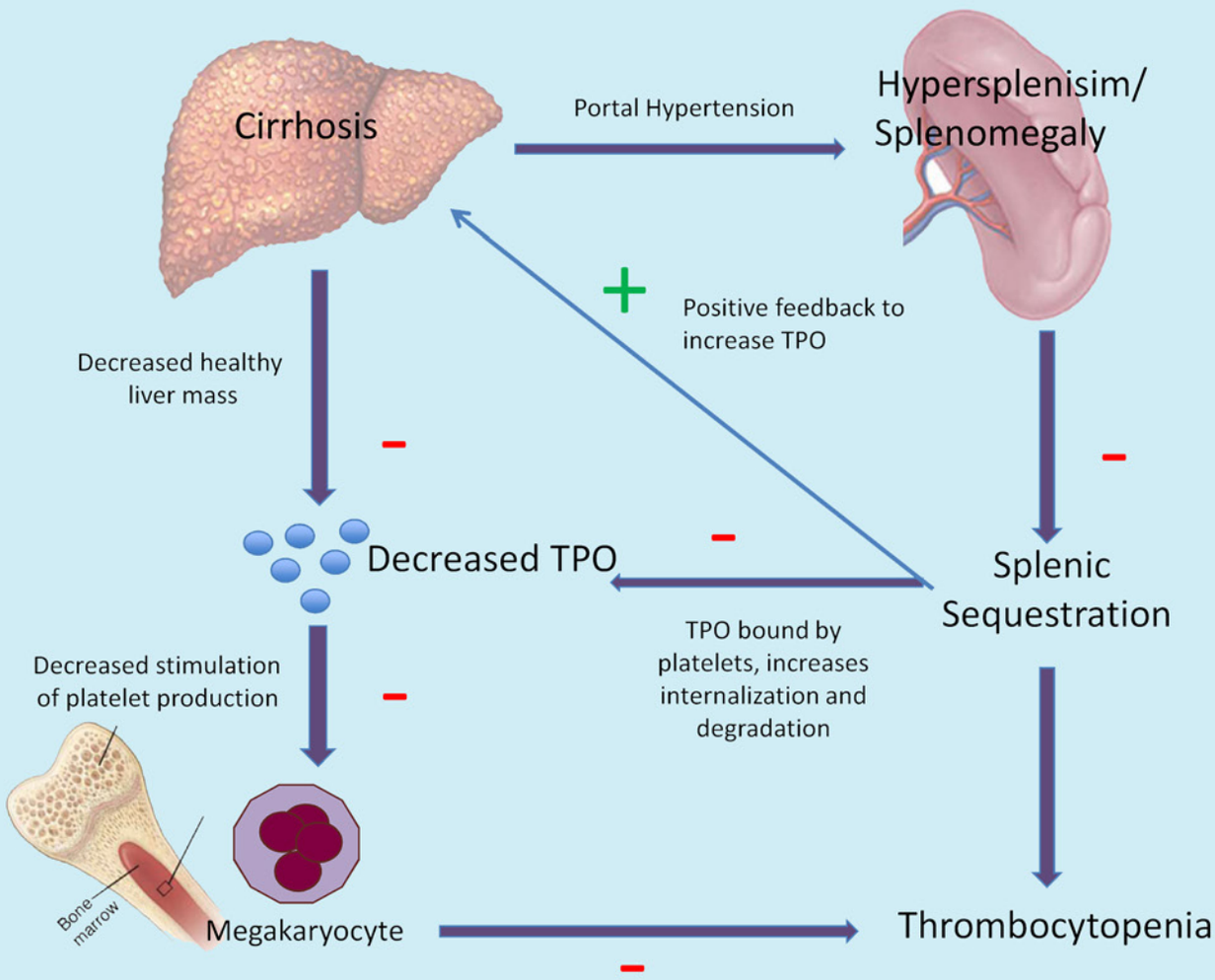
ISSN 2225-0719

2020

Volume 8 Issue 2
April/May/June

THE SECOND AFFILIATED HOSPITAL
CHONGQING MEDICAL UNIVERSITY

Journal of Clinical and Translational Hepatology



JCTH

OWNED BY THE SECOND AFFILIATED HOSPITAL OF CHONGQING MEDICAL UNIVERSITY
PUBLISHED BY XIA & HE PUBLISHING INC.
pISSN: 2225-0719; eISSN: 2310-8819
Frequency: Quarterly
Launch date: September 28, 2013 (Volume 1, Issue 1)

Current Issue: Volume 8, Issue 2
Publication date: June 25 2020

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Publisher

Xia & He Publishing Inc.
Website: www.xiahepublishing.com
E-mail: service@xiahepublishing.com
Postal Address: 14090 Southwest Freeway, Suite 300, Sugar Land, Texas, 77478, USA

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Editorial

What Has the COVID-19 Pandemic Taught Us so Far? Addressing the Problem from a Hepatologist's Perspective

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Citation of this article: Méndez-Sánchez N, Valencia-Rodríguez A, Qi X, Yoshida EM, Romero-Gómez M, George J, *et al.* What has the COVID-19 pandemic taught us so far? Addressing the problem from a hepatologist's perspective. J Clin Transl Hepatol 2020;8(2):109–112. doi: 10.14218/JCTH.2020.00024.

As of today, March 30, 2020, when this Editorial is being written, the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), causal agent of the coronavirus disease (COVID-19) has been confirmed in more than 745,000 cases worldwide and has claimed the lives of more than 35,000 people.[1] In addition to the morbidity and mortality associated with COVID-19, this betacoronavirus has placed several of the world's major economies in strife, mainly in Western Europe and North America, paralyzing travel and regular social interactions, making COVID-19 undoubtedly one of the most important pandemics in human history.

While we are in the midst of battling this pandemic, we have already learned some lessons from a cruel teacher: a) the importance of a strong association that must exist between governments and the scientific community in implementing a broad range of measures to contain and, in the future, prevent this type of epidemic; b) the potential for this pandemic to indirectly, due to less available resources, increase liver-related outcomes morbidity and mortality, including liver transplantation; c) the necessity to develop new working practices in multidisciplinary teams that will provide appropriate levels of care for patients from intensive care units to the outpatient setting. COVID-19 may make virtual clinic visits through telemedicine the norm and not the exception in some parts of the world. However, there are many questions that have yet to be answered. One of the most important to resolve is the understanding of the devastating impact of SARS-CoV-2 in specific geographic regions, such as Spain and Italy.

