)	0.286	
Acute rejection	4 (12.5)	63 (16.8)	0.525	
Post-LT renal replacement therapy	8 (25.0)	11 (2.9)	< 0.001	
Glucocorticoidse ≥ 1500 mg	19 (59.4)	237 (63.4)	0.653	
Basiliximab use ≥ 40 mg	10 (31.3)	149 (39.8)	0.339	
Anti-thymocyte globulin use	1 (3.1)	17 (4.5)	1.000	
Multivariate analysis				
Female sex			0.031	2.829 (1.098-7.288)
Intraoperative RBC transfusion ≥ 12 U			0.016	3.466 (1.259-9.543)
Creatinine on day 3 after LT ≥ 2 mg/dL			< 0.001	9.724 (4.077-23.194)
Post-LT infections due to CRKP			0.008	5.330 (1.534-18.524)
Post-LT mechanical ventilation			0.001	4.118 (1.790-9.476)

ALT: Alanine aminotransferase; CI: Confidence intervals; LT: Liver transplantation; RBC: Red blood cell; MELD: Model for End-Stage Liver Disease; OR: Odds ratios; BMI: Body mass index.

Table 7 Univariate and multivariate Logistic regression analysis of risk factors for intensive care unit stay after liver transplantation ≥ 7, *n* (%)

Variables	ICU stay after LT ≥ 7 d (150)	ICU stay after LT < 7 d (256)	*P* value	OR (95%CI)
Total				
Univariate analysis				
Female sex	34 (22.7)	38 (14.8)	0.046	
Recipient age ≥ 55 yr	45 (30.0)	56 (21.9)	0.068	
Recipient BMI ≥ 25	38 (25.3)	68 (26.6)	0.785	
MELD score at LT ≥ 22	98 (65.3)	131 (51.2)	0.005	
Hospital stay prior to LT ≥ 7 d	98 (65.3)	141 (55.1)	0.043	
Viral cirrhosis/necrosis/tumor	112 (74.7)	192 (75.0)	0.940	
Alcoholic cirrhosis	11 (7.3)	20 (7.8)	0.861	
Pre-LT diabetes	17 (11.3)	31 (12.1)	0.815	
Pre-LT creatinine ≥ 2 mg/dL	18 (12.0)	11 (4.3)	0.004	
Infection within 2 months prior to LT	57 (38.0)	103 (40.2)	0.657	
Pre-LT WBC count ≥ 10×10^9/L	27 (18.0)	123 (48.0)	0.129	
Pre-LT lymphocyte count ≤ 0.5×10^9/L	34 (22.7)	64 (25.0)	0.596	
Pre-LT platelet count ≤ 50×10^9/L	46 (30.7)	89 (34.8)	0.397	
Pre-LT albumin level < 30 g/L	28 (18.7)	123 (48.0)	0.687	
Donor age ≥ 50 yr	46 (30.7)	88 (34.4)	0.443	
Steatosis ≥ 30%	16 (10.7)	26 (10.2)	0.871	
Cold ischemia time ≥ 360 min	78 (52.0)	136 (53.1)	0.827	
Duration of surgery ≥ 450 min	31 (20.7)	40 (15.6)	0.197	
Intraoperative bleeding ≥ 3000 ml	102 (68.0)	135 (52.7)	0.003	
Intraoperative RBC transfusion ≥ 12 U	92 (61.3)	129 (50.4)	0.033	
ALT on day 1 after LT ≥ 1500 U/L	41 (27.3)	39 (15.2)	0.003	
Creatinine on day 3 after LT ≥ 2 mg/dL	30 (20.0)	31 (12.1)	0.032	
Albumin level on day 1 after LT < 30 g/L	12 (8.0)	16 (6.3)	0.502	
Post-LT infections due to *Klebsiella pneumoniae*	18 (12.0)	14 (5.5)	0.018	
Post-LT infections due to CRKP	15 (10.0)	6 (2.3)	0.001	
Post-LT mechanical ventilation	59 (39.3)	35 (13.7)	< 0.001	
Reoperation	11 (7.3)	6 (2.3)	0.015	
Acute rejection	28 (18.7)	39 (15.2)	0.369	
Post-LT renal replacement therapy	14 (9.3)	5 (2.0)	0.001	
Glucocorticoidse ≥ 1500 mg	102 (68.0)	154 (60.2)	0.114	
Basiliximab use ≥ 40 mg	55 (36.7)	104 (40.6)	0.430	
Anti-thymocyte globulin use	7 (4.7)	11 (4.3)	0.861	
Multivariate analysis				
MELD score at LT ≥ 22			0.020	1.695 (1.086-2.645)

Intraoperative bleeding ≥ 3000 ml	0.012	1.790 (1.139-2.813)
ALT on day 1 after LT ≥ 1500 U/L	0.017	1.915 (1.123-3.265)
Post-LT renal replacement therapy	0.014	4.058 (1.327-12.409)
Post-LT mechanical ventilation	< 0.001	3.402 (2.052-5.639)

ICU: Intensive care unit; ALT: Alanine aminotransferase; CI: Confidence intervals; LT: Liver transplantation; RBC: Red blood cell; MELD: Model for End-Stage Liver Disease; OR: Odds ratios; CRKP: Carbapenem-resistant *Klebsiella pneumoniae*; BMI: Body mass index.

CONCLUSION

The homogeneity of infections caused by *K. pneumoniae* may lead to an accurate analysis of the risk factors for KPIs and mortality. Although our study included a relatively large cohort of LT recipients, the effect of KPIs, particularly CRKP infections, on patient outcomes emphasizes the need for further prospective studies. Given that the antimicrobial treatment of KPIs, especially CRKP infections, remains an ongoing challenge, knowledge of the risk factors for these infections and implementation of enhanced infection control measures are essential for successful LT.

ARTICLE HIGHLIGHTS

Research background

Liver transplantation (LT) is the only curative treatment available for end-stage liver disease. However, LT recipients are prone to many types of infections, which are the most common cause of early mortality after LT. Recent studies have demonstrated that LT recipients suffer from bloodstream infections caused by *K. pneumoniae*. In addition, there has been little discussion on the adverse impacts of *K. pneumoniae* infections (KPIs) or carbapenem-resistant *K. pneumoniae* (CRKP) infections among LT recipients.

Research motivation

The key to retrospective cohort studies is to explore the risk factors for the development of KPIs in patients after LT and analyze drug resistance. Careful follow-up is required to minimize the occurrence of KPIs in patients with LT, reduce the development of drug resistance, and improve patient survival and prognosis.

Research objectives

The primary objective of this study was to assess the incidence, timing, distribution, drug resistance, and risk factors of KPIs within 3 months of LT. The secondary objective was to evaluate the impact of KPIs, particularly CRKP, on outcomes.

Research methods

In total, 406 patients undergoing LT between January 2015 and January 2023 were included in the present retrospective study to investigate the risk factors for KPIs and assess the impact of KPIs and CRKP on the prognosis of LT recipients using logistic regression.

Research results

Of the 406 LT recipients recruited, 32 (7.9%) were infected with 44 strains of *K. pneumoniae* within 3 months post-LT. Of the 32 patients, 21 (65.6%) were infected with CRKP. The median time from LT to KPI onset was 7.5 d. KPIs (18.8%, 6/32) and CRKP infection (18.8%, 6/32) rates were significantly higher in patients who died than in those who survived (7.0%, 26/374 and 4.0%, 15/374, respectively). The multivariate analysis identified female sex [odds ratio (OR) = 2.827, 95% confidence interval (CI): 1.256-6.364, P = 0.012], pre-LT diabetes [OR = 2.794, 95%CI: 1.070-7.294, P = 0.036], day 1 post-LT alanine aminotransferase levels ≥ 1500 U/L (OR = 3.645, 95%CI: 1.671-7.950, P = 0.001), and post-LT urethral catheter durations > 4 d (OR = 2.266, 95%CI: 1.016-5.054, P = 0.046) were independently associated with the development of post-LT KPIs. On the prognosis of patients with LT, patients with KPIs were more likely to stay in the intensive care unit ≥ 7 d after LT than those without KPIs (56.3% *vs* 35.3%; P = 0.018). Patients with KPIs had a higher 6-month all-cause mortality rate than those without KPIs (17.6% *vs* 5.0%; P = 0.017). The multivariate analysis showed that KPIs were not risk factors for 6-month all-cause mortal-ity after LT. However, infections caused by CRKP (OR = 1.534-18.524, 95%CI: 5.330, P = 0.008), female sex (OR = 2.829, 95%CI: 1.098-7.288, P = 0.031), intraoperative red blood cell transfusion ≥ 12 U (OR = 3.466, 95%CI: 1.259-9.543, P = 0.016), day 3 post-LT creatinine levels ≥ 2 mg/dL (OR = 9.724, 95%CI: 4.077-23.194, P < 0.001) and post-LT mechanical ventilation (OR = 4.118, 95%CI: 1.790-9.476, P = 0.001) were risk factors for 6-month all-cause mortality after LT.

Research conclusions

This novel retrospective assessment explored key factors in the prevention of KPIs or CRKP. Many risk factors play crucial roles in the development of KPIs after LT and in recipient prognosis. This study explored the role of KPIs in the

prognosis of LT recipients and the risk factors for all KPIs after LT. By analyzing the distribution of KPIs and drug resistance, we demonstrated that risk factors are associated with surgical variables. Identifying these risk factors provides a basis for the prevention of KPIs, thereby improving the prognosis of LT recipients.

Research perspectives

In future studies, we should obtain more data to more accurately identify other potential correlates of KPIs in patients with LT to reduce the occurrence of KPIs. In addition, monitoring *K. pneumoniae*, especially CRKP, colonization before LT may provide new insights.

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FOOTNOTES

Author contributions: Guo L, Wan QQ, Peng WT, and Zhao J collected and analyzed the data; Wan QQ and Peng P wrote the original manuscript, revised the paper, and approved the final version.

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ORIGINAL ARTICLE

Retrospective Study

Development and validation of a nomogram for predicting in-hospital mortality of intensive care unit patients with liver cirrhosis

Xiao-Wei Tang, Wen-Sen Ren, Shu Huang, Kang Zou, Huan Xu, Xiao-Min Shi, Wei Zhang, Lei Shi, Mu-Han Lü

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Abstract

BACKGROUND

Liver cirrhosis patients admitted to intensive care unit (ICU) have a high mortality rate.

AIM

To establish and validate a nomogram for predicting in-hospital mortality of ICU patients with liver cirrhosis.

METHODS

We extracted demographic, etiological, vital sign, laboratory test, comorbidity, complication, treatment, and severity score data of liver cirrhosis patients from the Medical Information Mart for Intensive Care IV (MIMIC-IV) and electronic ICU (eICU) collaborative research database (eICU-CRD). Predictor selection and model building were based on the MIMIC-IV dataset. The variables selected through least absolute shrinkage and selection operator analysis were further screened through multivariate regression analysis to obtain final predictors. The final predictors were included in the multivariate logistic regression model, which was used to construct a nomogram. Finally, we conducted external validation using the eICU-CRD. The area under the receiver operating characteristic curve (AUC), decision curve, and calibration curve were used to assess the efficacy of the models.

RESULTS
Risk factors, including the mean respiratory rate, mean systolic blood pressure, mean heart rate, white blood cells, international normalized ratio, total bilirubin, age, invasive ventilation, vasopressor use, maximum stage of acute kidney injury, and sequential organ failure assessment score, were included in the multivariate logistic regression. The model achieved AUCs of 0.864 and 0.808 in the MIMIC-IV and eICU-CRD databases, respectively. The calibration curve also confirmed the predictive ability of the model, while the decision curve confirmed its clinical value.

CONCLUSION
The nomogram has high accuracy in predicting in-hospital mortality. Improving the included predictors may help improve the prognosis of patients.

Key Words: Liver cirrhosis; Intensive care unit; Nomogram; Predicting model; Mortality

Core Tip: Liver cirrhosis patients admitted to the intensive care unit have a high mortality rate. In this study, we collected clinical data from patients with liver cirrhosis and constructed a nomogram predictive model that gained high accuracy in predicting in-hospital mortality. The accuracy was also confirmed by external validation, which suggests that the model can help us identify high-risk patients.

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INTRODUCTION

Liver cirrhosis is the terminal stage of various chronic liver diseases[1]. In this stage, the liver undergoes diffuse liver fibrosis, and the normal structure is replaced by regenerated nodules[2]. As a global public health problem, the most common cause of liver cirrhosis includes alcohol-related liver disease, nonalcoholic fatty liver disease (NAFLD), and chronic viral hepatitis B and C[1]. In Africa and Asia, the leading cause of liver cirrhosis is chronic viral hepatitis B, while NAFLD has become the main cause of chronic liver disease in Western countries[3,4]. With the control of viral hepatitis and the increase in obesity and metabolic syndrome, NAFLD is likely to become the major cause of liver cirrhosis[5]. Notably, as the 11th leading cause of death and the third most common cause of death among people aged 45-64 years, liver cirrhosis leads to more than one million deaths annually, which accounts for half of all liver disease deaths[6].

Liver cirrhosis can be divided into compensated and decompensated stages depending on the course of the disease. In the compensated phase, the patient is asymptomatic. In contrast, in the decompensated phase, patients suffer from a variety of complications, such as ascites, portal hypertension-related bleeding, nonobstructive jaundice, and hepatic encephalopathy (HE)[1]. Complications are the cause of repeated hospital admissions and seriously affect the quality of life and prognosis of patients[7]. The risk of death in patients with compensated liver cirrhosis is 4.7 times greater than that in the general population, while the risk increases sharply to 9.7 times greater in the decompensated stage[7]. In the decompensated stage, patients often suffer from hepatic and extrahepatic organ failure[1]. This group of patients often requires intensive care support. A meta-analysis highlighted the importance of receiving intensive care support before patients develop excessive extrahepatic failure[8]. The Model for End-stage Liver Disease (MELD), MELD and Sodium, Chronic Liver Failure-Sequential Organ Failure Assessment, and Child-Turcotte-Pugh were used to assess liver disease and determine patient prognosis[9-11]. However, patients with cirrhosis admitted to the intensive care unit (ICU) may have a more complex situation. Therefore, in this study, we constructed a nomogram suitable for liver cirrhosis patients admitted to the ICU, which aims to identify high-risk patients early and administer intervention.

MATERIALS AND METHODS

Data source

The Medical Information Mart for Intensive Care IV (MIMIC-IV) database is a publicly available and freely accessible database. It was established in 2003 with funding from the National Institutes of Health by the Massachusetts Institute of Technology (MIT) Laboratory of Computational Physiology (LCP) and the Beth Israel Deaconess Medical Center of Harvard Medical School and Philips Healthcare. Clinical data from more than 190000 patients and 450000 hospitalizations are detailed in the MIMIC-IV database. The eICU collaborative research database (eICU-CRD) is a large public

database created by the Philips Group in collaboration with the MIT Laboratory of LCP. The eICU-CRD includes patient information from 335 ICU units in 208 hospitals across the United States using a stratified random sample covering more than 200000 patients admitted to ICUs in 2014 and 2015. The above two databases record detailed information on patient demographics, laboratory test results, medication administration, vital signs, surgical operations, diagnosis, *etc.* All the data in this study were extracted from the MIMIC-IV and eICU-CRD. We completed the Collaborative Institutional Training Initiative Program course and obtained access to the database (Record ID: 52439741).

Participants

The diagnosis of disease was based on the International Classification of Diseases code. Patients diagnosed with hepatic cirrhosis and admitted to the ICU were enrolled in the study. The following conditions were excluded: (1) had liver cancer or other malignant cancers; (2) were admitted to the ICU less than 24 h; (3) were aged < 18 years; and (4) had missing outcomes or missing data for more than 20% of the patients. Overall, 2730 and 841 patients were enrolled from the MIMIC-IV and eICU-CRD, respectively (Figure 1).