To better understand the current pandemic, we have to start by analyzing the mechanisms that COVID-19 possesses to infect humans and cause disease. We can observe the close similarity of SARS-CoV-2 with the SARS-CoV virus of 2002, that shares the same interaction between the viral protein Spike (commonly referred to as 'S') and the angiotensin-converting enzyme-2 receptor (ACE2) in the host.[2] ACE2 is a protein with an important role in the regulation of cardiovascular, renal and liver function. It is highly expressed in the lungs and in other tissues, such as the liver (discussed later), intestine, and oral mucosa.[3] Further, ACE2 is expressed more in Asian males compared to females and to other ethnic groups, possibly contributing to the susceptibility of Asians for developing respiratory infections by coronavirus species.[4]

Interestingly, in SARS-CoV there was a correlation between the susceptibility to infection of airway epithelia with the state of cell differentiation, and with ACE2 expression and location. SARS-CoV replication has been observed in polarized epithelia, exiting mainly in the apical zone.[2] In this context, tobacco use is strongly associated with many lung diseases and cancer development. Although, strictly speaking no relationship has yet been proven to exist between COVID-19 and tobacco use, smoking is well-recognized to upregulate the expression of the epidermal growth factor receptor in polarized airway epithelial cells. It may therefore increase the susceptibility to infection by SARS-CoV-2 by deregulating

Abbreviations: ACE2, angiotensin-converting enzyme-2 receptor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCG, Bacillus Calmette-Guerin; COVID-19, coronavirus disease; HLA, human leukocyte antigen; NCP, COVID-19 pneumonia; S, Spike; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

Received: 4 April 2020; Accepted: 5 April 2020

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the ACE2 receptor.[4] Considering that the European Region has the highest prevalence of tobacco use (29%) worldwide,[5] this may provide a possible explanation for the significant burden of COVID-19, as opposed to asymptomatic or mildly symptomatic infection seen in other parts of the world.

Moreover, the human genome is the basis for a large component of inter-individual phenotypic variability to disease. The discovery of the human leukocyte antigen (HLA) in the early 1970s clarified our understanding of the basis for many human diseases, especially in the field of infectious diseases,[6] through a more well-founded comprehension of the interaction between environmental and host factors.

During the 2002 epidemic of SARS-CoV, some studies found an association between the severity of disease and the HLA-B*46:01 and HLA-B*07:03 alleles in Taiwanese and mainland Chinese populations,[7,8] respectively. In contrast, expression of the HLA-DRB1*03:01 allele conferred protection against the disease in both Taiwanese and mainland Chinese populations.[8,9] Surprisingly, a recent *in silico* analysis of viral peptide-HLA interaction that awaits publication suggests that the HLA-B*46:01 allele could also impact the severity of COVID-19 and that the HLA-B*15:03 allele could confer immunity to the disease.[10]

Vaccination policies between countries could be an important factor in susceptibility or protection against COVID-19. For example, it has been reported that the Bacillus Calmette-Guerin (commonly referred to as BCG) vaccine may confer protection against respiratory infections. Consistently, a recent study reported that countries that do not utilize the BCG vaccination routinely (Italy, the Netherlands, USA) have been most affected by COVID-19.[11]

From a hepatologist's perspective, COVID-19 must be of concern, especially since infection with this virus has led to complications in other organs. In the case of the liver, the first study conducted in patients diagnosed with COVID-19 pneumonia (NCP) found that 43 of the 99 patients developed a degree of liver injury characterized by an increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT);[12] one patient developed severe liver injury (ALT 7590 U/L, AST 1445 U/L). In the latter case, acute liver injury secondary to hypoxic hepatitis could not be excluded. In the study, 97 patients (98%) had a decrease in serum albumin and 75 (76%) had increased levels of lactate dehydrogenase — a generalized systemic inflammatory response to the virus could explain these findings. Guan *et al.*[13] carried out the largest study in NCP patients that included 1099 patients from 552 Chinese hospitals. They noted that patients with severe pneumonia were more likely to develop abnormal aminotransferase levels as compared with those with mild and moderate disease. A recent meta-analysis of four studies has also suggested an increased level of ALT is observed in 29% of patients with COVID-19.[14] Yao *et al.*[15] undertook a multiple regression analysis and suggested that the appearance of liver injury was probably related to the critical illness itself, with elevations of aminotransferases in the first week and hypoalbuminemia from the second week; both appear to be reversible with resolution of infection.

We anticipate a question that hepatologists will be asked is whether COVID-19 directly targets the liver?

It is a matter of debate whether COVID-19 is directly responsible for the development of liver injury, or whether the observed changes are secondary to the systemic inflammation triggered by infection (Fig. 1). The Chinese Digestion Association of the Chinese Medical Doctor Association and the Chinese Society of Hepatology of the Chinese Medical Association recently stated that the development of liver injury in COVID-19 patients might be related to any of the following: 1) a direct hit from this virus; 2) systemic inflammation; 3) hepatic ischemia and hypoxia; 4) pre-existing liver disease; and 5) drug-related liver injury (especially the use of antibiotics or other hepatotoxic drugs in critically ill patients).[16] As mentioned previously, ACE2 is expressed in other tissues, such as the liver.[3] Interestingly, hepatocytes do not express the ACE2 receptor as much as bile duct epithelial cells, suggesting that, at least in theory, biliary tract could be more susceptible to SARS-CoV-2-driven injury. However, in the studies conducted so far, no increase in bile duct injury markers, such as gamma-glutamyl transferase and alkaline phosphatase, has been observed. Moreover, SARS-CoV-2 viremia seems to be uncommon, despite severe pneumonia with high viral replication in the lung. The virus has been detected in feces, even several days after clearance on nasopharyngeal swaps. Patients with more advanced liver diseases, including hepatitis B, and those with lower serum albumin levels showed impaired prognosis. A viral translocation from the gut to the liver could allow the virus to reach the liver and promote hepatic injury.

A recent mouse model of acute liver injury with partial hepatectomy found that at day 1 post-hepatectomy, hepatic expression of ACE2 in rats was downregulated. From day 3, there was an up to two-fold increased expression of this receptor, with a normalization of values on day 7, when the liver recovered and hepatocyte proliferation ceased.[17] With this information, the authors proposed that the systemic inflammation derived from significant NCP could trigger liver necrosis and hepatocyte proliferation, upregulating the expression of ACE2 and therefore worsening liver injury. Similarly, liver biopsies from SARS-CoV patients showed a significant increase in mitotic cell numbers, with eosinophilic bodies and balloon-like hepatocytes,[18] supporting a role for SARS-CoV-2 in the development of liver injury.

Systemic inflammation and the subsequent multiple organ failure triggered by severe NCP would undoubtedly act synergistically in the development of liver injury. COVID-19 has been reported to upregulate the expression of pro-inflammatory cytokines, like IL-1β, IL-6, and tumor necrosis factor-alpha.[2] These cytokines are upregulated in a wide number of liver disorders, including viral hepatitis, metabolic associated fatty liver disease, and alcoholic liver disease. The use of antipyretics, such as paracetamol, and certain antivirals, like oseltamivir, lopinavir and ritonavir, for the management of COVID-19 has been widely reported to be associated with the development of liver injury.[19]

What precautions, then, should patients with chronic liver disease follow during this pandemic? Due to the exponential increase in cases of infection worldwide, it is very likely that gastroenterologists and hepatologists will encounter patients with chronic liver disease and concomitant COVID-19 infection.