Data collection

We used the Structured Query Language query tool Navicat Premium to extract the data. The following information of patients were collected: Demographic data (gender, age), etiology, complications [HE, variceal hemorrhage (VH), acute kidney injury (AKI)], comorbidities [chronic obstructive pulmonary disease (COPD), heart failure (HF), myocardial infarct, Rena disease, Diabetes], the first laboratory tests after admitted to ICU [bicarbonate, calcium, chloride, sodium, potassium, blood urea nitrogen (BUN), creatinine, albumin, alanine aminotransferase, aspartate aminotransferase (AST), total bilirubin, international normalized ratio (INR), prothrombin time (PT), hemoglobin, platelets, white blood cells (WBC), red cell distribution width (RDW)], mean vital signs in first day admitted to ICU [heart rate (HR), respiratory rate (RR), systolic blood pressure (SBP), diastolic blood pressure], treatment [invasive ventilation, renal replacement therapy (RRT), vasopressor use] and prognostic scoring system [sequential organ failure assessment (SOFA) and MELD]. The MELD score was calculated as MELD = 9.6 × In (creatinin) + 3.8 × In (total bilirubin) + 11.2 × In (INR) + 6.4 × cause (cholestatic liver disease or alcoholic cirrhosis score is 0; other causes are 1)[12]. To avoid negative numbers in the calculation, if the value of creatinine, total bilirubin or the INR was less than 1, then the value was taken as 1 in the calculation. The diagnosis of AKI met the KDIGO criteria[13]. The official code for the corresponding view is provided (https://github.com/MIT-LCP/mimic-code/). Table 1 shows the baseline data of the patients in the two databases. Table 2 compares the baseline data between the MIMIC-IV and eICU-CRD.

Predictor selection model construction

We used least absolute shrinkage and selection operator (LASSO) regression to select the candidate variables (Figure 2). The LASSO algorithm adds a penalty function, which continuously shrinks the coefficients, to achieve the goals of simplifying the model and avoiding collinearity and overfitting. The selected predictors were subjected to multivariate logistic regression. Predictors with $P < 0.05$ and odds ratios not containing 1 were considered final predictors (Table 3). The final predictors were included in the multivariate logistic regression model, which was used to construct a nomogram.

Statistical analysis

Continuous variables are expressed as medians with interquartile ranges and were tested using the Mann-Whitney U test. Categorical variables are expressed as counts and percentages and were tested using the chi-square test. For variables missing less than 20% of the data, we used the method of imputation to fill in the missing values.

RESULTS

Patient characteristics

A total of 2730 and 814 patients were included in this study from the MIMIC-IV and eICU-CRD, respectively. The mortality rates in the MIMIC-IV and eICU-CRD cohorts were 20.842% and 20.809%, respectively. Although the data comes from different database, compared with survival group, the none-survival group have higher incidence of HE, higher stage of AKI, lower level of bicarbonate and albumin, higher level of BUN, creatinine, total bilirubin, AST, INR, PT, WBC and RDW, higher usage of invasive ventilation and vasopressor, higher HR, RR, lower level of blood pressure, and higher score of SOFA, and MELD.

Variable selection and model construction

Thirty-six variables were included in the variable screening process. We used LAASO regression to screen variables with the aim of minimizing the occurrence of covariance and overfitting. To simplify the model as much as possible while ensuring model fitting, we identified the variables at one standard deviation from the minimum penalty coefficient (lambda.min). Variables selected by LASSO regression were included in multivariate regression for secondary screening.

Variables screened by LASSO regression and multivariate regression were used to construct a predictive model. The final model included 11 predictors: SOFA score (OR: 1.082, 95%CI: 1.044-1.121); RR_mean (OR: 1.055, 95%CI: 1.026-1.085); SBP_mean (OR: 0.982, 95%CI: 0.973-0.99); HR_mean (OR: 1.017, 95%CI: 1.009-1.024); WBC (OR: 1.029, 95%CI: 1.015-1.044); INR (OR: 1.230, 95%CI: 1.106-1.371); total bilirubin (OR: 1.047, 95%CI: 1.033-1.062); age (OR: 1.039, 95%CI: 1.029-1.051);

Table 1 Baseline characteristics of two cohorts

Variables		MIMIC-IV cohort			*P* value		eICU cohort			*P* value
		All	Survivors	Non-survivors			All	Survivors	Non-survivors	
		(*n* = 2730)	0 (*n* = 2161)	1 (*n* = 569)			(*n* = 841)	0 (*n* = 666)	1 (*n* = 175)	
Demographics										
Age, median [IQR], year		59.043 [51.654, 67.468]	58.865 [51.466, 67.150]	60.412 [52.537, 69.440]	0.012		56.000 [50.000, 64.000]	56.000 [50.000, 64.000]	56.000 [50.000, 64.000]	0.820
Gender, *n* (%)	Female (0)	1027 (37.619)	813 (37.621)	214 (37.610)	0.996	Female (0)	324 (38.526)	265 (39.790)	59 (33.714)	0.142
	Male (1)	1703 (62.381)	1348 (62.379)	355 (62.390)		Male (1)	517 (61.474)	401 (60.210)	116 (66.286)	
Etiology and complications										
Etiology, *n* (%)	Alcoholic (0)	1448 (53.040)	1129 (52.244)	319 (56.063)	0.104	Alcoholic (0)	290 (34.483)	231 (34.685)	59 (33.714)	0.81
	Others (1)	1282 (46.960)	1032 (47.756)	250 (43.937)		Others (1)	551 (65.517)	435 (65.315)	116 (66.286)	
HE, *n* (%)	No (0)	2171 (79.524)	1752 (81.074)	419 (73.638)	< 0.001	No (0)	605 (71.938)	503 (75.526)	102 (58.286)	< 0.001
	Yes (1)	559 (20.476)	409 (18.926)	150 (26.362)		Yes (1)	236 (28.062)	163 (24.474)	73 (41.714)	
VH, *n* (%)	No (0)	2407 (88.168)	1896 (87.737)	511 (89.807)	0.174	No (0)	740 (87.990)	585 (87.838)	155 (88.571)	0.79
	Yes (1)	323 (11.832)	265 (12.263)	58 (10.193)		Yes (1)	101 (12.010)	81 (12.162)	20 (11.429)	
AKI_stage_max, *n* (%)	Without (0)	646 (23.663)	625 (28.922)	21 (3.691)	< 0.001	Without (0)	431 (51.249)	391 (58.709)	40 (22.857)	< 0.001
	Stage I (1)	333 (12.198)	296 (13.697)	37 (6.503)		Stage I (1)	164 (19.501)	114 (17.117)	50 (28.571)	
	Stage II (2)	779 (28.535)	683 (31.606)	96 (16.872)		Stage II (2)	29 (3.448)	23 (3.453)	6 (3.429)	
	Stage III (3)	972 (35.604)	557 (25.775)	415 (72.935)		Stage III (3)	217 (25.803)	138 (20.721)	79 (45.143)	
Comorbidities										
Renal_disease, *n* (%)	No (0)	2096 (76.777)	1688 (78.112)	408 (71.705)	0.001	No (0)	688 (81.807)	552 (82.883)	136 (77.714)	0.115
	Yes (1)	634 (23.223)	473 (21.888)	161 (28.295)		Yes (1)	153 (18.193)	114 (17.117)	39 (22.286)	
Diabetes, *n* (%)	No (0)	1872 (68.571)	1479 (68.441)	393 (69.069)	0.774	No (0)	642 (76.338)	507 (76.126)	135 (77.143)	0.778
	Yes (1)	858 (31.429)	682 (31.559)	176 (30.931)		Yes (1)	199 (23.662)	159 (23.874)	40 (22.857)	
COPD, *n* (%)	No (0)	2563 (93.883)	2027 (93.799)	536 (94.200)	0.722	No (0)	752 (89.417)	598 (89.790)	154 (88.000)	0.493
	Yes (1)	167 (6.117)	134 (6.201)	33 (5.800)		Yes (1)	89 (10.583)	68 (10.210)	21 (12.000)	
HF, *n* (%)	No (0)	2148 (78.681)	1724 (79.778)	424 (74.517)	0.006	No (0)	751 (89.298)	595 (89.339)	156 (89.143)	0.94
	Yes (1)	582 (21.319)	437 (20.222)	145 (25.483)		Yes (1)	90 (10.702)	71(10.661)	19(10.857)	
MI, *n* (%)	No (0)	2462 (90.183)	1968 (91.069)	494 (86.819)	0.002	No (0)	806 (95.838)	639(95.946)	167(95.429)	0.76

	Yes (1)	268 (9.817)	193 (8.931)	75 (13.181)		Yes (1)	35 (4.162)	27(4.054)	8(4.571)	
Treatment										
Vasopressor, *n* (%)	No (0)	1569 (57.473)	1432 (66.266)	137 (24.077)	< 0.001	No (0)	630 (74.911)	535 (80.330)	95 (54.286)	< 0.001
	Yes (1)	1161 (42.527)	729 (33.734)	432 (75.923)		Yes (1)	211 (25.089)	131 (19.670)	80 (45.714)	
Invasive_ventilation, *n* (%)	No (0)	1499 (54.908)	1333 (61.684)	166 (29.174)	< 0.001	No (0)	651 (77.408)	538 (80.781)	113 (64.571)	< 0.001
	Yes (1)	1231 (45.092)	828 (38.316)	403 (70.826)		Yes (1)	190 (22.592)	128 (19.219)	62 (35.429)	
RRT, *n* (%)	No (0)	2314 (84.762)	1940 (89.773)	374 (65.729)	< 0.001	No (0)	730 (86.801)	578 (86.787)	152 (86.857)	0.98
	Yes (1)	416 (15.238)	221 (10.227)	195 (34.271)		Yes (1)	111 (13.199)	88 (13.213)	23 (13.143)	
Laboratory tests										
Bicarbonate, median [IQR], mmol/L		22.000 [19.000, 25.000]	22.000 [19.000, 25.000]	20.000 [17.000, 24.000]	< 0.001		22.000 [18.000, 25.000]	22.700 [19.000,26.000]	21.000 [17.000, 24.000]	< 0.001
Calcium, median [IQR], mg/dL		8.300 [7.700, 8.900]	8.300 [7.700, 8.800]	8.300 [7.700, 9.000]	0.328		8.200 [7.700, 8.700]	8.200 [7.700, 8.700]	8.200 [7.700, 8.700]	0.953
Chloride, median [IQR], mmol/L		102.000 [97.000, 107.000]	103.000 [97.000, 107.000]	101.000 [95.000, 106.000]	< 0.001		102.000 [98.000, 107.000]	102.000 [98.000, 107.000]	102.000 [97.000, 108.000]	0.938
Sodium, median [IQR], mmol/L		137.000 [133.000, 140.000]	137.000 [133.000, 140.000]	136.000 [132.000, 140.000]	0.015		136.000 [131.000, 140.000]	136.000 [131.000, 139.700]	135.000 [130.000, 140.000]	0.768
Potassium, median [IQR], mmol/L		4.200 [3.700, 4.800]	4.200 [3.700, 4.700]	4.200 [3.700, 4.900]	0.149		4.100 [3.600, 4.600]	4.020 [3.500, 4.600]	4.300 [3.800, 4.900]	0.003
BUN, median [IQR], mg/dL		26.000 [15.000, 45.000]	24.000 [14.000, 40.000]	36.000 [20.000, 60.000]	< 0.001		25.000 [14.000, 45.000]	24.000 [13.000, 43.000]	32.000 [19.000, 54.000]	< 0.001
Creatinine, median [IQR], mg/dL		1.200 [0.800, 2.100]	1.100 [0.800, 1.800]	1.800 [1.000, 3.100]	< 0.001		1.250 [0.800, 2.200]	1.100 [0.760, 2.040]	1.600 [1.100, 2.800]	< 0.001
Albumin, median [IQR], g/dL		3.000 [2.600, 3.400]	3.000 [2.600, 3.400]	2.900 [2.400, 3.400]	< 0.001		2.500 [2.100, 3.067]	2.500 [2.100, 3.100]	2.300 [1.900, 2.800]	< 0.001
ALT, median [IQR], IU/L		31.000 [20.000, 59.500]	31.000 [20.000, 58.000]	34.000 [20.000, 65.000]	0.115		36.000 [23.000, 60.000]	34.000 [23.000, 57.000]	38.000 [24.000, 70.000]	0.045
AST, median [IQR], IU/L		63.000 [38.000, 125.000]	60.000 [37.000, 117.000]	79.000 [42.000, 149.000]	< 0.001		70.000 [43.000, 130.000]	67.000 [42.000, 118.000]	86.000 [48.000, 150.000]	0.004
Bilirubin_total, median [IQR], mg/dL		2.500 [1.100, 6.200]	2.100 [1.000, 5.000]	4.800 [1.900, 15.100]	< 0.001		3.100 [1.400, 7.000]	2.800 [1.300, 5.700]	5.700 [2.400, 14.000]	< 0.001
Inr, median [IQR]		1.600 [1.300, 2.100]	1.600 [1.300, 2.000]	2.000 [1.600, 2.700]	< 0.001		1.600 [1.300, 2.100]	1.500 [1.300, 2.000]	1.900 [1.500, 2.500]	< 0.001
Pt, median [IQR], sec		17.800 [14.600, 22.700]	17.000 [14.200, 21.100]	21.850 [17.800, 28.400]	< 0.001		18.300 [15.500, 23.400]	17.800 [15.200, 22.000]	21.700 [17.400, 27.633]	< 0.001
Hemoglobin, median [IQR], g/dL		9.500 [8.100,11.100]	9.600 [8.200,11.200]	9.100 [7.800, 10.600]	< 0.001		9.500 [8.000, 11.300]	9.400 [7.800, 11.300]	9.600 [8.200, 11.200]	0.434
Platelets, median [IQR], 109/L		108.000 [68.000, 170.000]	109.000 [70.000, 171.000]	100.000 [62.000, 161.000]	0.012		97.000 [63.000, 154.000]	99.000 [66.000, 155.000]	89.000 [58.000, 145.000]	0.094

WBC, median [IQR], 109/L	9.000 [5.800, 13.600]	8.400 [5.600, 12.700]	11.500 [7.600, 16.900]	< 0.001	9.400 [5.900, 14.300]	8.700 [5.600, 13.100]	12.100 [8.400, 17.800]	< 0.001
RDW, median [IQR], %	16.800 [15.100, 18.900]	16.600 [15.000, 18.700]	17.800 [15.800, 20.000]	< 0.001	17.300 [15.500, 19.600]	17.100 [15.280, 19.300]	18.000 [16.400, 20.100]	< 0.001
Vital signs								
HR_mean, median [IQR]	86.800 [76.237, 98.769]	85.360 [75.040, 96.875]	93.304 [80.826, 103.724]	< 0.001	89.029 [78.045, 100.000]	87.105 [76.676, 99.333]	94.796 [84.556, 102.423]	< 0.001
SBP_mean, median [IQR], mmHg	110.120 [101.694, 122.500]	112.292 [103.125, 124.917]	104.828 [97.667, 113.741]	< 0.001	108.920 [99.750, 121.000]	109.963 [100.654, 122.314]	103.855 [97.103, 115.954]	< 0.001
DBP_mean, median [IQR], mmHg	60.320 [53.963, 68.038]	61.520 [55.000, 69.080]	57.095 [50.625, 63.045]	< 0.001	59.310 [53.225, 67.000]	60.231 [53.638, 67.970]	56.455 [51.579, 63.857]	< 0.001
RR_mean, median [IQR]	18.243 [15.958, 21.200]	17.872 [15.774, 20.577]	19.900 [16.846, 23.318]	< 0.001	18.640 [16.533, 21.896]	18.321 [16.277, 20.964]	20.649 [17.852, 23.911]	< 0.001
Prognostic scoring system								
SOFA, median [IQR]	8.000 [5.000, 10.000]	7.000 [5.000, 9.000]	11.000 [8.000, 14.000]	< 0.001	7.000 [5.000, 10.000]	7.000 [4.000, 9.000]	9.000 [7.000, 12.000]	< 0.001
MELD, median [IQR]	16.060 [10.225, 23.595]	14.287 [9.338, 21.346]	23.674 [16.662, 30.045]	< 0.001	17.887 [12.060, 26.087]	16.699 [10.941, 24.147]	24.499 [16.194, 32.895]	< 0.001

HE: Hepatic encephalopathy; VH: Variceal hemorrhage; AKI: Acute kidney injury; COPD: Chronic obstructive pulmonary disease; HF: Heart failure; MI: Myocardial infarct; BUN: Blood urea nitrogen; ALT: Aminotransferase alanine; AST: Aminotransferase aspartate; INR: International Normalized Ratio; Pt: Prothrombin Time; WBC: White blood cells; RDW: Red cell distribution width; RRT: Renal replacement therapy; HR: Heart rate; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; RR: Respiratory rate; SOFA: Sequential Organ Failure Assessment; max: Maximum; MELD: Model for end-stage liver disease; IQR: Interquartile range; MIMIC-IV: Medical Information Mart for Intensive Care IV.