In the case of viral hepatitis, there is still no reliable information that supports synergism with SARS-CoV-2. However, it is known that SARS-CoV patients with viral hepatitis are more prone to develop liver damage and severe hepatitis. This was likely due to enhanced replication of the hepatitis virus during SARS-CoV infection.[20] In this context, some HLA class II haplotypes, like DRB1*1302, HLA-DR13, DQA1*0501, and DQB1*0301, have been related

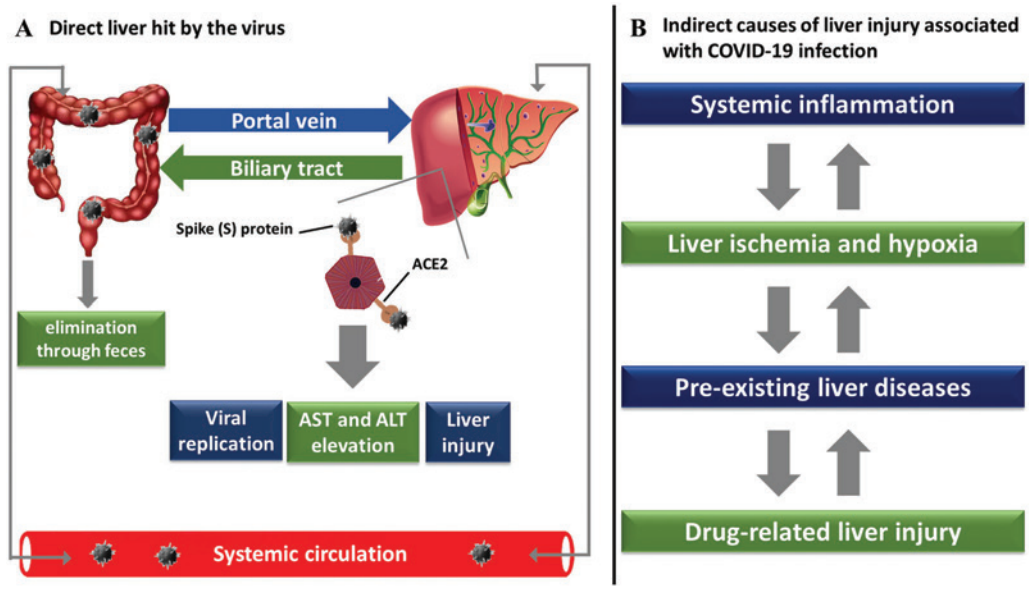

Fig. 1. Mechanisms of liver injury induced by SARS-CoV-2.
Abbreviations: ACE2, angiotensin converting enzyme 2; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

to acute and chronic hepatitis B virus persistence.[21] Thus, it would be useful to study risk-sharing alleles that might exist between viral hepatitis and COVID-19.

Patients with cholestatic diseases are another group that could present with more liver injury in the context of COVID-19 illness, as ACE2 is expressed in the epithelial cells of bile ducts. Moreover, immunocompromised states, such as those of individuals with cirrhosis and on immunosuppressive therapy, may place the patient at higher risk of severe illness. It has therefore been suggested that COVID-19 could accelerate the onset of complications in patients with compensated cirrhosis.[22] To date, we are uncertain about the effects of immunosuppression in these patients and in post-transplant liver patients. During previous outbreaks of coronavirus infections, it was suggested that immunocompromised patients (adults and children) are not at increased risk of infection compared to the general population.[23] However, if this pandemic has taught us anything, it is that the knowledge from previous experiences may not be generalizable to this new infection. A recent case report described a perioperative presentation of COVID-19 in a liver transplant recipient that jeopardized the graft.[24] The groups of people who need to take extra precautions may expand as we learn more about SARS-CoV-2.

Even though there is no consensus in the management of these types of situations, an expert panel of physicians from Wuhan, China provided a list of suggestions in terms of precautions to follow in patients with chronic liver disease.[25] They suggested avoiding home visits and going to crowded places, following a balanced diet, frequent handwashing of not less than 20 s, as well as adequate ventilation and cleaning of the home. For inpatients, they suggested treatment by only one physician and a nurse, with strict handwashing and disinfection after any rounds or procedures, provision of adequate personal protective equipment, and establishing a clean care area.

We are experiencing the full impact of the SARS-CoV-2 pandemic on our countries, our profession, and our patients. As we meet this crisis head-on, the analysis of epidemiology data can provide a valuable tool to assess the current evolution of the COVID-19 outbreak worldwide and to evaluate the impact of the countermeasures adopted, leading to the development of appropriate healthcare policies, targeting precious resources and equipment to all the clinical situations we encounter. While we wait for advances in treating the virus and the development of a vaccine, changes in personal behavior and widely available testing for the virus could really make a difference in the outcomes for our patients and healthcare workers, while preventing further spread within our communities. In other words, "be safe and use common public health sense". We are all in this crisis together.

Funding

None to declare.

Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

Conception and design of the study (NM-S), data acquisition (AV-R, XQ, EMY, MR-G, JG, RT, LA, APK), writing of the manuscript, critical revision of the manuscript and approval of the final version to be published (NM-S, AV-R, XQ, EMY, MR-G, JG, RT, LA, APK).

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Original Article

FibroScan Detection of Fatty Liver/Liver Fibrosis in 2266 Cases of Chronic Hepatitis B

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Abstract

Background and Aims: FibroScan is used to determine liver stiffness and controlled attenuation parameter (referred to as CAP) scores in patients, including those with chronic hepatitis B (CHB). We used FibroScan to detect the incidence of fatty liver and fibrosis in CHB patients, and to assess the correlation of FibroScan measurements with blood chemistry tests. ***Methods:*** CHB patients enrolled in this study were divided independently for three separate analyses (of fibrosis, cirrhosis, and fatty liver) based on FibroScan results. Basic information, blood chemistry test results, liver fibrosis parameters, and FibroScan results were collected. T-tests and Pearson's analyses were used to analyze the correlations between FibroScan liver stiffness measurement/CAP values and liver function, blood fat, uric acid metabolite, fibrosis, and hepatitis B virus load. ***Results:*** A total of 2266 CHB patients were enrolled in the study and divided into three groups: non-significant and significant fibrosis; non-cirrhosis and early cirrhosis; and non-fatty and fatty liver. Spearman's statistical analyses showed that liver stiffness measurement or CAP values correlated with sex (r=0.137), age (r=0.119), glutamic-pyruvic transaminase (r=0.082), glutamic-oxaloacetic transaminase (r=−0.172), gamma-glutamyltransferase (r=0.225), albumin (r=0.150), globulin (r=−0.107), total bilirubin (r=−0.132), direct bilirubin (r=−0.145), white blood cell count (r=0.254), hemoglobin (r=0.205), platelets (r=0.206), total cholesterol (r=0.214), high density lipoprotein (r=−0.243), low density lipoprotein (r=0.255), apolipoprotein B (r=0.217), hyaluronic acid (r=−0.069), laminin (r=−0.188), procollagen type IV (r=−0.067)and hepatitis B viral DNA load (r=−0.216). ***Conclusions:*** FibroScan is a non-invasive device that can detect the occurrence of fatty liver or liver fibrosis in CHB patients.

Keywords: FibroScan; Chronic hepatitis B; Fatty liver; Liver fibrosis; Cirrhosis.

Abbreviations: Alb, albumin; ALD, alcoholic fatty liver disease; ALT, glutamic-pyruvic transaminase; AST, glutamic-oxaloacetic transaminase; CAP, controlled attenuation parameter; CHB, chronic hepatitis B; DB, direct bilirubin; EC, early cirrhosis; FL, fatty liver; GGT, gamma-glutamyltransferase; Hb, hemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein; LSM, liver stiffness measurement; NAFLD, non-alcoholic fatty liver disease; NC, non-cirrhosis; NFL, non-fatty liver; NSF, non-significant fibrosis group; Pl, platelet; SF, significant fibrosis; TB, total bilirubin; TE, transient elastography; WBC, white blood cell.

Received: 14 October 2019; Revised: 18 March 2020; Accepted: 31 March 2020

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Citation of this article: He T, Li J, Ouyang Y, Lv G, Ceng X, Zhang Z, *et al.* FibroScan detection of fatty liver/liver fibrosis in 2266 cases of chronic hepatitis B. J Clin Transl Hepatol 2020;8(2):113–119. doi: 10.14218/JCTH.2019.00053.

Introduction

The liver is the largest digestive organ in the human body and participates in the metabolism of most substances. Therefore, liver damage, which can occur due to a variety of reasons, impacts a large proportion of the bodily metabolism, including metabolization of blood lipids, blood sugar, uric acid, and proteins.[1] Fatty liver disease refers to steatosis, in which the weight of liver fat accounts for more than 5% of the total liver weight, or where the histological appearance of fat accounts for 30% or more of liver volume.[2] Fatty liver includes alcoholic fatty liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). Recently, NAFLD has become a particular area of interest in research, as this disease is increasingly seen in the clinic, but it could be improved through relatively simple and cost-effect changes in lifestyle.[3]

Studies have found that chronic hepatitis B (CHB) is a critical cause of fatty liver. As there is a high incidence of CHB in China, it is critical to determine whether CHB is associated with either liver fibrosis or fatty liver in these patients.[4,5] Recently, a novel non-invasive technique called transient elastography (TE) was developed to assess liver fibrosis/fatty liver. This technique induces a shear wave in the liver and measures the velocity of the wave in real time. Based on this technique, the FibroScan 502 device was developed in 2001 by Echosens (Paris, France), and has since been used in hospitals.[6] Using FibroScan, values for liver stiffness measurement(LSM) and controlled attenuation parameter(CAP), indicating liver fibrosis and fatty liver respectively, can now be obtained non-invasively and in real-time. Therefore, FibroScan is an advanced non-invasive quantitative detection device for liver hardness and steatosis, which greatly improves early detection rates of these injuries. FibroScan is, thus, suitable for the diagnosis of fatty liver, liver fibrosis, and cirrhosis, which commonly exist alongside chronic viral hepatitis, alcoholic hepatitis, autoimmune liver diseases, and other liver conditions.[7–9]

In this clinical study, we collected data and information from 2266 CHB patients to assess the value of using FibroScan as a non-invasive tool for the diagnosis of fatty liver/liver fibrosis, and to determine the correlation between FibroScan results and blood chemistry test results.

Methods

Study design and patients

A total of 2266 patients with CHB were enrolled in this study between December 2014 and August 2018 in the Department of Infectious Diseases, Shunde Hospital, Southern Medical University. These patients were divided into three different groups according to their FibroScan results, combined with measured clinical manifestations. Group 1 was divided by the degree of fibrosis. Group 2 was stratified by the level of cirrhosis. Group 3 was separated based on the absence (3a) or presence (3b) of fatty liver.

Blood chemical tests

Blood biochemical indicators (lipids, glucose, liver function, fibrosis) were quantified using an Olympus Au1000 automatic biochemical analyzer (Shanghai Kehua - Dongling Diagnostic Products Co., Ltd., Shanghai, China).

HBV DNA load in sera

Blood HBV DNA load was measured with a quantitative real-time PCR kit (DA AN Gene, Guangzhou, China) using the LightCycler 96 system (Roche Molecular Systems, Basel, Switzerland).[10]

LSM and CAP values detected by FibroScan

We quantified LSM and CAP values using FibroScan 502 to assess the degree of fatty liver or liver fibrosis in CHB patients. According to the Operator's instructions, patients with CAP scores ≥237.7 db/m were defined as fatty liver patients, in which 237.7–259.3db/m was defined as mild fatty liver, 259.4–292.3db/m was defined as moderate fatty liver, and >292.3db/m was defined as severe fatty liver. LCM values of 7 kPa, 9.5 kPa, and 12.5 kPa demarcated the boundaries between no significant fibrosis (S1 of the METAVIR classification system), significant fibrosis (S2), severe fibrosis (S3), and cirrhosis (S4).[11]

Liver tissues

Liver biopsy samples were obtained from CHB in-patients. Human healthy liver tissue samples were provided by Xi'an Alenabio Inc. (Xi'an, China) and used as the negative control.

Sirius Red staining on CHB liver paraffin sections

A small number of patients (10.4%) underwent liver biopsy for histopathological analysis. Sirius Red staining is a common histochemical method for the detection of liver fibrosis. The Sirius Red staining materials used included: Solution A: 0.5 g Sirius Red F3B in 500 mL saturated picric acid; Solution B: 5 mL acetic acid in 1 L of distilled water. The staining procedure was carried out per standard methods. Briefly, the sample was warmed to room temperature for 5m. The paraffin sections were dewaxed, hydrated, and soaked in 100% alcohol for 10 m. The sections were then washed twice in phosphate-buffered saline, stained in Solution A for 1 h, washed twice in Solution B, and examined under a light microscope to assess fibrosis.

Statistical analyses

We used SPSS 19.0 for statistical analysis (IBM Corp., Armonk, NY, USA). Means ± standard deviations (x±s) were used for normal distribution measurements, while non-normal distribution measurement data were expressed as median ± standard deviation (25^{th} and 75^{th} percentile). The mean values between two groups were compared by t-tests or by non-parametric methods if the data was not normally distributed. Count data was analyzed using the chi-square test. P values less than 0.05 were considered statistically significant. For correlation studies, we carried out Pearson's statistical analyses. We classified absolute correlation values as follows: very weak, 0.00-0.19; weak, 0.20-0.39; moderate, 0.40-0.59; strong, 0.60-0.79; and very strong, 0.80-1.00.

Ethics

The authors declare that this study fully complied with all relevant ethical standards. Informed consent was acquired from all of the patients. This study was approved by the Human Ethics Committee of Shunde Hospital, Southern Medical University, according to the Declaration of Helsinki.

Results

Clinical features

Fibrosis is the earliest pathological sign of CHB, and cirrhosis represents the last stage of fibrosis, at which point liver transplantation may be required. In our study, patients were divided into three groups. Group 1 was divided by the degree of fibrosis, consisting of group 1a (S1) with non-significant fibrosis, and group 1b (S2-4) with significant fibrosis. Group 1a (S1) consisted of 1082 male patients (72.8%) and 404 female patients (27.2%), with an average age of 40.0±10.5 years. Group 1b (S2-4) contained of 618 male patients (79.2%) and 162 female patients (20.8%), with an average age of 44.8±12.1 years. Group 2 was stratified by the level of cirrhosis. Group 2a (S1-3) was the non-cirrhosis group, with an average age of 41.0±10.9 years and composed of 1540 male patients (74.4%) and 531 female patients (25.6%). Group 2b (S4) was the early cirrhosis group (average age of 48.4±12.8 years), which included 160 male patients (82.1%)and 35 female patients (17.9%). Group 3 was divided into non-fatty liver group (3a) and fatty liver group (3b). The non-fatty liver group had an average age of 42.8±10.9 years and contained 659 male patients (69.2%) and 294 female patients (30.8%). The fatty liver group had 1041 male patients (79.3%) and 272 female patients (20.7%), with an average age of 40.1±11.6 years. In all three groups, the incidence of fatty liver or liver fibrosis was markedly higher in men than in women (p<0.05). Groups 1b, 2b, and 3b suffered from worse or abnormal liver function, fibrosis parameters, and hepatitis B viral load than groups 1a, 2a, and 3a respectively.