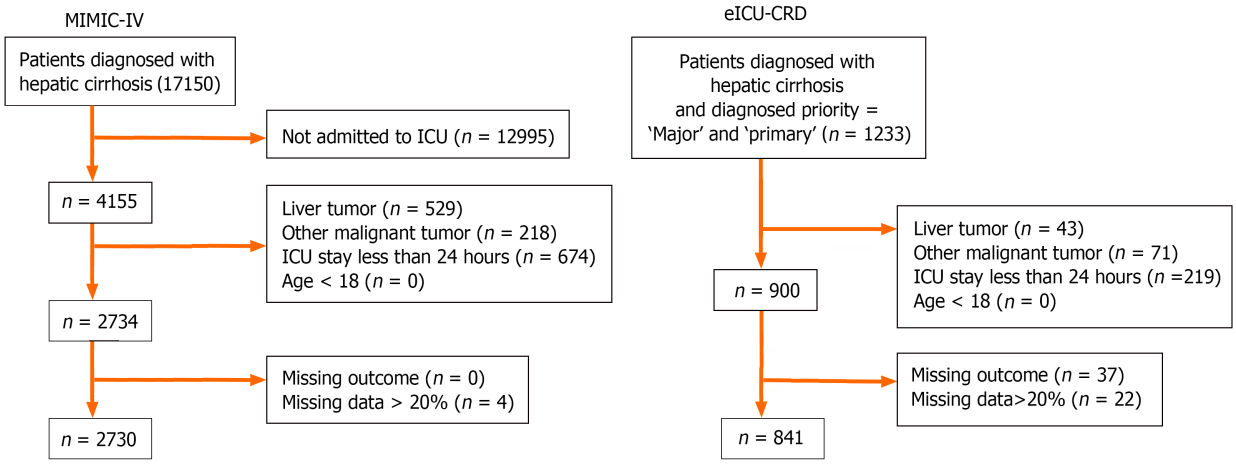

Figure 1 Flowchart of the data extraction procedure. MIMIC-IV: Medical Information Mart for Intensive Care IV; ICU: intensive care unit; eICU-CRD: Electronic intensive care unit collaborative research database.

invasive_ventilation (OR: 1.82, 95%CI: 1.385-2.397); vasopressor (OR: 1.718, 95%CI: 1.291-2.290); and AKI_stage_max = 1 (OR: 1.851, 95%CI: 1.031-3.387), AKI_stage_max = 2 (OR: 2.031, 95%CI: 1.237-3.472), AKI_stage_max = 3 (OR: 5.729, 95%CI: 3.585-9.585). The nomogram showed the scores of the predictors at different values and risk of death according to the total score (Figure 3).

Model performance and validation

Based on the nomogram scores, we constructed ROC curves (Figure 4). The nomogram model had AUCs of 0.864 and 0.808 in the training and test datasets, respectively. These findings showed that the nomogram has good discrimination

Table 2 Baseline comparison between the two databases

Variables		All	MIMIC	eICU	*P* value
		(*n* = 3571)	0 (*n* = 2730)	1 (*n* = 841)	
Hospital_expire_flag, *n* (%)	0	2827 (79.165)	2161 (79.158)	666 (79.191)	0.983
	1	744 (20.835)	569 (20.842)	175 (20.809)	
Demographics					
Age, median [IQR], yr		58.641 [51.114, 66.693]	59.043 [51.654, 67.468]	56.000 [50.000, 64.000]	< 0.001
Gender, *n* (%)	Female (0)	1351 (37.833)	1027 (37.619)	324 (38.526)	0.636
	Male (1)	2220 (62.167)	1703 (62.381)	517 (61.474)	
Etiology and complications					
Etiology, *n* (%)	Alcoholic (0)	1738 (48.670)	1448 (53.040)	290 (34.483)	< 0.001
	Others (1)	1833 (51.330)	1282 (46.960)	551 (65.517)	
HE, *n* (%)	No (0)	2776(77.737)	2171 (79.524)	605 (71.938)	< 0.001
	Yes (1)	795(22.263)	559 (20.476)	236 (28.062)	
VH, *n* (%)	No (0)	3147(88.127)	2407 (88.168)	740 (87.990)	0.889
	Yes (1)	424(11.873)	323 (11.832)	101 (12.010)	
AKI_stage_max, *n* (%)	Without (0)	1077 (30.160)	646 (23.663)	431 (51.249)	< 0.001
	Stage I (1)	497 (13.918)	333 (12.198)	164 (19.501)	
	Stage II (2)	808 (22.627)	779 (28.535)	29 (3.448)	
	Stage III (3)	1189 (33.296)	972 (35.604)	217 (25.803)	
Comorbidities					
Renal_disease, *n* (%)	No (0)	2784 (77.961)	2096 (76.777)	688 (81.807)	0.002
	Yes (1)	787 (22.039)	634 (23.223)	153 (18.193)	
Diabetes, *n* (%)	No (0)	2514 (70.400)	1872 (68.571)	642 (76.338)	< 0.001
	Yes (1)	1057 (29.600)	858 (31.429)	199 (23.662)	
COPD, *n* (%)	No (0)	3315 (92.831)	2563 (93.883)	752 (89.417)	< 0.001
	Yes (1)	256 (7.169)	167 (6.117)	89 (10.583)	
HF, *n* (%)	No (0)	2899 (81.182)	2148 (78.681)	751 (89.298)	< 0.001
	Yes (1)	672 (18.818)	582 (21.319)	90 (10.702)	
MI, *n* (%)	No (0)	3268 (91.515)	2462 (90.183)	806 (95.838)	< 0.001
	Yes (1)	303 (8.485)	268 (9.817)	35 (4.162)	
Treatment					
vasopressor, *n* (%)	No (0)	2199 (61.579)	1569 (57.473)	630 (74.911)	< 0.001
	Yes (1)	1372 (38.421)	1161 (42.527)	211 (25.089)	
invasive_ventilation, *n* (%)	No (0)	2150 (60.207)	1499 (54.908)	651 (77.408)	< 0.001
	Yes (1)	1421 (39.793)	1231 (45.092)	190 (22.592)	
RRT, *n* (%)	No (0)	3044 (85.242)	2314 (84.762)	730 (86.801)	0.145
	Yes (1)	527 (14.758)	416 (15.238)	111 (13.199)	
Laboratory tests					
Bicarbonate, median [IQR], mmol/L		22.000 [19.000, 25.000]	22.000 [19.000, 25.000]	22.000 [18.000, 25.000]	0.291
Calcium, median [IQR], mg/dL		8.300 [7.700, 8.800]	8.300 [7.700, 8.900]	8.200 [7.700, 8.700]	0.005
Chloride, median [IQR], mmol/L		102.000 [97.000, 107.000]	102.000 [97.000, 107.000]	102.000 [98.000, 107.000]	0.108

Sodium, median [IQR], mmol/L	137.000 [133.000, 140.000]	137.000 [133.000, 140.000]	136.000 [131.000, 140.000]	< 0.001
Potassium, median [IQR], mmol/L	4.100 [3.700, 4.700]	4.200 [3.700, 4.800]	4.100 [3.600, 4.600]	< 0.001
BUN, median [IQR], mg/dL	26.000 [15.000, 45.000]	26.000 [15.000, 45.000]	25.000 [14.000, 45.000]	0.49
Creatinine, median [IQR], mg/dL	1.200 [0.800, 2.100]	1.200 [0.800, 2.100]	1.250 [0.800, 2.200]	0.193
Albumin, median [IQR], g/dL	2.900 [2.400, 3.350]	3.000 [2.600, 3.400]	2.500 [2.100, 3.067]	< 0.001
ALT, median [IQR], IU/L	32.000 [20.000, 60.000]	31.000 [20.000, 59.500]	36.000 [23.000, 60.000]	0.001
AST, median [IQR], IU/L	65.000 [39.000, 126.000]	63.000 [38.000, 125.000]	70.000 [43.000, 130.000]	0.007
Bilirubin_total, median [IQR], mg/dL	2.623 [1.200, 6.400]	2.500 [1.100, 6.200]	3.100 [1.400, 7.000]	< 0.001
INR, median [IQR]	1.600 [1.300, 2.100]	1.600 [1.300, 2.100]	1.600 [1.300, 2.100]	0.272
Pt, median [IQR], sec	18.000 [14.800, 22.900]	17.800 [14.600, 22.700]	18.300 [15.500, 23.400]	0.005
Hemoglobin, median [IQR], g/dL	9.500 [8.000, 11.100]	9.500 [8.100, 11.100]	9.500 [8.000, 11.300]	0.88
Platelets, median [IQR], 10^9/L	105.000 [67.000, 166.000]	108.000 [68.000, 170.000]	97.000 [63.000, 154.000]	< 0.001
WBC, median [IQR], 10^9/L	9.100 [5.800, 13.700]	9.000 [5.800, 13.600]	9.400 [5.900, 14.300]	0.113
RDW, median [IQR], %	17.000 [15.200, 19.100]	16.800 [15.100, 18.900]	17.300 [15.500, 19.600]	< 0.001
Bicarbonate, median [IQR], mmol/L				
Hr_mean, median [IQR]	87.182 [76.588, 99.122]	86.800 [76.237, 98.769]	89.029 [78.045, 100.000]	0.002
SBP_mean, median [IQR], mmHg	109.842 [101.208, 122.292]	110.120 [101.694, 122.500]	108.920 [99.750, 121.000]	0.002
Dbp_mean, median [IQR], mmHg	60.143 [53.750, 67.810]	60.320 [53.963, 68.038]	59.310 [53.225, 67.000]	0.031
Rr_mean, median [IQR], mmHg	18.363 [16.047, 21.286]	18.243 [15.958, 21.200]	18.640 [16.533, 21.896]	< 0.001
Prognostic scoring system				
Meld, median [IQR]	16.588 [10.602, 24.153]	16.060 [10.225, 23.595]	17.887 [12.060, 26.087]	< 0.001
SOFA, median [IQR]	7.000 [5.000, 10.000]	8.000 [5.000, 10.000]	7.000 [5.000, 10.000]	0.017

HE: Hepatic encephalopathy; VH: Variceal hemorrhage; AKI: Acute kidney injury; COPD: Chronic obstructive pulmonary disease; HF: Heart failure; MI: Myocardial infarct; BUN: Blood urea nitrogen; ALT: Aminotransferase alanine; AST: Aminotransferase aspartate; INR: International Normalized Ratio; Pt: Prothrombin Time; WBC: White blood cells; RDW: Red cell distribution width; RRT: Renal replacement therapy; HR: Heart rate; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; RR: Respiratory rate; SOFA: Sequential Organ Failure Assessment; max: Maximum; MELD: Model for end-stage liver disease; IQR: Interquartile range; MIMIC-IV: Medical Information Mart for Intensive Care IV.

ability in the MIMIC-IV and eICU-CRD cohorts. We also compared the nomogram with the traditional prognostic scoring system. The nomogram model outperformed the MELD score and SOFA score in both the training and test sets. The calibration curve showed good agreement between the predicted probability and the actual observation, which also confirmed the predictive ability of the model (Figure 5). We plotted decision curves to demonstrate the value of the clinical application of the model (Figure 6). The model has net benefits at almost the full range of threshold probabilities. Compared to traditional prognostic scoring systems, nomogram-guided clinical interventions also have greater net benefits.

DISCUSSION

Liver cirrhosis, a global public health problem, is the 11th leading cause of death and the third most common death among people aged 45-64 years[6]. Patients in the decompensated stage of liver cirrhosis develop a variety of complications, often accompanied by hepatic and extrahepatic organ failure[1]. The ICU provides treatment, including respiratory support, circulatory support, RRT and antibiotics, needed by critically ill patients. Timely detection and early intervention for organ failure may improve patient prognosis.

In this study, we developed a nomogram model for predicting in-hospital mortality in patients with liver cirrhosis admitted to the ICU. A total of 11 variables were included in the prediction model after screening. The AUC of the model in the training set (MIMIC-IV) and test set (eICU-CRD) were 0.864 and 0.808, respectively, which indicated that the model

Table 3 Multivariate logistic regression model of in-hospital mortality

	Multivariable analysis based on LASSO regression result			Multivariable logistics model		
Predictor	β	*P* value	Odds ratio (95%CI)	β	*P* value	Odds Ratio
SOFA	0.074	0	1.076 (1.037-1.118)	0.078	0	1.082 (1.044-1.121)
RR_mean	0.05	0	1.052 (1.022-1.082)	0.054	0	1.055 (1.026-1.085)
DBP_mean	-0.009	0.226	0.991 (0.976-1.006)			
SBP_mean	-0.014	0.006	0.986 (0.976-0.996)	-0.019	0	0.982 (0.973-0.99)
HR_mean	0.02	0	1.021 (1.012-1.029)	0.017	0	1.017 (1.009-1.024)
RDW	0.029	0.161	1.029 (0.988-1.072)			
WBC	0.027	0	1.027 (1.012-1.042)	0.029	0	1.029 (1.015-1.044)
INR	0.203	0	1.226 (1.102-1.366)	0.207	0	1.230 (1.106-1.371)
Bilirubin_total	0.043	0	1.044 (1.029-1.059)	0.046	0	1.047 (1.033-1.062)
ALT	0	0.029	1 (0.999-1)			
BUN	0.004	0.049	1.004 (1-1.008)			
Age	0.033	0	1.034 (1.022-1.045)	0.039	0	1.039 (1.029-1.051)
AKI_stage_max 1	0.588	0.052	1.801 (1.002-3.3)	0.616	0.041	1.851 (1.031-3.387)
AKI_stage_max 2	0.683	0.01	1.981 (1.2-3.398)	0.709	0.007	2.031 (1.237-3.472)
AKI_stage_max 3	1.701	0	5.48 (3.402-9.231)	1.746	0	5.729 (3.585-9.585)
RRT1	0.002	0.987	1.002 (0.743-1.35)			
Invasive_ventilation1	0.653	0	1.922 (1.456-2.543)	0.599	0	1.820 (1.385-2.397)
Vasopressor1	0.536	0	1.709 (1.279-2.288)	0.541	0	1.718 (1.291-2.290)
MI1	0.299	0.113	1.349 (0.928-1.949)			
HF1	0.206	0.169	1.229 (0.915-1.646)			

SOFA: Sequential Organ Failure Assessment; RR: Respiratory rate; SBP: Systolic blood pressure; HR: Heart rate; WBC: White blood cells; RDW: Red cell distribution width; INR: International Normalized Ratio; BUN: Blood urea nitrogen; HE: Hepatic encephalopathy; MI: Myocardial infarct; HF: Heart failure; max: Maximum.

had good predictive ability. Recently, a nomogram predictive model was established to predict in-hospital mortality in patients with alcoholic liver cirrhosis based on the MIMIC-III and eICU-CRD[14]. Compared to this study, our study was not limited to patients with alcoholic cirrhosis, and we used the updated MIMIC database MIMIC-IV, which represents a larger sample size. Consistent with their study, our study also concluded that the nomogram had better performance than did the MELD score. In previous studies, the MELD score performed well and outperformed the Child-Pugh score and the Simplified Acute Physiology Score II[15-17]. However, the MELD score did not perform well in our study. Both bilirubin and the INR, as indicators of liver function, reflect the severity of cirrhosis[18]. According to the definition of ACLF developed by the Asian Pacific Association, patients with a serum bilirubin concentration > 5 mg/dL and an INR > 1.5 should be considered for liver failure[19]. As important components of the MELD score, bilirubin concentration and the INR were also included as predictors[20]. According to the multivariate logistic regression analysis, the INR and bilirubin concentration had OR of 1.23 (95%CI: 1.106-1.371) and 1.047 (95%CI: 1.033-1.062), respectively.