In our cohort of 2266 patients, we detected a total of 780 cases with significant liver fibrosis (34.42%). In men, the incidence of fibrosis detection was higher than in women (36.35% of men showed fibrosis [618/1700], as opposed to 28.62% of women [162/566]). This sex difference was statistically significant (p<0.001). All patients were then divided into either a non-significant fibrosis group (further referred to NSF, 1486 patients) or a significant fibrosis group (further referred to as SF, 780 patients). The clinical characteristics of the two groups are shown in Table 1. A comparative

analysis of the two groups (Table 1) shows age, glutamic-pyruvic transaminase (ALT), glutamic-oxaloacetic transaminase (AST), gamma-glutamyltransferase (GGT), albumin (Alb), globin, total bilirubin (TB), direct bilirubin (DB), white blood cell (WBC) count, hemoglobin (Hb), platelet (Pl), blood urea nitrogen, creatinine, uric acid, glucose, total cholesterol, triglycerides, high-density and low-density lipoprotein (HDL and LDL respectively), apolipoprotein A and B, hyaluronic acid, laminin, type III and IV procollagen, HBV DNA load, as well as CAP and LSM scores for each patient. Unsurprisingly, most liver function parameters were significantly higher in the SF group than in the NSF group (p<0.001). Blood type III and IV procollagen levels, which represent liver fibrosis severity and HBV DNA load, were furthermore significantly higher in the SF group than in the NSF group (p<0.001). There were no significant differences in age, kidney function, levels of triglycerides, HDL, or apolipoprotein A between the two groups (p>0.05).CAP scores were 251.2±54.3 db/m and 251.4±59.6 db/m for the NSF group and SF group respectively, indicating no significant difference in CAP values between the two groups (p>0.05). However, there was a significant difference in LSM values (p<0.001); the SF group had a significantly increased LSM value (15.8±12.8 kPa) compared to the NSF group (5.0±1.1 kPa).

In total, 195 cases of early cirrhosis were detected among the 2266 patients, representing a rate of 8.61%. Early cirrhosis was more frequently found in men compared to women (9.41% of men [160/1700] compared to 6.18% of women [35/56]). Similar to fibrosis, this sex-specific difference in incidence was statistically significant (p<0.001). Patients were then divided into the non-cirrhosis group (further referred to as NC group, 2071 patients) and early cirrhosis group (further referred to as EC group, 195 patients), according to presence or absence of cirrhosis. The clinical characteristics and comparative analysis of the two groups can be found in Table 2. Most liver function parameters in the EC group were higher than in the NC group (p<0.001). Blood type III and IV procollagen levels, which

Table 1. Clinical features of patients with non-significant fibrosis (S1 group) and significant fibrosis (S2-4 group)

	S1 *n*=1486	S2-4 *n*=780	*p* value
Male, *n* (%)	1082 (68.4)	618 (79.2)	<0.001
Female, *n* (%)	404 (31.6)	162 (20.8)	<0.001
Age in years	40.0±10.5	44.8±12.1	0.942
Glutamic-pyruvic transaminase in U/L	32 (22, 49)	53 (31, 111)	<0.001
Glutamic-oxaloacetic transaminase in U/L	30 (23, 39)	46 (32, 81)	<0.001
Gamma-glutamyltransaminase in U/L	23 (15, 35)	45 (26, 83)	<0.001
Albumin in G/L	51.0±33.0	45.5±15.4	<0.001
Globin in G/L	29.6±11.2	32.0±20.2	0.003
Total bilirubin in μmol/L	13.9 (10.9, 17.2)	15.7 (12.0, 21.1)	<0.001
Direct bilirubin in μmol/L	3.3 (2.5, 4.4)	4.2 (3.0, 6.2)	<0.001
White blood cells in G/L	7.0±3.3	6.4±2.2	<0.001
Hemoglobin in G/L	147.5±20.7	142.8±20.3	<0.001
Platelets in G/L	218.4±60.2	171.2±69.0	<0.001
Blood urea nitrogen in μmol/L	5.1±2.8	5.2±3.1	0.723
Creatinine in μmol/L	81.8±32.6	84.5±62.6	0.199
Uric acid in μmol/L	397.0±118.9	389.2±117.4	0.173
Glucose in mmol/L	7.2±25.2	7.6±28.7	0.727
Total cholesterol in mmol/L	4.9 (4.4, 5.7)	4.7 (4.1, 5.5)	<0.001
Triglycerides in mmol/L	1.1 (0.8, 1.6)	1.0 (0.8, 1.5)	0.054
High-density lipoprotein in mmol/L	1.3±0.4	1.3±0.7	0.552
Low-density lipoprotein in mmol/L	3.1 (2.5, 3.7)	2.8 (2.2, 3.5)	<0.001
Apolipoprotein A in mmol/L	1.3±0.3	1.3±0.9	0.830
Apolipoprotein B in mmol/L	1.1±0.4	1.0±0.5	0.014
Hyaluronic acid	51.7 (28.7, 79.5)	88.5 (49.8, 160.4)	<0.001
Laminin	35.1 (21.7, 49.8)	48.5 (30.5, 78.7)	<0.001
Type III procollagen	9.2 (7.3, 11.9)	12.1 (9.1, 17.5)	<0.001
Type IV procollagen	30.6 (23.3, 39.7)	53.7 (34.7, 98.8)	<0.001
HBV DNA load as log10 IU/mL	3.4±2.1	4.1±2.2	<0.001
Controlled attenuation parameter	251.2±54.3	251.4±59.6	0.942
Liver stiffness measurement	5.0±1.1	15.8±12.8	<0.001

represent liver fibrosis severity and HBV DNA load, were higher in the EC group than in the NC group (p<0.001)but no significant differences were found in kidney function, triglycerides, HDL and apolipoprotein B between the two groups (p>0.05). Both CAP and LSM values were significantly different between the two groups (p<0.001; NC: CAP of 237.4±57.9, LSM of 32.7 ±16.0 kPa; EC: CAP of 252.6±55.9, LSM of 6.5±2.8 kPa).

In total, 1313 fatty liver cases were detected among the 2266 patients, marking an overall incidence of 57.94%. Again, incidence was higher in males than in females, with 61.24% (1041/1700) of men showing fatty liver compared to 48.06% of women (272/566). The difference in incidence between the two sexes was statistically significant (p<0.001). Patients were divided into a non-fatty liver group (NFL, 953 patients) and fatty liver group (FL, 1313 patients). The clinical characteristics and comparative analysis of the two groups are shown in Table 3. Blood glucose levels, total cholesterol, triglycerides, LDL, apolipoprotein B, laminin and type III procollagen were significantly higher in NFL patients compared to FL patients (p<0.05). On the other hand, blood HDL, hyaluronic acid and type IV procollagen levels and HBV DNA load were significantly lower in the NFL group than in the FL group (p<0.05). Here, we found significant differences in the CAP score between the groups (p<0.001). NFL CAP scores were 284 (265, 313) db/m, while FL CAP scores were 203(182, 220) db/m. LSM values were 5.9 (4.6, 8.3) kPa and 6.0 (4.6, 9.3) kPa for NFL and FL respectively. There were no significant differences in LSM values between the two groups (p>0.05).

Sirius Red staining of liver paraffin sections

We obtained liver sections from 10.4% of the patients (235/2266) who underwent biopsy. Fig. 1 shows representative pictures from the Sirius Red staining of liver sections at different stages of liver fibrosis, indicating that the staining matched the FibroScan results very well.

Table 2. Clinical features of patients with non-cirrhosis (S1-3 group) and early cirrhosis (S4 group)

	S1-3 *n*=2071	S4 *n*=195	*p* value
Male, *n* (%)	1540 (74.4)	160 (82.1)	0.018
Female, *n* (%)	531 (25.6)	35 (17.9)	<0.001
Age in years	41.0±10.9	48.4±12.8	<0.001
Glutamic-pyruvic transaminase in U/L	35 (23, 60)	66 (36, 196)	<0.001
Glutamic-oxaloacetic transaminase in U/L	32 (24, 45)	66 (45, 158)	<0.001
Gamma-glutamyltransaminase in U/L	26 (17, 43)	86 (51, 166)	<0.001
Albumin in G/L	49.9±29.3	40.0±9.9	<0.001
Globin in G/L	30.1±13.9	34.3±23.2	0.015
Total bilirubin in μmol/L	14.1 (11.0, 17.7)	22.3 (16.4, 30.9)	<0.001
Direct bilirubin in μmol/L	3.5 (2.6, 4.7)	6.7 (4.6, 12.3)	<0.001
White blood cells in G/L	6.9±3.1	5.6±2.0	<0.001
Hemoglobin in G/L	147.3±19.5	131.6±25.7	<0.001
Platelets in G/L	209.3±36.1	127.6±63.1	<0.001
Blood urea nitrogen in μmol/L	5.2±2.9	5.3±3.6	0.621
Creatinine in μmol/L	82.2±33.7	88.8±110.8	0.457
Uric acid in μmol/L	395.6±118.1	380.1±122.8	0.140
Glucose in mmol/L	5.7 (5.3, 6.1)	5.6 (5.1, 6.6)	0.709
Total cholesterol in mmol/L	4.9 (4.3, 5.6)	4.6 (3.9, 5.2)	<0.001
Triglycerides in mmol/L	1.1 (0.8, 1.6)	1.0 (0.8, 1.6)	0.054
High-density lipoprotein in mmol/L	1.3±0.5	1.3±0.4	0.074
Low-density lipoprotein in mmol/L	3.0 (2.5, 3.6)	2.5 (1.9, 3.0)	<0.001
Apolipoprotein A in mmol/L	1.4±0.7	1.1±0.4	0.017
Apolipoprotein B in mmol/L	1.1±0.5	1.0±0.3	0.184
Hyaluronic acid	56.1 (32.9, 88.8)	213.0 (114.7, 411.5)	<0.001
Laminin	36.4 (23.7, 53.3)	80.5 (55.9, 140.4)	<0.001
Type III procollagen	9.6 (7.5, 12.8)	17.5 (13.4, 24.2)	<0.001
Type IV procollagen	33.3 (25.0, 47.4)	146.6 (80.3, 244.7)	<0.001
HBV DNA load as log10 IU/mL	3.6±2.1	4.5±2.4	<0.001
Controlled attenuation parameter	252.6±55.9	237.4±57.9	<0.001
Liver stiffness measurement	6.5±2.8	32.7±16.0	<0.001

Table 3. Clinical features of patients with non-fatty liver and fatty liver

	Non-fatty liver *n*=953	Fatty liver *n*=1313	*p* value
Male, *n* (%)	659 (69.2)	1041 (79.3)	<0.001
Female, *n* (%)	294 (30.8)	272 (20.7)	<0.001
Age in years	42.8±10.9	40.1±11.6	<0.001
Glutamic-pyruvic transaminase in U/L	38 (25, 61)	34 (22, 68)	0.033
Glutamic-oxaloacetic transaminase in U/L	31 (23, 44)	37 (27, 59)	<0.001
Gamma-glutamyltransaminase in U/L	31 (20, 51)	22 (14, 44)	<0.001
Albumin in G/L	50.6±34.3	46.9±16.6	0.003
Globin in G/L	29.5±9.3	31.7±20.3	0.004
Total bilirubin in μmol/L	14.0 (10.8, 17.5)	15.3 (11.9, 20.0)	<0.001
Direct bilirubin in μmol/L	3.4 (2.5, 4.6)	3.9 (2.8, 5.4)	<0.001
White blood cells in G/L	7.2±3.6	6.2±1.8	<0.001
Hemoglobin in G/L	148.5±20.4	142.1±20.4	<0.001
Platelets in G/L	212.3±65.9	187.8±66.3	<0.001
Blood urea nitrogen in μmol/L	5.3±3.4	5.0±2.2	0.032
Creatinine in μmol/L	82.3±26.4	83.2±62.5	0.617
Uric acid in μmol/L	414.2±119.0	365.8±116.7	<0.001
Glucose in mmol/L	5.8 (5.4, 6.3)	5.5 (5.2, 5.9)	<0.001
Total cholesterol in mmol/L	5.1 (4.4, 5.8)	4.7 (4.0, 5.4)	<0.001
Triglycerides in mmol/L	1.3 (0.9, 1.8)	0.9 (0.7, 1.2)	<0.001
High-density lipoprotein in mmol/L	1.3±0.4	1.4±0.7	<0.001
Low-density lipoprotein in mmol/L	3.2 (2.6, 3.8)	2.8 (2.3, 3.3)	<0.001
Apolipoprotein A in mmol/L	1.3±0.8	1.3±0.4	0.791
Apolipoprotein B in mmol/L	1.1±0.5	1.0±0.3	<0.001
Hyaluronic acid	56.1 (32.0, 98.7)	64.3 (38.9, 101.3)	0.038
Laminin	65.1 (21.1, 52.0)	44.1 (28.4, 66.5)	<0.001
Type III procollagen	10.3 (7.8, 14.7)	9.8 (7.5, 13.5)	0.046
Type IV procollagen	34.7 (25.0, 51.7)	35.7 (27.0, 58.3)	0.032
HBV DNA load as log10 IU/mL	3.3±2.0	4.1±2.2	<0.001
Controlled attenuation parameter	284 (265, 313)	203 (182, 220)	<0.001
Liver stiffness measurement	5.9 (4.6, 8.3)	6.0 (4.6, 9.3)	0.119

Statistical analysis results

Spearman's correlation analyses showed that CAP scores and LSM values correlated with sex (r=0.137), age (r=0.119), ALT (r=0.082), AST (r=−0.172), GGT (r=0.225), Alb (r=0.150), globulin (r=−0.107), TB (r=−0.132), DB (r=−0.145), WBC count (r=0.254), Hb (r=0.205), PI (r=0.206), total cholesterol (r=0.214), HDL (r=−0.243), LDL (r=0.255), apolipoprotein B (r=0.217), hyaluronic acid (r=−0.069), laminin (r=−0.188), procollagen type IV (r=−0.067), and hepatitis B viral DNA load (r=−0.216).