The SOFA score assesses illness severity in six organ systems (nervous, respiratory, cardiovascular, renal, liver, and coagulation)[21]. The Sepsis-3 criteria also use the SOFA score to define sepsis[22]. In fact, patients with decompensated cirrhosis are at high risk of bacterial infections and developing sepsis, which greatly increases the mortality rate of liver cirrhosis patients[23,24]. The level of WBC confirmed this. According to both the MIMIC-IV and eICU-CRD, the death group had a greater WBC than the nondeath group. This means that the death group had more severe infections. According to the model, WBC is a risk factor for death, with an OR of 1.029 (95%CI: 1.015-1.044). As prognostic scoring system, both the score of MELD and SOFA in non-death group are higher. In our study, MELD and SOFA scores had close performance and are inferior to nomogram in the MIMIC-IV and eICU-CRD. This may be because the 11-variable nomogram can better reflect the complexity of liver cirrhosis patients admitted to the ICU.

In our study, age was a risk factor for patient death. This may be due to the fact that elderly patients often have a combination of chronic diseases such as hypertension, diabetes mellitus, HF, COPD, *etc*. For liver cirrhosis patients, older age is associated with a longer disease course and a greater likelihood of entering the decompensated phase of liver cirrhosis. Moreover, circulatory dynamics, immune function and organ function gradually begin to deteriorate as individuals age[25]. This may explain why older patients have a worse prognosis.

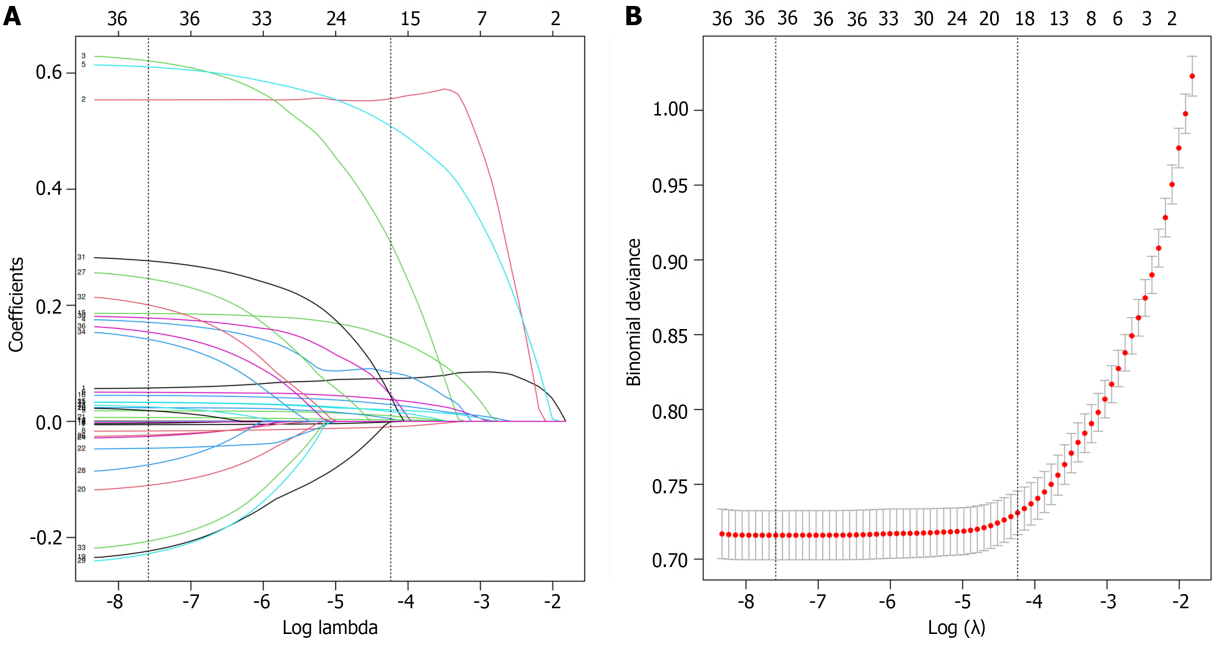

Figure 2 Clinical feature selection based on least absolute shrinkage and selection operator logistic regression. A: Selection of the optimal lambda according to least absolute shrinkage and selection operator (LASSO) logistic regression. Each line represents the change in the coefficient of each feature; B: LASSO coefficient profiles of features. The left and right black vertical lines were drawn at the lambda with minim deviance and 1 standard error to the lambda with minim deviance.

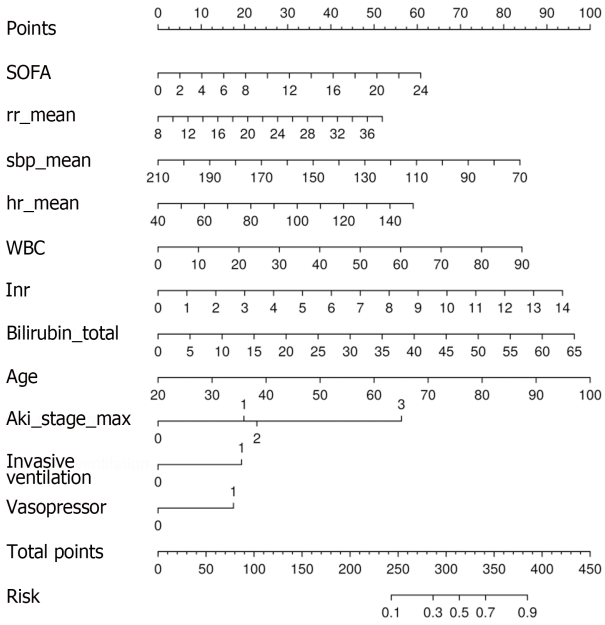

Figure 3 Nomogram based on the logistic regression model. The score of each predictor was summed to obtain the total points. The total points were used to determine the risk of death. SOFA: Sequential organ failure assessment.

Unstable circulatory status is an important reason patients are admitted to the ICU. There is an interaction between heart function and liver function[26]. Hepatic cardiomyopathy has started to receive increased amounts of attention in recent years. Impaired liver function and portal hypertension lead to arterial vasodilatation in patients with cirrhosis, which causes hemodynamic disturbances, including hyperdynamic circulation; increased cardiac output and HR; and impaired myocardial structure and function[27]. Patients suffering from cirrhosis have a weakened immune system, increasing vulnerability to various infections[28]. Severe infection can cause septic shock. Patients with cirrhosis may also develop hypovolemic shock due to VH[29]. Whatever the cause of the shock, the patient is in a critical condition. Patients with shock may have a higher RR and HR and lower pressure and may require vasopressors to maintain pressure. In our study, a higher RR and HR, lower SBP and the use of vasopressors were risk factors for hospital death.

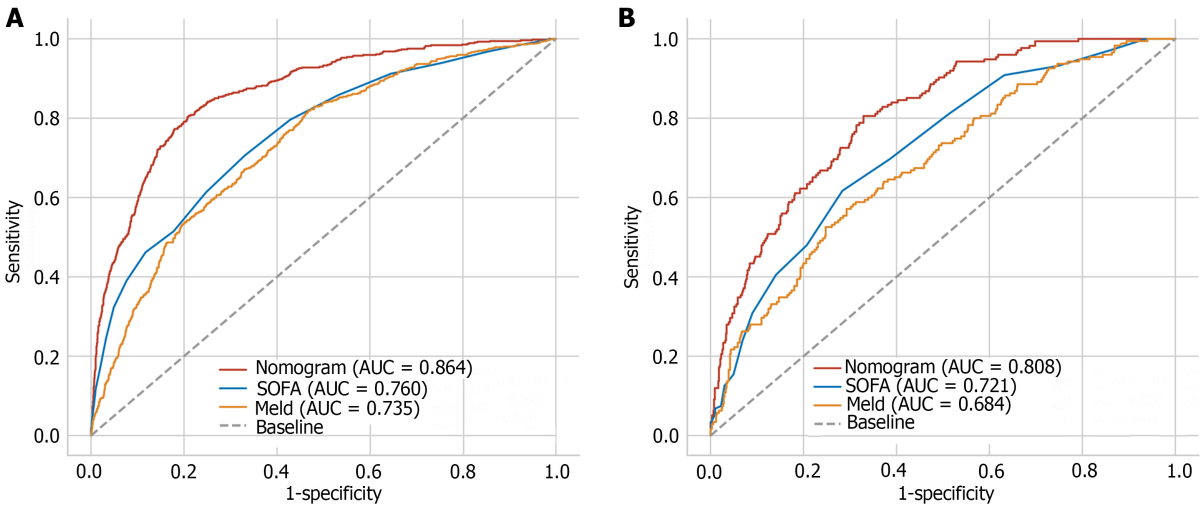

Figure 4 Receiver operating characteristic curves. A: The training dataset; B: The test dataset. SOFA: Sequential organ failure assessment; AUC: The area under the receiver operating characteristic curve.

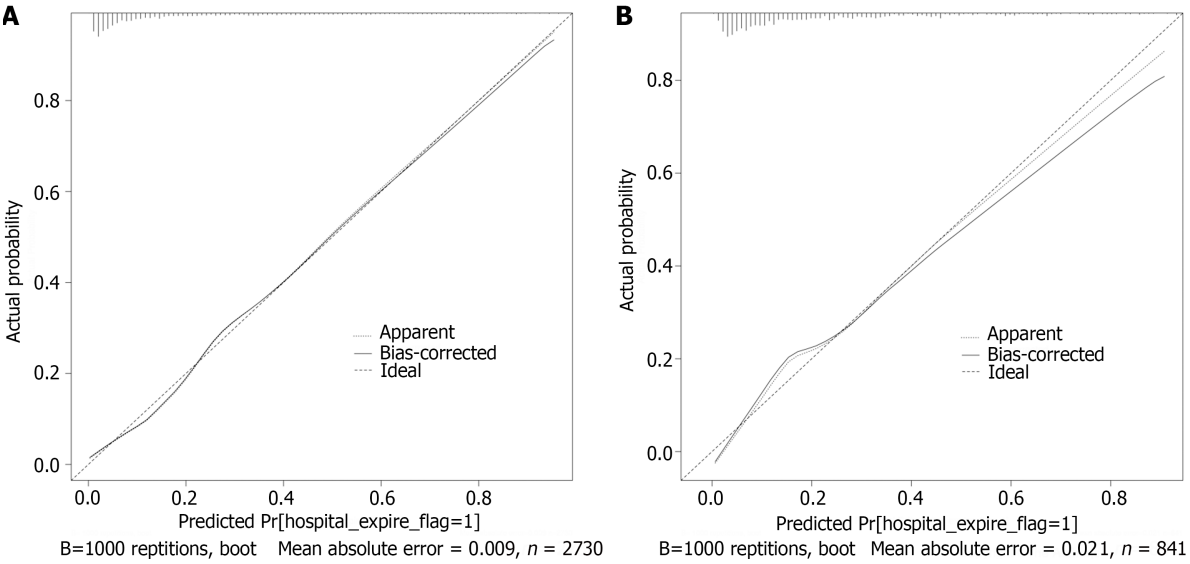

Figure 5 Calibration curves. A: The training dataset; B: The test dataset. The X-axis and Y-axis represent the predicted and actual probability of hospital mortality, respectively. The apparent and bias-corrected lines show that the predicted probability and adjusted predicted probability fit the actual probability.

Acute renal failure is a common complication in patients with cirrhosis and is associated with a poorer prognosis and chronic kidney disease[1,30,31]. For patients with liver cirrhosis, prerenal injury, acute tubular necrosis and hepatorenal syndrome are the main causes of AKI[32]. AKI has been reported to occur in 10%-15% of hospitalized patients and more than 50% of ICU patients[33]. In this study, AKI occurred in 70% of the cohort from the MIMIC-IV database and 49% of the cohort from the eICU-CRD. AKI was a significant predictor of hospital mortality in this study. Notably, the mortality group had a greater percentage of patients with stage III AKI in both the MIMIC-IV and the eICU-CRD cohorts. The OR for stage III AKI was as high as 5.729 (95%CI: 3.585-9.585), which was much greater than that for stage I and stage II AKI. Previous studies have also confirmed that a higher AKI stage indicates a worse prognosis[34,35]. Therefore, we should pay attention not only to the occurrence of AKI but also to the stage of AKI. Prevention of AKI development and progression may improve the prognosis of patients with liver cirrhosis.

The need for airway protection due to hepatic coma and respiratory failure resulting from lung infection, pleural effusion, hepatopulmonary syndrome, *etc*., are the main reasons why liver cirrhosis patients are admitted to the ICU for respiratory support[36,37]. Mechanical ventilation has been demonstrated to be associated with poorer prognosis in several studies[38,39]. Mechanical ventilation (OR: 1.82, 95%CI: 1.385-2.397) was also a risk factor for in-hospital mortality in our study, which is consistent with the findings of previous studies. The length of mechanical ventilation also affects the prognosis of patients. Levesque *et al*[39] found that the length of ventilation was an independent risk factor for one-year survival [OR: 1.1 (95%CI: 1.0-1.2)]. For patients who are not intubated, aggressive intervention is needed to avoid tracheal intubation. For patients with mechanical ventilation, actively treat the cause of tracheal intubation is needed in order to extubate as early as possible.

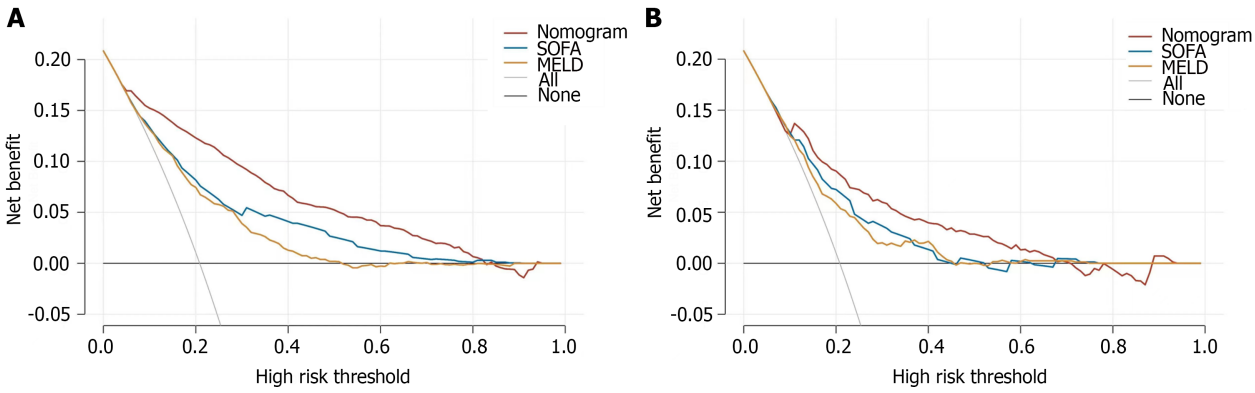

Figure 6 The decision curves. A: The training dataset; B: The test dataset. SOFA: Sequential organ failure assessment; MELD: Model for End-stage Liver Disease.

Decompensated cirrhosis can affect multiple systems and lead to multiple-organ failure. The prognosis of patients with cirrhosis worsens as the number of organ failures increases[40]. Therefore, cirrhosis is not just a liver disease but also a systemic disease. The complexity of cirrhosis is particularly pronounced in patients admitted to the ICU. Therefore, integrated and comprehensive management is needed for these patients.

There are several limitations of our study. First, several important variables were not included in this study because of the large number of missing data. Second, although external validation was performed for this study, both the training and test sets were from the United States. Therefore, data from other regions are needed to validate the model.

CONCLUSION

We developed and validated a nomogram model for predicting in-hospital mortality in liver cirrhosis patients admitted to the ICU. The nomogram has high accuracy in predicting hospital mortality. This helps us to identify patients at high risk timely and give intervention actively.

ARTICLE HIGHLIGHTS

Research background

Liver cirrhosis patients in decompensated stage often suffer from hepatic and extrahepatic organ failure and part of them requires intensive care support.

Research motivation

Liver cirrhosis patients admitted to intensive care unit have a high mortality rate.

Research objectives

To identify patients at high risk timely and give intervention actively.

Research methods

We extracted clinical data of liver cirrhosis patients from the Medical Information Mart for Intensive Care IV and electronic intensive care unit (eICU) collaborative research database. Predictors after selection were used to construct a nomogram prediction model. The efficacy of the model was tested by external validation.

Research results

The model gained the area under the receiver operating characteristic curve of 0.864 and 0.808 in the Medical Information Mart for Intensive Care IV and eICU collaborative research respectively. The calibration curve also confirmed the predictive ability of the model, while the decision curve confirmed the clinical use value.

Research conclusions

The nomogram model has high accuracy in predicting in-hospital mortality.

Research perspectives

The model helps us identify patients at high risk timely and give intervention actively, which may help improve the prognosis of the patient.

FOOTNOTES

Co-first authors: Xiao-Wei Tang and Wen-Sen Ren.

Author contributions: Tang XW and Ren WS contributed equally to this work; Ren WS, Lü MH, Tang XW, and Huang S designed the research study; Ren WS, Zou K, Xu H and Shi XM collected the data; Ren WS, Zhang W and Shi L analyzed the data and constructed the nomogram model; Ren WS, Lü MH and Tang XW wrote the manuscript. All authors have read and approve the final manuscript.

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Institutional review board statement: This study was reviewed and approved by the Institutional Review Committee of the Affiliated Hospital of Southwest Medical University (approval No. KY2023387).

Informed consent statement: This is an informed consent exemption statement. All data were downloaded from the Medical Information Mart for Intensive Care IV and the eICU collaborative research database. The two databases are publicly available. Before extracting data from the database, we completed the Collaborative Institutional Training Initiative Program course and were authorized to use the database.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: Data is available on the website (https://physionet.org/).

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Prospective Study

Prospective study of hepatitis B and D epidemiology and risk factors in Romania: A 10-year update

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Abstract

BACKGROUND

The global burden of hepatitis D virus (HDV) infection represents a major medical challenge and a public health crisis worldwide. However, there is a lack of accurate data on the epidemiology and risk factors for HDV. Hepatitis B virus (HBV) and HDV coinfection causes the most severe form of viral hepatitis, leading to a higher cumulative incidence of liver-related events compared with HBV monoinfection, including the need for liver transplantation and death.

AIM

To investigate the epidemiology, natural history, risk factors and clinical management of HBV and HDV coinfection in Romanian patients.

METHODS

This prospective study was conducted between January and July 2022 in six tertiary gastroenterology and hepatology referral centres in Romania. All consecutive adults admitted for any gastroenterology diagnosis who were HBV-positive were enrolled. Patients with acute hepatitis or incomplete data were excluded. Of the 25390 individuals who presented with any type of gastroenterology diagnosis during the study period, 963 met the inclusion criteria. Testing for anti-HDV antibodies and HDV RNA was performed for all participants. Demographic and risk factor data were collected by investigators using medical charts and patient questionnaires. All data were stored in an anonymized online database during the study.

RESULTS

The prevalence of HBV was 3.8%; among these patients, the prevalence of HBV/HDV coinfection was 33.1%. The median age of the study population was 54.0 years, and it consisted of 55.1% men. A higher prevalence of HBV/HDV coinfection was observed in patients 50–69 years old. Patients with HBV/HDV coinfection were significantly older than those with HBV monoinfection ($P = 0.03$). Multivariate multiple regression analysis identified female gender ($P = 0.0006$), imprisonment ($P < 0.0001$), older age at diagnosis ($P = 0.01$) and sexual contact with persons with known viral hepatitis ($P = 0.0003$) as significant risk factors for HDV.

CONCLUSION

This study shows that HDV infection among those with HBV remains endemic in Romania and updates our understanding of HDV epidemiology and associated risk factors. It emphasizes the need for systematic screening for HDV infection and collaborative initiatives for controlling and preventing HBV and HDV infection.

Key Words: Epidemiology; Hepatitis B; Hepatitis D; Natural history; Risk factors; Romania

Core Tip: In this study, we investigated the epidemiology, natural history, risk factors and clinical management of hepatitis B virus (HBV) and hepatitis D virus (HDV) coinfection in Romanian patients. We found that HDV infection among those with HBV remains endemic and identified the following significant risk factors associated with HBV/HDV chronic hepatitis: female gender, older age at diagnosis, sexual contact with persons with known viral hepatitis and imprisonment. This study emphasizes the need for systematic screening for HDV infection, subsequent reflex testing of HDV RNA and collaborative initiatives for controlling, treating and preventing HBV and HDV infection.

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INTRODUCTION

Hepatitis D virus (HDV) requires hepatitis B virus (HBV) to infect humans; it uses the envelope proteins of HBV, which acts as a helper virus for HDV entry and infection of hepatocytes[1]. Chronic HDV infection causes the most severe form of viral hepatitis, associated with accelerated progression to cirrhosis and a higher cumulative incidence of events including hepatic decompensation, liver failure, hepatocellular carcinoma, liver transplantation and liver-related death[1-5]. The global burden of HDV infection represents a major medical challenge and a public health crisis worldwide. Data on prevalence and health burden are patchy and heterogeneous owing to a lack of awareness, systematic population-based screening and accurate diagnostic assays[6]. Three large meta-analyses estimated the pooled global seroprevalence of HDV infection to be 0.2%-1.0% among the general population, 4.5%-14.6% among people who are hepatitis B surface antigen (HBsAg)-positive and 14.6%-18.6% among patients with chronic liver disease attending hepatology clinics[2,7,8]. These figures would correspond to an estimated burden of 12-72 million people living with serological evidence of HDV infection worldwide.

There is significant geographic variability in the prevalence of HDV infection, driven by various factors such as coverage of HBV vaccination, routes of transmission, hygiene, socio-economic conditions, migration and viral heterogeneity[1,9,10]. HDV infection is hyperendemic in certain geographic hotspots and populations called 'endemic pockets', including Eastern European countries such as Romania[1,9]. Mass migration from these HDV endemic areas in the early 2000s has prompted a rise in the HDV prevalence in some Western European countries[3,11-13]. Therefore, efforts to update our understanding of the HDV prevalence, particularly in endemic pockets, will guide strategies to decrease HDV infection Europe-wide.

The availability of efficacious and specific treatment options for HDV is limited. The World Health Organization (WHO) recommends pegylated interferon-α therapy for HDV infection; however, treatment efficacy is low and side effects are common[14,15]. Nucleos(t)ide analogues are recommended for control of HBV infection in patients with HBV/HDV coinfection if there is evidence of ongoing HBV replication[6]. The antiviral bulevirtide, which inhibits HBV and HDV entry into hepatocytes, received conditional European marketing authorization in 2020 for HDV infection in adults with compensated liver disease[16]. However, pegylated interferon-α remains the only HDV treatment recommended by the Romanian National Health Insurance House, suggesting that there may be issues with treatment access in endemic pockets[17].

With the development and emerging availability of dedicated antiviral therapeutics for HDV, an updated understanding of the epidemiology and clinical management of HDV infection is needed to allow more accurate targeting of high-risk populations for diagnosis and treatment. Epidemiological data on HBV/HDV coinfection in Romania have been published previously[18-20]. However, these data were collected more than 10 years ago and may no longer be accurate owing to healthcare policy changes, including the implementation of double reflex testing following an internal policy agreement between gastroenterology centre hepatologists and virologists. Double reflex testing refers to the testing of anti-HDV antibodies in patients with HBV, followed by HDV RNA testing in patients with a positive anti-HDV antibodies test result[21]. Therefore, this study aims to update our understanding of the epidemiology, natural history, risk factors and clinical management of HBV and HDV coinfection in patients in Romania.

MATERIALS AND METHODS

This short-term prospective study was conducted between January and July 2022 in six tertiary gastroenterology and hepatology referral centres in Romania, covering approximately 70%-80% of the population from all geographical regions of the country [Bucharest (two referral centres), Craiova, Iasi, Oradea, Timisoara]. All adults (≥ 18 years) admitted for any gastroenterology diagnosis who were HBV-positive were eligible, and the specific disease stage of each participant upon enrolment was classified using International Classification of Diseases-10 codes[22]. If a participant was hospitalized multiple times during the study period, data were collected only during their first admission. Patients with acute hepatitis or incomplete data were excluded. The number of admissions of any gastroenterology diagnosis during the study period was also recorded.

All HBV-positive participants were tested for anti-HDV immunoglobulin G antibodies by enzyme-linked immunosorbent assays (HDV antibody ELISA kit, Dia.Pro, Milan, Italy), following implementation of a policy agreement between hepatologists and virologists at our gastroenterology centres. If the test result for anti-HDV antibodies was positive, subsequent reflex testing of HDV RNA was also performed by single and nested polymerase chain reaction amplifications of a highly conserved region of the HDV genome, using primers selected from genotype 1 of HDV (RoboGene HDV RNA Quantification Kit 2.0, Roboscreen GmbH, Leipzig, Germany). Demographic data on gender, age, area of residence, education, and partner status were collected by the participating investigators using a patient

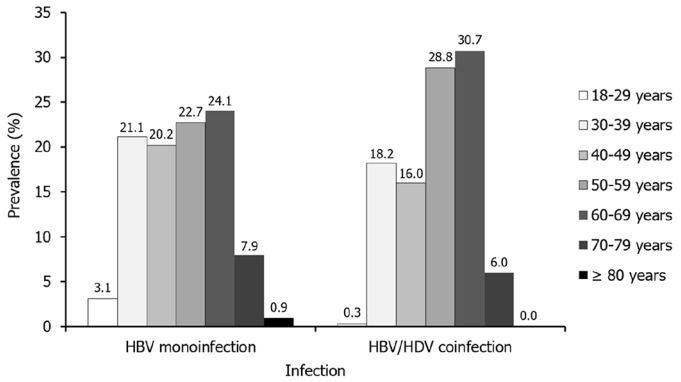

Figure 1 Prevalence of hepatitis B virus monoinfection and hepatitis B virus/hepatitis D virus coinfection across different age groups. A statistically significant difference in overall prevalence was identified between those with hepatitis B virus (HBV) monoinfection and HBV/hepatitis D virus coinfection (P = 0.001). HBV: Hepatitis B virus; HDV: Hepatitis D virus.

questionnaire. Data were collected on disease stage and therapeutic history from the admission medical charts. Risk factors for HBV and HDV infection were collected using both patient questionnaires and medical charts. All data were stored in an anonymized online database during the study.

The study was approved by the institutional ethics committees and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Fundeni Clinical Institute obtained the ethical approval to enrol patients across all hospitals included in this study. Written informed consent was obtained from each participant before enrolment.

The prevalence of HBV monoinfection and HBV/HDV coinfection was calculated with a 95%CI. Qualitative or quantitative variables were analysed using non-parametric Chi-square, Kruskal–Wallis or Mann–Whitney U tests, as appropriate. Using multivariate multiple regression analysis, variables identified as risk factors for HBV/HDV coinfection from the univariate multiple regression analysis were investigated. These variables included sociodemographic factors [participant age, gender, residence (urban or rural) and education level [no or elementary school, high school, college/university] and medical history (previous documented coronavirus disease 2019 or comorbid diabetes mellitus). The HBV vaccination status of participants and their life partners was also included, as were the existence of any known family members positive for HBV/hepatitis C virus (HCV)/HDV (monoinfection or coinfection) and sexual contact with a partner positive for HBV/HCV/HDV (monoinfection or coinfection). In addition, exposure to healthcare procedures was considered; variables included were an occupational risk of exposure to blood products, history of blood transfusion, haemodialysis in antecedents (long-term or incidental owing to complications in an intensive care unit), any surgery before diagnosis (excluding dental surgery), at least one hospitalization before diagnosis and any dental surgery before diagnosis. Other risk factors included as variables were any history of severe accidents (work, traffic, domestic), record of accidents with blood-contaminated objects, history of injections at home or at an outpatient unit, imprisonment (current or previous), tattoos or any body piercing, injecting drug use, multiple sexual partners in the past three years, previous sexually transmitted diseases and history of abortions in improper conditions. All statistical tests were two-sided, and a P value of less than 0.05 was used to indicate statistical significance.

RESULTS

During the study period, 25390 individuals with any gastroenterology disease were admitted to the six study centres, of whom 963 individuals were HBV-positive, were eligible, provided informed consent and were enrolled into the study. Therefore, the hospital-based prevalence of HBV infection was 3.8% (95%CI: 1.8-5.8). The relative per-centre distribution of patients with HBV enrolled in the study was as follows: Bucharest (40.3%), Iasi (29.3%), Oradea (19.0%), Timisoara (7.8%) and Craiova (3.6%). Among those 963 patients who were HBV-positive, the prevalence of HBV/HDV coinfection was 33.1% (95%CI: 31.2-35.1).

A comparison of patient characteristics between those with HBV monoinfection and those with HBV/HDV coinfection is shown in Table 1. Overall, the median age of the study population was 54.0 years. Participants with HBV/HDV coinfection were significantly older than participants with HBV monoinfection (mean age ± SD, 53.5 ± 11.7 *vs* 51.6 ± 13.6 years, P = 0.03; Table 1). Upon assessment of prevalence data within 10-year age brackets, a relatively equal distribution of HBV monoinfection was noted among patients aged between 30 years and 69 years (20.2%-24.1%, Figure 1). In contrast, there was an unequal distribution of HBV/HDV coinfection among the age groups, with the majority of those with HBV/HDV coinfection aged between 50 years and 69 years (59.5%).

The study population consisted of 531 men (55.1%) with a median age of 53.0 years, and 432 women (44.9%) with a median age of 54.0 years (P = 0.16). The proportion of men was lower in those with HBV/HDV coinfection than in those with HBV monoinfection (48.3% *vs* 58.5%, P = 0.002). Women with HBV/HDV coinfection were older than women with HBV monoinfection (mean ± SD, 55.5 ± 11.4 years *vs* 51.2 ± 13.4 years, P = 0.002). In contrast, ages were similar between men with HBV monoinfection and HBV/HDV coinfection (P = 0.67).

Table 1 Demographic and clinical data for patients with hepatitis B virus monoinfection or hepatitis B virus/hepatitis D virus coinfection, %

Variable	HBV monoinfection (*n* = 644)	HBV/HDV coinfection (*n* = 319)	*P* value
Sex			
Female	41.5	51.7	0.002[a]
Male	58.5	48.3	0.002[a]
Age, mean ± SD, yr			
Total	51.6 ± 13.6	53.5 ± 11.7	0.03[a]
Female	51.2 ± 13.4	55.5 ± 11.4	0.002[a]
Male	51.8 ± 13.6	51.3 ± 11.7	0.67
Time since HBV diagnosis, mean ± SD, months	86.2 ± 3.4	112.8 ± 7.1	0.36
Stage of disease at diagnosis			< 0.0001[a]
Chronic hepatitis	87.9	73.1	
Compensated liver cirrhosis	9.9	19.4	
Decompensated liver cirrhosis	2.2	7.5	
Hepatocellular carcinoma at diagnosis	2.6	4.1	0.22
HBeAg-positive	5.1	3.3	0.19
Liver stiffness measurement at therapy initiation, mean ± SD, kPa	8.7 ± 3.3	10.9 ± 5.7	0.003[a]
HBV DNA serum level at diagnosis, mean ± SD, IU/mL	2994542.8 ± 3014.7	610025.3 ± 158.9	< 0.0001[a]
Past or current pegylated interferon-α therapy	14.6	42.5	< 0.0001[a]
Current nucleos(t)ide analogue therapy	70.3	36.4	< 0.0001[a]
Previous documented COVID-19	40.8	34	0.0003[a]
Associated diabetes mellitus	11.2	7.5	0.07

[a]$P < 0.05$, statistically significant P values.

COVID-19: Coronavirus disease 2019; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HDV: Hepatitis D virus.