Discussion

CHB is a chronic hepatophagocytic viral infection of the human liver, which induces deleterious protracted immune responses and ultimately leads to progressive liver fibrosis or cirrhosis as well as a greatly increased risk for hepatocellular carcinoma.[12,13] Accurately determining the staging and severity of CHB is therefore extremely important for selecting the appropriate therapeutic interventions. Currently, such estimations are made largely based on serial serological biochemical tests that are intended to reflect liver injury and repair responses in patients, but these tests are limited in their ability to fully reflect the entirety of liver function in CHB patients.[14,15] Liver biopsies followed by pathological staining—including Sirius Red staining, Masson's Trichrome staining, and Oil Red O staining—are commonly used for diagnosis of liver fibrosis and hepatic steatosis. However, these methods are limited as screening tools in medical practice because of the invasive nature of the biopsies.[16]

FibroScan is already used worldwide in the pathological analysis of various liver diseases, including CHB and NFALD. The LSM and CAP values measured by FibroScan were previously found to accurately reflect liver steatosis and fibrosis.[17–19] However, a larger amount of CHB patient

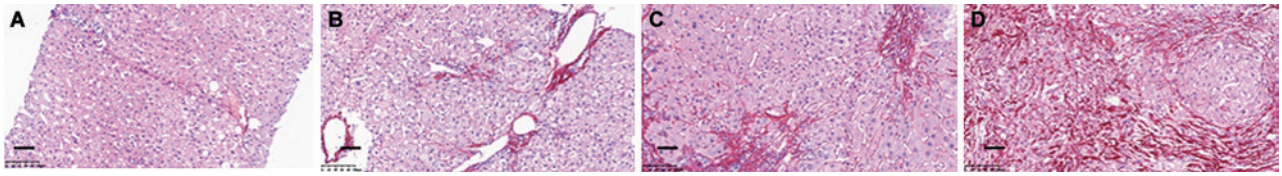

Fig. 1. Comparison of Sirius Red staining with LSM values on CHB liver tissues. (A) Normal control, LSM <7.0 kPa. (B) Significant fibrosis,LSM:7.0 kPa-9.5 kPa. (C) Severe fibrosis, LSM: 9.5 kPa-12.5 kPa. (D) Cirrhosis, LSM>12.0 kPa. Scale bar: 100μm.

Abbreviations: CHB, chronic hepatitis B; LSM, liver stiffness measurement.

FibroScan data was required to fully assess its utility in detecting human fatty liver or liver fibrosis diseases. In the current study, we found that the two FibroScan measurements, LSM and CAP, taken from CHB patients' livers were weakly or very weakly correlated to blood chemical test results, indicating that both LSM and CAP are independent indicators of disease. LSM values were furthermore found to be closely associated with the results of fibrosis staining. Our findings are consistent with several previous studies. Christiansen *et al.*[20] conducted a 5-year prospective study for CHB and hepatitis C patients. They found that patients with LSM values ≥17 kPa have an increased risk of liver diseases, suggesting that a single LSM value cannot be used alone to make clinical decisions.

After analysis of 307 patients who underwent liver biopsy and LSM determination, Li *et al.*[21] found that LSM was an independent indicator for different stages of fibrosis ($p<0.001$). LSM values combined with biochemical indices showed potential value for assessment of CHB-related liver fibrosis.

Performing a multivariate analyses, Zhang *et al.*[22] showed that the cumulative probability of hepatocellular carcinoma development in patients with lower liver stiffness was significantly lower compared to patients with increased liver stiffness ($p<0.05$).

Fatty liver is caused by excessive accumulation of fat in hepatocytes but its exact pathogenesis is still unclear. Similarly, the pathomechanisms underlying fibrosis remain to be elucidated. Eddowes *et al.*[11] collected data from 450 suspected NAFLD patients at seven medical centers in the UK and found that CAP scores and LSM values determined by FibroScan were an efficient non-invasive means to assess hepatic steatosis and fibrosis respectively.

In conclusion, our study as well as previous work have shown that FibroScan is a rapid and non-invasive method for detection of fatty liver and liver fibrosis. Its results are not strongly correlated to blood chemistry tests, and may therefore be used to independently assess the presence and development of fatty liver or liver fibrosis.

Funding

Medical Scientific Research Foundation of Guangdong Province of China (No: A2016450 and A2013695).

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study design (JD), data collection (TH, JL, YO), data analysis (JD, ZZ), pathological staining (GL, XC), statistical analysis (ZZ), and drafting the article (JD).

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Original Article

Effect of Exercise on NAFLD and Its Risk Factors: Comparison of Moderate versus Low Intensity Exercise

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Abstract

Background and Aims: Lifestyle (exercise and dietary) modification is the mainstay of treatment for non-alcoholic fatty liver disease (NAFLD). However, there is paucity of data on effect of intensity of exercise in management of NAFLD, and we aimed to study the effect of variable intensities of exercise on NAFLD. ***Methods:*** The study was performed in the Department of Gastroenterology of the SCB Medical College, Cuttack and the Biju Patnaik State Police Academy, Bhubaneswar. The subjects were police trainees [18 in a moderate intensity exercise group (MIG) and 19 in a low intensity exercise group (LIG)] recruited for a 6-month physical training course (261.8 Kcalorie, 3.6 metabolic equivalent in MIG and 153.6 Kcalorie, 2.1 metabolic equivalent in LIG). NAFLD was diagnosed by ultrasonography, with exclusion of all secondary causes of steatosis. All participants were evaluated by anthropometry (weight, height, body mass index (BMI), waist circumference), assessed for blood pressure and biochemical parameters (blood glucose, liver function test, lipid profile, serum insulin), and subjected to transabdominal ultrasonography before and after 6 months of physical training, and the results were compared. ***Results:*** Both the groups had similar BMI, fasting plasma glucose, AST, gamma-glutamyl transpeptidase, insulin, and homeostatic model assessment-insulin resistance (known as HOMA-IR) ($p>0.05$). However, subjects in the LIG were older and had lower alanine transaminase, higher triglycerides and lower high-density lipoproteins than MIG subjects. There was a significant reduction in BMI (27.0±2.1 to 26.8±2.0; $p=0.001$), fasting blood glucose (106.7±21.6 to 85.8±19.0; $p<0.001$), serum triglycerides (167.5±56.7 to 124.6±63.5; $p=0.017$), total cholesterol (216.8±29.2 to 196.7±26.6; $p=0.037$), low-density lipoprotein cholesterol (134.6±21.4 to 130.5±21.9; $p=0.010$), serum aspartate transaminase (39.3±32.2 to 30.9±11.4; $p<0.001$), serum alanine transaminase (56.6 ±28.7 to 33.0±11.3; $p<0.001$) and HOMA-IR (2.63±2.66 to 1.70±2.59; $p<0.001$) in the MIG. However, changes in these parameters in the LIG were non-significant. Hepatic steatosis regressed in 66.7% of the NAFLD subjects in the MIG but in only 26.3% of the LIG NAFLD subjects ($p=0.030$). ***Conclusions:*** Moderate rather than low intensity physical activity causes significant improvement in BMI, serum triglycerides, cholesterol, serum transaminases and HOMA-IR, and regression of ultrasonographic fatty change in liver among NAFLD subjects.