Diagnosis of HBV/HDV coinfection was obtained by HDV antibody reflex testing in 75.7% of patients. In the remaining patients with coinfection, the HDV diagnosis was delayed by a mean time of 34.7 months. Regarding the serological profile of HBV infection, 59.2% of patients positive for HBsAg had anti-hepatitis B e antigen-positive antibodies. A positive HDV RNA viral load at diagnosis was observed in 86.5% of patients; the median HDV viral load was 16200 IU/mL (range: Undetectable to 3570742 IU/mL). HBV viremia was less than 20 IU/mL in 1.79% and 27.9% of patients with HBV monoinfection and HBV/HDV coinfection at diagnosis, respectively.

Liver stiffness at therapy initiation was higher in patients with HBV/HDV coinfection than in those with HBV monoinfection (mean ± SD, 10.9 ± 5.7 kPa *vs* 8.7 ± 3.3 kPa, $P = 0.003$). The distribution of fibrosis stages (F) according to the METAVIR score[23] in patients with HBV/HDV coinfection who had received antiviral therapy with pegylated interferon-α was as follows: F0 2.7%, F1 11.8%, F2 32.6%, F3 41.2% and F4 11.8%. There were statistically significant differences in disease stage at diagnosis, with patients with HBV/HDV coinfection having an increased likelihood of compensated and decompensated liver cirrhosis compared with patients with HBV monoinfection ($P < 0.0001$).

More than 90% of patients with HBV/HDV coinfection were treated, with 42.5% and 36.4% of patients receiving pegylated interferon-α therapy and nucleos(t)ide analogues, respectively (Table 1). Combination therapy of pegylated interferon-α and nucleos(t)ide analogues was received by 49.6% of patients with HBV/HDV coinfection.

Independent risk factors for HDV infection were identified from analysis of medical chart and patient questionnaire data. Female gender ($P = 0.002$) and older age at HBV/HDV diagnosis ($P = 0.03$) were identified from the medical chart data as risk factors for coinfection, while statistical analysis of the patient questionnaire data identified the following significant risk factors (Table 2): Education level ($P = 0.0006$), sexual contact with a partner positive for HBV/HCV/HDV ($P = 0.0001$), blood transfusion ($P = 0.0004$), haemodialysis in antecedents ($P < 0.0001$), at least one hospitalization before diagnosis ($P < 0.0001$), any dental surgery before diagnosis ($P < 0.0001$), serious accidents [work, traffic, domestic ($P < 0.0001$)], accidents with blood-contaminated objects ($P < 0.0001$), injections at home/outpatient unit ($P < 0.0001$), imprisonment [current or previous ($P < 0.0001$)], tattoos/any body piercing ($P < 0.0001$), injecting drug use ($P < 0.0001$), multiple sexual partners in the past 3 years ($P = 0.001$) and sexually transmitted diseases ($P < 0.0001$).

Multivariate regression analysis identified the following independent risk factors for HBV/HDV coinfection in Romanian patients: female gender ($P = 0.0006$), imprisonment (current or previous) ($P < 0.0001$), older age at diagnosis (P

Table 2 Risk factors for hepatitis D virus infection based on the patient questionnaire data

Variable, %	HBV monoinfection (*n* = 644)	HBV/HDV coinfection (*n* = 319)	*P* value
Urban area	59.0	51.5	0.06
Education level			0.0006[a]
No or elementary school (0 to 8 yr)	17.6	24.3	
High school (12 yr)	48.7	55.7	
College/university	33.7	20	
Vaccination against HBV (any dose)	15.6	12.5	0.25
Life partner vaccinated against HBV (any dose)	26.5	30.3	0.41
Known family members positive for HBV/HCV/HDV (monoinfection or coinfection)	18.4	20.5	0.48
Sexual contact with a partner positive for HBV/HCV/HDV (monoinfection or coinfection)	4.6	12.5	0.0001[a]
Occupation with risk of exposure to blood products	3.9	6.2	0.17
Blood transfusion	18.4	30	0.0004[a]
Haemodialysis in antecedents (long-term or incidental owing to a complication in ICU)	2.5	15.9	< 0.0001[a]
Any surgery before diagnosis (excluding dental surgery)	58.1	52.7	0.16
At least one hospitalization before diagnosis	82.3	65	< 0.0001[a]
Any dental surgery before diagnosis	83	67	< 0.0001[a]
Serious accidents (work, traffic, domestic)	8.4	21.3	< 0.0001[a]
Accidents with blood-contaminated objects	5.5	19	< 0.0001[a]
Injections at home/outpatient unit	4.8	16.7	< 0.0001[a]
Imprisonment (current or previous)	0.2	15.5	< 0.0001[a]
Tattoos/any body piercing	15	29.5	< 0.0001[a]
Injecting drug use	0.5	10.5	< 0.0001[a]
Multiple sexual partners in the past 3 years	15.9	25.6	0.001[a]
Previous sexually transmitted diseases	2.3	8.9	< 0.0001[a]
Abortions (improper conditions)[1]	3.3	5.9	0.14

[a]$P < 0.05$, statistically significant *P* values.
[1]Abortion was restricted between 1966 and 1989 in Romania.
HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; ICU: Intensive care unit.

= 0.01) and sexual contact with a partner positive for either HBV/HCV/HDV ($P = 0.0003$).

DISCUSSION

This short-term, prospective study updates our understanding of the epidemiology, natural history, risk factors, diagnostic methodology and treatments for HBV/HDV coinfection in Romania. Our study data suggest that Romania is still an HDV endemic pocket as the prevalence of HBV/HDV coinfection was high, with 33.1% anti-HDV antibody positivity among patients with HBV. In comparison, a prospective study of nearly 900 participants conducted in 2019

reported that the prevalence of anti-HDV antibodies among patients with HBV in Italy was more than threefold lower than the Romanian data reported here, at 9.9%[24]. Interestingly, the prevalence of HBV infection varied widely between the different sites in this study (3.6%-40.3%), probably influenced by regional variations in the quality of healthcare services across the country, exposure to risk factors for HBV infection and per-centre addressability[25].

We observed a change in the demographic characteristics of those with HBV/HDV coinfection compared with our previous epidemiological study population from 2011[18]. In this study conducted in 2022, there were proportionally more female patients with HDV, and patients were older compared with the previous study, with the prevalence peaking among those aged 60-69 years, an increase of 10 years from the previous study. In the present study, there was a significant difference in prevalence between those with HBV monoinfection and HBV/HDV coinfection. A relatively equal distribution in the prevalence of HBV monoinfection was noted among participants aged between 30 years and 70 years, whereas the prevalence of HBV/HDV coinfection was markedly higher in those aged 50–69 years than in those younger than 50 years. This may reflect the different modalities of acquiring HBV compared with HDV infection. The observed age-related trends suggest a cumulative risk of HDV exposure over time, as well as a cohort phenomenon of HDV infection in Romania. These demographic data highlighting age as a risk factor are similar to studies from our group and others on HCV, HBV and HDV infection[25-27]. The profile of risk factors for HDV coinfection has changed from the previous epidemiological study and now includes both nosocomial and sexually transmitted infections, similar to several Western European countries[18,28-30].

A higher proportion of women had HBV/HDV coinfection than HBV monoinfection in the current study. Abortion was restricted between 1966 and 1989 in Romania[31]. Unsafe abortion practices, particularly in settings where access to safe reproductive healthcare services was limited, may have posed a significant risk of viral transmission. Historical practices, policies and societal conditions may have shaped patterns of infection transmission and healthcare practices, leading to disparities between genders.

HBV vaccination was noted in 15.6% and 12.5% of people with HBV monoinfection and HBV/HDV coinfection, respectively. The effectiveness of the HBV vaccine can be reduced in people with certain risk factors, including older age, obesity or other chronic illnesses[32]. Some study participants may not have received all doses of the vaccine required for full protection[33]. The difference in HBV vaccination rates between these groups probably reflects a combination of factors related to healthcare access, provider practices, patient characteristics and the complex interplay between HBV and HDV infections.

Although guideline recommendations for HDV screening vary, the recently published European Association for the Study of the Liver (EASL) clinical practice guidelines have highlighted the importance of universal screening and double reflex testing in patients who are HBsAg-positive[6,34,35]. Owing to the high prevalence of HBV/HDV in Romania, this strategy is now standard practice following a policy agreement between hepatologists and virology specialists from the tertiary gastroenterology centres where testing is performed. Reflecting this, in our study diagnosis of HDV coinfection was obtained by the above approach in 75.7% of participants. The virological profile of the helper virus was similar to other observational studies: predominantly hepatitis B e antigen-negative, with an undetectable or low HBV viral load and significant fibrosis (≥ F2 METAVIR) in most individuals[36-38]. Compared with our previous epidemiological study, HDV viral load was positive in a higher proportion of patients, probably due to the extensive use of double reflex testing and the improved sensitivity of the kits used for quantification of HDV viremia[6,18]. Our data, therefore, support the adoption of double reflex testing policies at a national level. If this is not possible, high-risk groups such as prisoners could be prioritized[39,40].

HDV infection is associated with various comorbidities. Our data confirm that chronic HDV infection is associated with advanced liver fibrosis, advanced liver disease, chronic progressive hepatitis, compensated and decompensated cirrhosis, and hepatocellular carcinoma, in line with the published literature in this area[4,19,41,42]. HBV monoinfection has a milder evolution and a decreased risk of liver transplantation for decompensated liver cirrhosis or hepatocellular carcinoma compared with HBV/HDV coinfection[19,43,44]. There are studies showing that HDV coinfection can constrain HBsAg evolution and modulate the emergence of drug-resistance profiles, thus highlighting the need to optimize the use of existing antiviral therapies and find new therapeutic targets against HDV infection[45,46].

Antiviral treatment of hepatitis D has been demonstrated to prevent cirrhosis, liver failure and hepatocarcinoma[47, 48]. Most patients were treated for HDV in our practice setting, with pegylated interferon-α therapy (42.5%) and nucleos(t)ide analogues (36.4%) being the most commonly used treatments, in line with the 2017 EASL clinical practice guidelines for HBV/HDV coinfection[49]. These data reflect both the severity of disease and the lack of available therapies. At present, approved treatment options for chronic HDV infection are limited to pegylated interferon-α in most countries, even though its efficacy has been demonstrated to be low and it is frequently associated with significant side effects[14,15]. The HBV/HDV entry inhibitor bulevirtide, approved by the European Medicines Agency for the treatment of adult patients with compensated liver disease when the presence of HDV RNA has been confirmed by blood tests, demonstrated its efficacy and safety as a monotherapy or combined with pegylated interferon-α in clinical trials and real-world studies[16,50,51]. Bulevirtide is the only anti-HDV therapeutic option approved within the past decade that may improve the long-term prognosis of these patients. Bulevirtide is also being investigated in patients with chronic HDV, with and without compensated cirrhosis[52,53]. However, there are still several issues to be addressed, such as the optimal duration of treatment, the rates of off-therapy responses, associated costs and the cost–benefit ratio in relation to the need for liver transplantation. Other promising investigational agents are in development, including the prenylation inhibitor lonafarnib, nucleic acid polymers and an interferon subtype distinct from interferon-α, interferon-λ[54].

This study has several limitations, which should be considered. A larger sample size would have increased the statistical power of the study for the detection of HDV infection risk factors. The use of patient questionnaires may have resulted in a bias in the reported data. Additionally, enrolling patients from a hospital-based cohort may have resulted in selection bias, and the HDV prevalence may be overestimated owing to the severity of chronic liver disease that requires

evaluation and admittance to hospital. However, the global HDV prevalence may be underestimated owing to the lack of universal HDV screening in the people who are HBsAg-positive or among selected high-risk populations. In contrast, the prevalence data presented in this study benefit from Romania's implementation in tertiary hepatology clinics of double reflex testing in patients who are HBsAg-positive. As this study was conducted in Romania, the findings may not be generalizable to other populations or settings. Cultural, socioeconomic, and healthcare system differences between Romania and other countries could affect the prevalence and risk factors for HBV/HDV coinfection. Extended efforts should be made to elucidate the true HDV disease burden across the globe to enable the development of public health strategies to achieve HDV elimination, one of the WHO's global health strategy targets[55]. The implementation of the recommendations regarding screening, characterization, therapy and monitoring of HDV infection in the latest EASL guidelines will facilitate this aim[6]. Continued implementation of preventive measures for HDV transmission, along with increasing coverage of HBV vaccination and further development of innovative, efficacious, targeted therapies for both HBV and HDV remain crucial for policy-makers and healthcare providers.

CONCLUSION

In conclusion, HBV/HDV coinfection remains endemic in Romania, with a heterogeneous distribution across the country. Demographic characteristics of patients with HBV/HDV coinfection have changed in comparison to a similar study conducted over 10 years ago, suggesting a cumulative risk of HDV exposure over time. Encouragingly, national policy decisions regarding double reflex testing have elevated HDV detection rates. Further rollout of preventive measures and development of treatments will aid efforts to eliminate HDV globally.

FOOTNOTES

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SYSTEMATIC REVIEWS

Relative carcinogenicity of tacrolimus *vs* mycophenolate after solid organ transplantation and its implications for liver transplant care

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Abstract

BACKGROUND

De novo malignancy is a leading cause of late morbidity and mortality in liver transplant recipients. Cumulative immunosuppression has been shown to contribute to post-transplant malignancy (PTM) risk. There is emerging evidence on the differential carcinogenic risk profile of individual immunosuppressive drugs, independent of the net effect of immunosuppression. Calcineurin inhibitors such as tacrolimus may promote tumourigenesis, whereas mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil, may limit tumour progression. Liver transplantation (LT) is relatively unique among solid organ transplantation in that immunosuppression monotherapy with either tacrolimus or MPA is often achievable, which makes careful consideration of the risk-benefit profile of these immunosuppression agents particularly relevant for this cohort. However, there is limited clinical data on this subject in both LT and other solid organ transplant recipients.

AIM

To investigate the relative carcinogenicity of tacrolimus and MPA in solid organ transplantation.

METHODS

A literature search was conducted using MEDLINE and Embase databases using the key terms "solid organ transplantation", "tacrolimus", "mycophenolic acid", and "carcinogenicity", in order to identify relevant articles published in English between 1st January 2002 to 11th August 2022. Related terms, synonyms and explosion of MeSH terms, Boolean operators and truncations were also utilised in

the search. Reference lists of retrieved articles were also reviewed to identify any additional articles. Excluding duplicates, abstracts from 1230 records were screened by a single reviewer, whereby 31 records were reviewed in detail. Full-text articles were assessed for eligibility based on pre-specified inclusion and exclusion criteria.

RESULTS

A total of 6 studies were included in this review. All studies were large population registries or cohort studies, which varied in transplant era, type of organ transplanted and immunosuppression protocol used. Overall, there was no clear difference demonstrated between tacrolimus and MPA in *de novo* PTM risk following solid organ transplantation. Furthermore, no study provided a direct comparison of carcinogenic risk between tacrolimus and MPA monotherapy in solid organ transplantation recipients.

CONCLUSION

The contrasting carcinogenic risk profiles of tacrolimus and MPA demonstrated in previous experimental studies, and its application in solid organ transplantation, is yet to be confirmed in clinical studies. Thus, the optimal choice of immunosuppression drug to use as maintenance monotherapy in LT recipients is not supported by a strong evidence base and remains unclear.

Key Words: Immunosuppression; Solid organ transplantation; Liver transplantation; Carcinogenicity; Tacrolimus; Mycophenolate

Core Tip: Cumulative immunosuppression exposure is an important risk factor for the development of post-transplant malignancy. There is emerging evidence on the differential carcinogenic risk profile of individual immunosuppressive drugs, independent of the net immunosuppression effect. This review demonstrates that the evidence on the relative carcinogenicity of tacrolimus and mycophenolic acid, the two agents most commonly used as maintenance monotherapy in liver transplant patients, remains unclear. Further studies are required to determine the clinical relevance of previous experimental findings to enable physicians to tailor immunosuppression regimens to minimize individual malignancy risk in solid organ transplantation.

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INTRODUCTION

Liver transplantation (LT) remains the only curative treatment for end-stage liver disease and some cases of hepatocellular carcinoma, with an overall median survival of 20 years[1]. Despite improvements in short-term survival with the decline in rates of rejection and graft failure with the advent of modern immunosuppression regimens, long-term complications including post-transplant malignancy (PTM), have risen. Liver transplant recipients incur a 2- to 3-fold increase in rates of *de novo* malignancy compared to the general population[2,3]. Indeed, PTM has become a leading cause of late mortality in LT recipients[4,5].

The cumulative exposure to immunosuppression and direct carcinogenicity of individual agents may contribute to the development of PTM[6]. Tacrolimus and mycophenolic acid (MPA) are the most commonly used backbone immunosuppressants post-LT, and are also utilised as maintenance monotherapy in 42% of LT recipients in the United States due to the relatively immune tolerant microenvironment of the liver[7,8]. Experimental data have demonstrated multiple pro-oncogenic effects of tacrolimus, whereas MPA may be protective against tumour growth and progression[9]. This systematic review aims to compare the relative carcinogenicity of tacrolimus and MPA in solid organ transplantation to assist clinicians in making informed decisions regarding choice of immunosuppression regimens for patients.

PTM immunology

The development of PTM is a consequence of complex interactions between genetic, lifestyle and transplant factors (Figure 1). The central role of the immune system in cancer surveillance is highlighted by the increased malignancy risk that results from congenital and acquired immunodeficiencies, as well as the efficacy of immunotherapy for a growing number of malignancies such as hepatocellular carcinoma, melanoma, and renal cell carcinoma[6].

An intact immune system prevents oncogenesis through 3 main mechanisms. Firstly, the immune system eliminates or suppresses viral infections to prevent virus-induced tumours, as seen in the role of Ebstein Barr virus infections in the development of early post-transplant lymphoproliferative disorder (PTLD)[6,10]. Secondly, inflammation resolution and

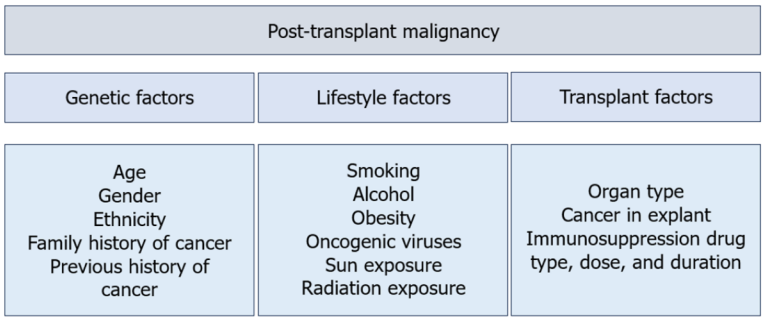

Figure 1 Risk factors for post-transplant malignancy.

pathogen elimination prevents the establishment of a pro-inflammatory environment conducive to tumourigenesis[6,10]. Thirdly, cells of the innate and adaptive immune system can identify and eliminate tumour cells based on the expression of tumour-specific antigens and danger signals[6,10]. Chronic immunosuppression exposure disrupts the integrity of cancer immunosurveillance. Furthermore, animal studies have suggested that tumours developing in an immunocompromised host are more immunogenic compared to an immunocompetent host, enabling tumour cells to evade immune recognition and destruction[10]. Unsurprisingly, the incidence of PTM is as high as 20% in solid organ transplant recipien -ts after 10 years of cumulative immunosuppression exposure[6].

Potential mechanisms of carcinogenicity of individual immunosuppression drugs

Individual immunosuppression drugs may also have direct carcinogenic effects, resulting in DNA damage and gene expression changes that promote cancer progression independent of the effects of overall immunosuppression exposure.

Tacrolimus

Calcineurin inhibitors (CNI) such as tacrolimus and cyclosporine suppress T cell activation and proliferation by inhibiting *interleukin-2* gene transcription (Figure 2)[11]. The reduced rate of cellular rejection and resultant improved graft and patient survival associated with tacrolimus-based immunosuppression has led to tacrolimus being the CNI of choice following solid organ transplantation[12]. However, experimental data suggest tacrolimus may promote cancer progression by creating a tumour-permissive microenvironment independent of its immunosuppressive effects.

Tacrolimus has a dose-dependent effect on the production of transforming growth factor β1 (TGF-β1), a cytokine implicated in tumour growth, metastatic spread and development of biologically aggressive cancers[13,14]. The microenvironment is further altered by TGF-β1 through inhibition of anti-tumour immune responses and promotion of extracellular matrix production and angiogenesis[15].

The direct effect of tacrolimus on tumour angiogenesis is not fully understood and may be tissue-dependent. *In vivo* studies have demonstrated tacrolimus enhanced lymphangiogenesis and invasion of hepatocellular carcinoma *via* increased vascular endothelial growth factor (VEGF)-C expression[16]. However, tacrolimus may also hinder angiogenesis through its indirect inhibition of nuclear factor of activated T cells, which has a critical role in mediating angiogenesis through its stimulation of VEGF and secreted frizzled-related protein 2[17,18]. This anti-angiogenic effect has an emerging therapeutic role in rheumatoid arthritis, breast cancer, corneal neovascularisation and hypertrophic scars [17,18].

Tacrolimus exposure may lead to alterations in gene expression that promote cancer development and progression. Tacrolimus has been found to activate the proto-oncogene, Ras, in human renal epithelial cells and renal cancer cells, contributing to renal cancer development[19]. Notably, the activation of Ras is critical for VEGF over-expression and subsequent angiogenesis[19]. Tacrolimus can also interfere with proline-oxidase and p53-mediated apoptosis, thus promoting tumour growth[20].

Experimental data on cyclosporine has similarly demonstrated its oncogenic effects through the over-expression of TGFβ and VEGF, impaired repair of radiation-induced DNA damage and promotion of apoptosis[21-25]. The shared mechanism of action between tacrolimus and cyclosporine possibly reflects a class-effect of CNIs on malignancy risk.

MPA

Mycophenolate mofetil is a key component of backbone immunosuppression following LT, allowing for CNI de-escalation or cessation and minimisation of renal and metabolic dysfunction. The active metabolite, MPA, inhibits inosine monophosphate dehydrogenase (IMPDH) which is a crucial enzyme involved in *de novo* guanosine nucleotide and DNA synthesis (Figure 2)[26]. This leads to the preferential depletion of lymphocytes due their dependency on *de novo* purine synthesis[26]. There is currently no experimental data linking MPA to increased carcinogenicity risk independent of its effects associated with overall immunosuppression. On the contrary, MPA has *in vitro* and *in vivo* anti-neoplastic properties which may confer a reduced risk of PTM.

MPA has been shown to inhibit the growth of a variety of *in vivo* tumour cell lines[27-31]. Upregulation of peroxisome proliferative-activated receptor gamma by MPA prevents tumour cell differentiation[32]. Reduced expression of adhesion molecules on lymphocytes and endothelial cells interferes with adhesion receptor-dependent tumour dissemination[33-36]. Furthermore, increased expression of subtypes of adhesion receptors from the β1 integrin family may induce re-

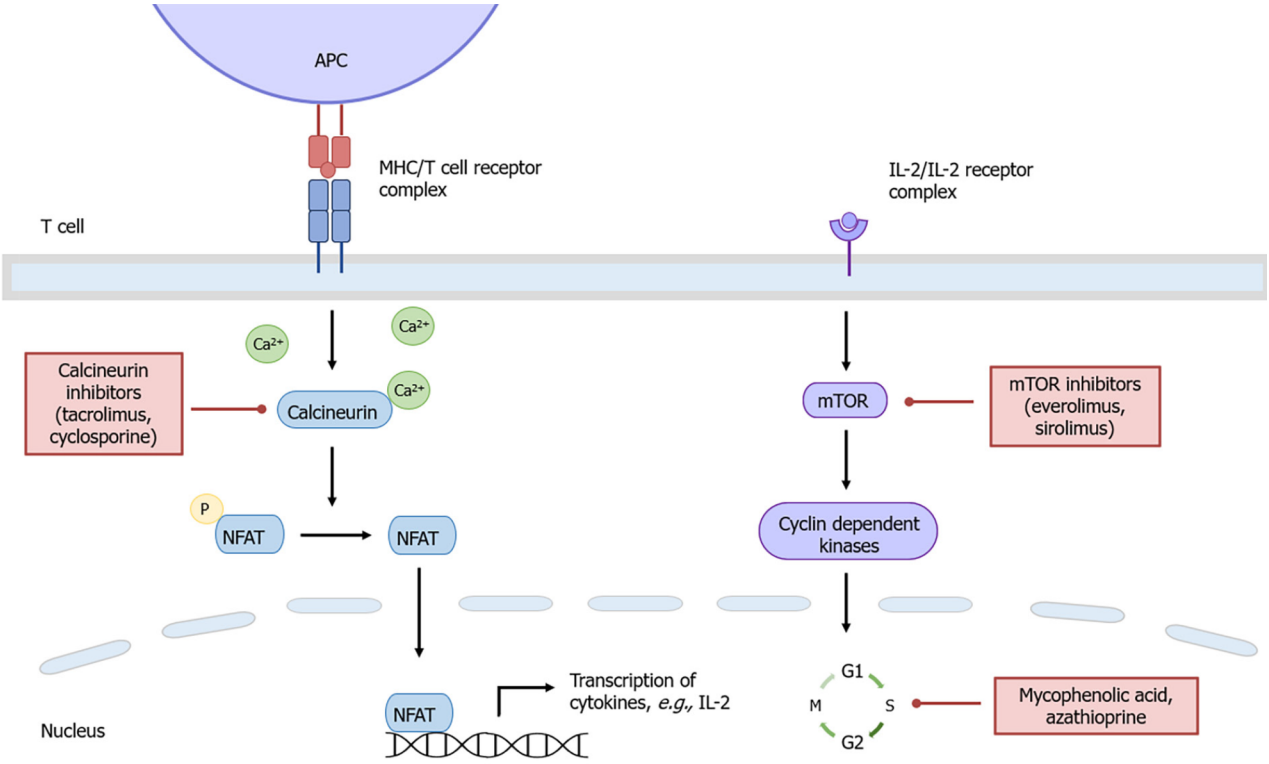

Figure 2 Mechanism of action of commonly used immunosuppression drugs following solid organ transplantation. APC: Antigen presenting cell; IL: Interleukin; MHC: Major histocompatibility complex; mTOR: Mammalian target of rapamycin; NFAT: Nuclear factor of activated T cells.

differentiation of tumour cells towards a lower invasive phenotype[36]. However, some cancer types have been found to be resistant to the anti-neoplastic properties of MPA[28,37].

The anti-neoplastic properties of MPA may also have a therapeutic potential. The enzyme IMPDH, the target of MPA, is over-expressed in cancer cells[26]. Furthermore, MPA-mediated inhibition of IMPDH has been demonstrated to induce tumour cell apoptosis, however these findings are yet to be confirmed *in vivo*[38].

Other immunosuppression agents

Azathioprine is a purine analogue that is incorporated into cellular DNA, where it inhibits purine nucleotide synthesis and interferes with RNA synthesis and metabolism (Figure 2)[15]. It is well known that azathioprine is a risk factor for the development of PTM, in particular, non-melanoma skin cancer. Multiple studies have demonstrated the synergistic effect between ultraviolet A radiation and the azathioprine metabolite, 6-thioguanine, in the generation of mutagenic oxidative DNA damage[39,40]. The carcinogenic effects of azathioprine have limited its use in transplantation in favour of MPA.

Sirolimus and everolimus inhibit mammalian target of rapamycin (mTOR), which subsequently downregulates cyclin-dependent kinases and mRNAs required for cell cycle progression, thus preventing interleukin-2-mediated lymphocyte proliferation (Figure 2)[9]. *In vivo* studies have shown mTOR inhibitors precipitate tumour cell cycle progression arrest and subsequent apoptosis[41,42]. Impaired VEGF production and signalling also restricts tumour angiogenesis and metastatic spread[43-45]. Interestingly, the simultaneous administration of sirolimus in these models can reverse the pro-angiogenic effects of cyclosporine[43-45]. The potential dual immunosuppressive and anti-neoplastic properties of mTOR inhibitors has led to its increasing utilisation in the transplantation setting.

MATERIALS AND METHODS

Multiple population and cohort studies have investigated the role of tacrolimus and MPA in the development of *de novo* PTM, however a direct causal relationship is difficult to establish. As the two most commonly used drugs for maintenance monotherapy post-LT, the oncogenic risk profile of tacrolimus and MPA warrants further review.

Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines were utilised to shortlist relevant articles for this narrative review to minimise bias. A comprehensive literature search was conducted through MEDLINE and Embase electronic databases between 1st January 2002 to 11th August 2022. This time period was selected to include relevant literature since the introduction and clinical use of MPA. The following terms were used, including synonyms and closely related words, as Medical Subject Headings (MeSH) and text words: "Solid Organ Transplantation", "Tacrolimus", "Mycophenolic Acid", and "Carcinogenicity". Explosion of MeSH terms, Boolean operators and truncations were also utilised throughout the search. Further articles were identified through reference lists of published systematic reviews in the area. Excluding duplicates, abstracts from 1230 records were screened by a single

reviewer, whereby 31 records were deemed appropriate for full-text review. Full-text articles were assessed for eligibility based on inclusion and exclusion criteria, leaving 6 studies for inclusion in this review (Table 1, Figure 3).

RESULTS

The 6 studies included in this review are summarised in Table 2. All studies were large population-based registries or cohort studies that analysed PTM risk in the presence or absence of tacrolimus or MPA use. No studies included data on individual drug dosages, plasma levels or duration to assess for cumulative drug exposure. There was heterogeneity amongst the studied populations in type of organ transplanted, transplantation era and immunosuppression regimens used. No studies provided a direct comparative risk of PTM with tacrolimus or MPA monotherapy.

Cutaneous and non-cutaneous malignancy

A Taiwanese population-based study evaluated risk factors for *de novo* cutaneous and non-cutaneous malignancy in 7852 liver, heart, and kidney transplant recipients[46]. Among 2127 liver transplant recipients, 111 (5.2%) malignancies were recorded during the mean follow-up period of 4.2 years[46]. Despite the majority of liver transplant recipients using tacrolimus (77.3%) or MPA (99.0%), neither immunosuppressant was associated with PTM risk[46].

Among 687 heart transplant patients, 31 (4.5%) *de novo* malignancies were reported[46]. Immunosuppression therapy was also not associated with PTM risk in this cohort[46]. However, the smaller number of malignancy outcomes may have contributed to attenuated risk estimates.

De novo malignancy was diagnosed in 470 out of 5038 (9.3%) kidney transplant recipients[46]. The use of MPA was an independent risk factor for PTM in kidney transplant recipients, compared to no MPA use [adjusted hazard ratio (HR): 1.5, 95% confidence interval (95%CI): 1.2-1.8; $P < 0.001$][46]. MPA exposure was also a risk factor for *de novo* transitional cell carcinoma (adjusted HR: 1.7, 95%CI: 1.2-2.4; $P < 0.01$) and renal cell carcinoma (adjusted HR: 1.7, 95%CI: 1.1-2.8; $P < 0.05$) in a sub-analysis of kidney transplant recipients without hypertension or diabetes as an underlying cause for renal failure[46].

On the contrary, a smaller Taiwanese population cohort study of 642 kidney transplant recipients did not demonstrate an association between MPA or tacrolimus exposure, and the development of 54 (8.4%) *de novo* malignancies[47]. However, the study's primary endpoint of hospitalisation due to malignancy as the primary coded diagnosis, likely underestimated the incidence of *de novo* PTM from the exclusion of malignancies coded as secondary diagnoses or those diagnosed in the community.

Differences in immunosuppression regimens and cumulative exposure to individual drugs may also contribute to the conflicting findings of the aforementioned studies, however this data was not available for analysis. Additionally, lifestyle factors known to influence malignancy risk such as smoking and alcohol consumption, were not included in either study.

Cutaneous malignancy

Three studies investigated the relationship between immunosuppression and post-transplant cutaneous malignancy.

A population-based study in the United Kingdom investigated the development of post-transplant melanoma and non-melanoma skin cancers in 2852 liver, kidney, pancreas, heart, and lung transplant recipients, compared to 13527 matched controls from the general population[48]. Among 437 liver transplant recipients, 19 (4.3%) skin cancers were diagnosed during the 6.2 year median follow-up period[48]. Liver transplant recipients had the lowest incidence of skin cancer compared to other solid organ transplant recipients [Incidence rate ratio (IRR): 4.34, 95%CI: 2.48-7.58, $P = 0.00$], possibly reflecting lower immunosuppression requirements and relative immune privilege[48]. Neither tacrolimus nor MPA use was associated with the development of *de novo* cutaneous malignancy across all solid organ transplantation[48]. However, these findings are limited by small outcome numbers. Additionally, the complex interaction between immunosuppression agents and other risk factors for skin cancer including smoking status and ultraviolet light exposure was not considered.

An American study compared 170 kidney, kidney/pancreas, and heart transplant recipients with *de novo* cutaneous squamous cell carcinoma (SCC) to 324 matched recipient controls[49]. Risk factors such as smoking status, family history of skin cancer and personal history of pre-cancerous skin lesions were adjusted for, however the cancer group were significantly older than the non-cancer group despite matching. In azathioprine naïve patients, MPA use was associated with lower cutaneous SCC risk, independent of tacrolimus exposure (OR: 0.52, 95%CI: 0.32-0.84)[49]. Current and previous MPA use was also inversely associated with the development of multiple cutaneous SCCs (previous MPA use: OR: 0.53, 95%CI: 0.3-0.94; current MPA use: OR: 0.52, 95%CI: 0.29-0.94)[49]. Conversely, cyclosporine-naïve patients treated with tacrolimus had no significant difference in cutaneous SCC risk compared to no tacrolimus use, when adjusted for MPA exposure[49]. Although the authors considered individual immunosuppression exposure risk in the clinical context of changing multi-drug regimens, this was limited by potential recall bias associated with self-reported questionnaires used to obtain immunosuppression data.

Finally, *de novo* lip SCC was evaluated in a large Australian and New Zealand registry study of 8162 kidney transplant patients[50]. Mycophenolate use was associated with reduced risk of SCC of the lower vermillion of the lip in univariate (IRR: 0.28, 95%CI: 0.12-0.69, $P = 0.006$), but not multivariate (IRR: 0.85, 95%CI: 0.28-2.60, $P = 0.774$) analyses[50]. There was no difference between tacrolimus use *vs* no use in the risk of lip SCC of the lower vermillion (IRR: 2.07, 95%CI: 0.45-9.50, $P = 0.35$)[50]. Of note, the study included patients transplanted between 1982 and 2003, with less use of tacrolimus (2/121, 1.7%) and MPA (5/121, 4.1%) during this transplant era, compared to cyclosporine and azathioprine, respectively.

Table 1 Inclusion and exclusion criteria utilised for literature search strategy

Inclusion criteria	Exclusion criteria
Involve human solid organ transplant recipients	Presents risk data on only one of the immunosuppressant medications
Independent malignancy risk analysis related to both immunosuppressants mycophenolic acid and tacrolimus	Does not specify type of immunosuppression
Contains a group of participants exposed to tacrolimus or mycophenolic acid, exclusive of the other	Mean follow up less than one year (given the slow growing nature of malignancy)
Greater than 100 participants	Not published in English
Greater than 5 cases of malignancy	Full text not available
Randomised controlled trials and observational studies	Systematic reviews and meta-analyses

This study was likely underpowered to draw conclusions between tacrolimus and MPA exposure and risk of SCC of the lower vermillion of the lip.

PTLD

A large population registry in France evaluated risk factors for PTLD occurrence in kidney and kidney/pancreas transplant recipients over a 10-year period[51]. Compared to 21170 control kidney transplant recipients, 327 cases of PTLD were recorded and 181 cases were included in the final analysis[51]. Tacrolimus and MPA use were not associated with overall PTLD risk, even when simultaneous kidney pancreas transplant recipients were excluded[51]. However, tacrolimus and MPA were negatively associated with graft site PTLD (tacrolimus: HR: 0.33, 95%CI: 0.16-0.68; MPA: HR: 0.44; 95%CI: 0.23-0.86), which may be attributed to fewer episodes of acute rejection and less immunosuppression exposure in this subgroup[51].

DISCUSSION

With long-term survival now commonplace following LT, there is an increasing need to improve non-hepatic health to avoid complications including metabolic derangements, renal impairment and *de novo* malignancy. *De novo* PTM accounts for approximately 16.4% of late deaths following LT[12,52]. Although immunosuppression exposure is a well-known contributor of PTM risk, there remains uncertainty regarding the carcinogenic effect of specific immunosuppression drugs, alone or in combination. This is the first narrative review that compares the relative carcinogenicity of tacrolimus and MPA in solid organ transplant recipients.

Existing *in vitro* and *in vivo* experimental data have portrayed a contrasting carcinogenic risk profile between tacrolimus and MPA. Tacrolimus promotes oncogenesis and tumour growth in its surrounding microenvironment with the activation of proto-oncogenes, production of TGF-β and inhibition of apoptosis[13,19,20]. The data on MPA is limited but suggests possible inhibition of tumour cell differentiation and prevention of vascular spread through alteration of cellular adhesion molecule expression[6]. However, there is currently no human data that directly compares the carcinogenic effects of tacrolimus and MPA in LT or other solid organ transplantation.

This review included a small number of studies that did not demonstrate a clear difference between tacrolimus and MPA in *de novo* PTM risk following solid organ transplantation. Our findings are in keeping with a recent systematic review and meta-analysis of kidney, liver, heart, and lung transplant recipients, whereby the risk of *de novo* malignancy did not differ between patients who received MPA and patients who received tacrolimus (OR: 0.88, 95%CI: 0.69-1.14, P = 0.33)[53]. However, the relationship between immunosuppression exposure and *de novo* PTM risk may vary based on transplant type. In liver transplant recipients, cumulative tacrolimus exposure has been associated with the development of PTM[54,55], although the high tacrolimus doses utilised in these studies are no longer aimed for in routine clinical practice. Furthermore, the conversion from CNI-based immunosuppression to MPA monotherapy post-LT results in either similar or lower rates of PTM[56,57]. Whether the reduction in PTM risk found in these studies is due to the effects of MPA or the reduction in tacrolimus exposure, is unknown. Thus, the differential carcinogenic risk profile of tacrolimus and MPA found in previous experimental studies is yet to be replicated in the clinical setting. Further clarification with large prospective studies is required.

There are inherent practical and financial difficulties in designing studies to compare the relative risk of *de novo* PTM between tacrolimus and MPA. Large prospective population-based studies of prolonged follow-up duration are required to ensure adequate statistical power. Variables that influence PTM risk such as age, gender, ethnicity, and smoking should be identified. However, there may be unidentifiable confounders that are difficult to capture, owing to the complex interaction between genetic, lifestyle and disease factors in oncogenesis. Population-based registries often rely on International Classification of Diseases coding for data collection, which can lead under-representation of malignancy incidence due to miscoding. Finally, longitudinal recording of drug dose, plasma levels and duration is required to capture changes in immunosuppression regimens frequently seen in routine clinical practice. The accurate calculation of cumulative immunosuppression exposure minimises drug exposure misclassification bias seen in current transplant

Table 2 Summary of studies comparing tacrolimus to mycophenolic acid in solid organ transplantation

Ref.	Transplant era	Organ	*n*	*De novo* malignancy	Time to malignancy (median)	TAC *vs* no TAC (OR/HR/IRR)	MPA *vs* no MPA (OR/HR/IRR)
Van Leeuwen *et al*[50], 2009	1982 to 2003	Kidney	8162	203 lip SCC; 121 lip SCC of the lower vermillion in first transplant)	6.1 yr	SCC of the lower vermillion of lip during first transplant [IRR: 2.07, 95%CI: 0.45-9.50 (P = 0.35)]	SCC of the lower vermillion of lip during first transplant [IRR: 0.85, 95%CI: 0.28-2.60 (P = 0.77)]
Caillard *et al* [51], 2012	1998 to 2007	Kidney ± pancreas	21351	181 PTLD; 43 graft PTLD	Not specified	Any PTLD [aHR: 0.66, 95%CI: 0.36-1.22 (P = 0.19)]; Graft PTLD [HR: 0.33, 95%CI: 0.16-0.68 (P = 0.003)[a]]	Any PTLD [aHR: 1.22, 95%CI: 0.74-2.02 (P = 0.44)]; Graft PTLD [HR: 0.44, 95%CI: 0.23-0.86 (P = 0.015)[a]]
Hsiao *et al*[47], 2014	2000 to 2008	Kidney	642	54 non-cutaneous malignancy	3.9 yr	HR: 1.99, 95%CI: 0.66-6.00 (P = 0.22)	HR: 1.00, 95%CI: 0.40-2.45 (P = 0.99)
Coghill *et al*[49], 2016	1995 to 2010	Kidney ± pancreas, and heart	2004	170 SCC	9.0 yr	Single SCC (OR: 1.11, 95%CI: 0.48-2.60)	Single SCC (OR: 0.52, 95%CI: 0.32-0.84[a])
Yeh *et al*[46], 2020	1997 to 2011	Liver, kidney, and heart	7852	612 cutaneous and non-cutaneous malignancy	Not specified	cHR (heart): 0.6, 95%CI: 0.1-2.7; cHR (kidney): 1.5, 95%CI: 0.8-2.6; aHR (liver): 0.6, 95%CI: 0.2-1.7	cHR (heart): 1.6, 95%CI: 0.7-3.3; aHR (kidney): 1.5, 95%CI: 1.2-1.8 (P < 0.001)[a]; cHR (liver): 1.5, 95%CI: 0.9-2.5
Gibson *et al*[48], 2021	2010 to 2018	Liver, kidney ± pancreas, heart, and lung	2852	242 cutaneous malignancy	4.7 yr	IRR: 0.83, 95%CI: 0.55-1.25 (P = 0.37)	IRR: 0.78, 95%CI: 0.54-1.12 (P = 0.18)

[a]P < 0.05.

aHR: Adjusted hazard ratio; cHR: Crude hazard ratio; HR: Hazard ratio; IRR: Incidence rate ratio; MPA: Mycophenolic acid; OR: Odds ratio; PTLD: Post-transplant lymphoproliferative disorder; SCC: Squamous cell carcinoma; TAC: Tacrolimus; 95%CI: 95% confidence interval.

cohort analyses that presume an unvarying drug regimen.

Immunosuppression minimisation is an important strategy to reduce PTM risk given the limited clinical data surrounding individual agents. There are currently no clear guidelines regarding immunosuppression drug choice to minimise PTM risk following LT. European LT guidelines state CNI-related *de novo* PTM risk may be due to dosage, and that there is no evidence to suggest MPA contributes to *de novo* PTM development[58]. In our centre, there is a preference for MPA, alone or in combination with everolimus, due to improved renal outcomes and experimental data suggesting higher PTM risk with tacrolimus. Overall, the choice of immunosuppression needs to be individualised based on recipient characteristics, liver disease aetiology, and alloimmune risk.

Routine cancer surveillance for all transplant recipients is recommended in addition to immunosuppression minimisation. Strict cancer surveillance strategies may lead to earlier cancer detection rates and improved non-cutaneous cancer patient survival in LT recipients[59,60]. As non-melanoma skin cancer is the leading cause of PTM in LT recipients, annual skin examinations by a dermatologist are recommended from 5 years or more after LT[5,61]. Recipients with primary sclerosing cholangitis and inflammatory bowel disease require annual colonoscopies for colorectal cancer surveillance[61]. Age and gender based cancer surveillance for all LT recipients is also recommended.

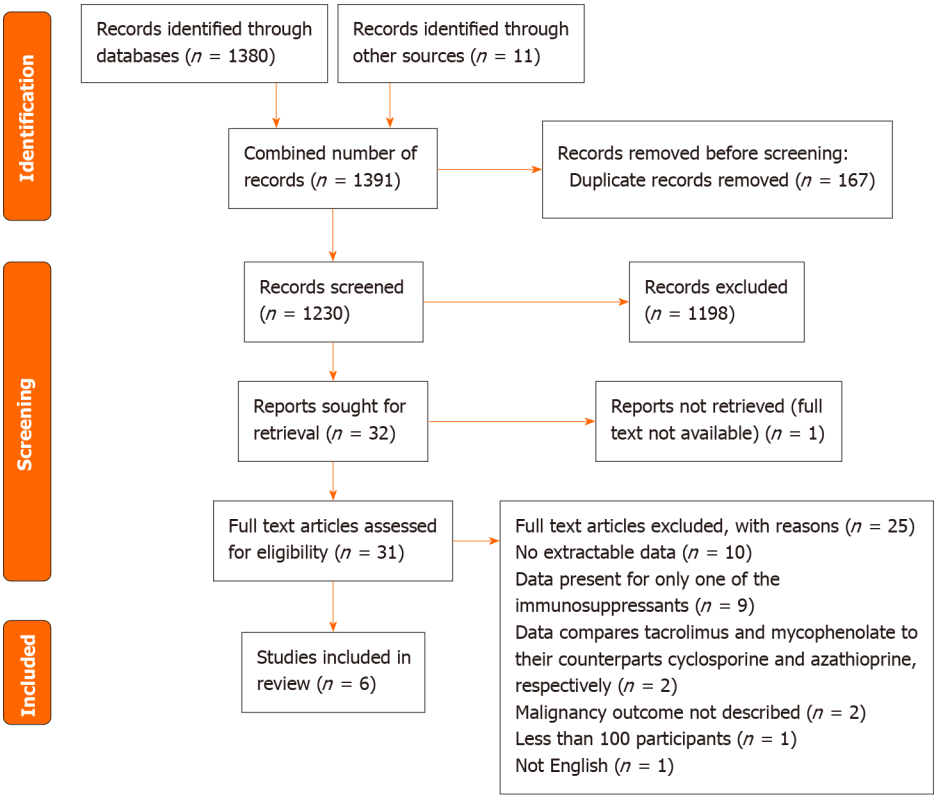

Figure 3 Search strategy utilised for article selection.

CONCLUSION

The clinical relevance of previous experimental studies on the relative carcinogenicity of tacrolimus and MPA, and its application in solid organ transplantation, is yet to be confirmed. Consequently, the choice of immunosuppressive agent to use as maintenance monotherapy in LT patients is not currently supported by a strong evidence base and remains unclear. Further studies are required to enable physicians to tailor immunosuppression regimens to minimise individual malignancy risk.

ARTICLE HIGHLIGHTS

Research background

Many liver transplant (LT) recipients are able to be maintained on long-term immunosuppressive monotherapy, most commonly with either tacrolimus or mycophenolate. In experimental studies, tacrolimus is associated with increased carcinogenicity, whereas mycophenolic acid (MPA) may have anti-neoplastic properties. However, there is minimal clinical data comparing the relative carcinogenicity of tacrolimus and MPA in LT or other solid organ transplant recipients.

Research motivation

Post-transplant malignancy (PTM) is a leading cause of late mortality in LT recipients. Thus, a clinically relevant difference in the carcinogenic risk profile between tacrolimus and MPA will affect the choice of immunosuppressive agent used as maintenance monotherapy in LT patients.

Research objectives

To determine the relative carcinogenicity of tacrolimus and MPA in solid organ transplantation.

Research methods

A systematic review was conducted using PRISMA guidelines with relevant articles published between 1st January 2002 to 11th August 2022 retrieved from MEDLINE and Embase databases for review.

Research results

A total of 6 studies were included in this systematic review, which did not demonstrate a clear difference between tacrolimus and MPA in the development of *de novo* PTM following solid organ transplantation.

Research conclusions

The relative carcinogenicity of tacrolimus and MPA, and its clinical relevance in solid organ transplantation, remains unclear.

Research perspectives

This review highlights the need for further large, population-based prospective studies to further assess the carcinogenic profiles of tacrolimus and MPA, to assist physicians in the choice of immunosuppressive agent to use as maintenance monotherapy in LT patients.

FOOTNOTES

Author contributions: Liu D was involved in data interpretation, drafting of the original and revised manuscripts, and prepared some of the figures and tables; Youssef MM performed data acquisition and prepared some of the figures and tables; Sinclair M was responsible for the study design; Grace JA and Sinclair M were involved in data interpretation and critical revision of the manuscript; and all authors have read and approved the final manuscript.

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