Citation of this article: Nath P, Panigrahi MK, Sahu MK, Narayan J, Sahoo RK, Patra AA, *et al.* Effect of exercise on NAFLD and its risk factors: Comparison of moderate versus low intensity exercise. J Clin Transl Hepatol 2020;8(2):120–126. doi: 10.14218/JCTH.2019.00012.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common hepatic disorders, with macrovesicular fat accumulation in more than 5% of hepatocytes in the absence of any secondary cause of hepatic steatosis, such as significant alcohol abuse (more than 10 g/day for women and 20 g/day for men), hepatotropic viral infection, drugs which can cause fatty liver, or any other etiologies.[1] In the last couple of decades, NAFLD has emerged as the most common liver disease in adults.[2] Its prevalence varies in different parts of the world, ranging from 5-40%. However, its occurrence is even higher in individuals with obesity (30-100%) and type 2 diabetes mellitus (42.6-69.5%).[3] Despite the benign natural course in the majority, 10% of the NAFLD patients may progress to cirrhosis later on in their lives.[4] It contributes to formation of a pro-inflammatory environment that accelerates atherosclerosis, increasing the risk of ischemic heart disease and its severity, which itself correlates with the degree of inflammation.[5] In addition, these individuals are at a high risk for developing diabetes mellitus. Hence, patient education about the entity, need for treatment and compliance are necessary to avert future complications.

Keywords: Exercise; NAFLD; Intensity; Physical activity.

Abbreviations: 1H-MRS, magnetic resonance spectroscopy; ALT, alanine transaminase; APRI, AST to platelet ratio; AST, aspartate transaminase; BMI, body mass index; FLI, fatty liver index; GGT, gamma-glutamyl transpeptidase; HOMA, homeostatic model assessment; IR, insulin resistance; LDL, low-density lipoprotein; LIG, low intensity exercise group; MET, metabolic equivalent; MIG, moderate intensity exercise group; NAFLD, non-alcoholic fatty liver disease.

Received: 10 March 2019; Revised: 27 December 2019; Accepted: 1 April 2020

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Lifestyle modification is still the treatment of first choice for NAFLD patients and is recommended for all patients with NAFLD.[6] This aims at weight loss and reduction of insulin resistance (IR). Further, it is also directed at managing obesity and the features of metabolic syndrome which are frequently associated with NAFLD. Low-calorie diet and increased physical activity are the cornerstones of lifestyle modification. Patients are encouraged to lose at least 10% of their initial body weight to achieve maximal benefits. Weight loss improves patients' cardiovascular risk profile and steatosis,[7] and probably reduces hepatic inflammation as well as hepatocellular injury.[8] Exercise is a major component of treatment for NAFLD, as recommended by the American Gastroenterological Association,[9] the American Association for the Study of Liver Diseases and the European Association of Study of Liver. As compared to type 2 diabetes mellitus, there is a paucity of data supporting the role of physical activity in management of NAFLD. This may be due to the invasive nature of grading hepatic steatosis by needle biopsy and histology, which limits the capacity for repeated measurement of hepatic steatosis and degree of necroinflammation. The federal guidelines of the USA's Department of Health and Human Services and the USA's Department of Agriculture recommend that adults should perform 150 min or more of moderate-intensity physical activity per week, 75 min or more of vigorous intensity physical activity per week, or a combination, to improve and maintain health.[10] The Centers for Disease Control and Prevention and the World Health Organization[11] recommendations are also the same. For additional health benefits, the amount of physical activity recommended should be doubled.[11] However, controversy remains over the role of exercise intensity and total volume of exercise responsible for final health outcomes.

The aim of this study was to compare the beneficial effects of low and moderate intensity exercise for 6 months, on NAFLD.

Methods

This study was performed in the Biju Patnaik State Police Academy, Bhubaneswar, Odisha, India which conducts 6 months of physical training before recruitment or promotion. The subjects were middle-aged male police recruits selected for 6 months of physical training.

Inclusion criteria

The police trainees who had fatty liver detected on ultrasonography were included in this study. These participants were in good health and had no abnormal findings on general and systemic physical examinations and for blood count and transabdominal ultrasonography (except for fatty liver).

Exclusion criteria

Patients with organic gastrointestinal disease revealed by ultrasonography or gastroduodenoscopy were excluded. Participants who had history of alcohol consumption exceeding 20 g/day, subjects with other liver diseases [hepatitis viruses A through E (by viral serologies: hepatitis B surface antigen, anti-hepatitis C virus antibody), autoimmune disease (autoimmune markers: anti-nuclear antibody, anti-smooth muscle antibody, anti-liver kidney microsome type 1 antibody) and Wilson's disease (serum ceruloplasmin)] and those on drugs which can induce fatty liver or insulin sensitization (estrogens, amiodarone, methotrexate, tamoxifen, glitazones, metformin) were also excluded from the study.

An informed consent was obtained from each participant. Detailed anthropometric assessment, including measurements of weight, height, and waist and hip circumferences, was conducted before and after 6 months of physical exercise. The waist circumference was measured at a level midway between the lowest rib and the iliac crest and hip circumference at the level of the greater trochanter. Body mass index (BMI) was calculated by the formula of weight (kg) / height^2 (m^2).

The measurements of fasting plasma glucose, 2-h post-glucose load plasma glucose, serum triglycerides, serum total cholesterol, serum high-density lipoprotein cholesterol and liver function markers were performed by standard laboratory methods both before the beginning and after the completion of 6 months of physical exercise. The estimation of serum insulin was performed by using the electrochemiluminescence method (Roche-Diagnostics, USA) with an autoanalyzer, Elecsys 2010 (Roche-Hitachi, Japan). IR was computed by using the homeostatic model assessment (HOMA) method via a mathematical model derived from fasting plasma glucose and plasma insulin. The value of HOMA was calculated by the following equation: [fasting insulin (μU/mL) $\times$ fasting blood glucose (mg/dL)]/405, and depicted as the HOMA-IR value.[12] For the purpose of the study, a HOMA-IR value above 2 was considered to indicate IR.[13]

Transabdominal ultrasonography was performed independently and blindly by two experienced radiologists (AAP and SJ; 5-10 years' experience) to identify and grade fatty changes in liver; in case of any conflict, the discrepancy was resolved by consensus after examination by a third senior radiologist (RKS; 15 years' experience). The diagnosis of fatty liver was made as per the standard criteria adopted by the American Gastroenterology Association,[14] i.e. an increase in liver echogenicity as compared to renal echogenicity as a reference, as well as lack of differentiation in periportal intensity and vascular wall due to hyperechogenicity of the liver parenchyma.

All the participants were subjected to aerobic physical training for 6 months, which included brisk walking, jogging, marching drill, 'lathi' drill, and yoga. The duration of each session of the physical activity was 50-60 m per day with a frequency of 5-6 sessions per week. The duration of physical activity and total calories burnt during exercise (volume of exercise) were estimated by accelerometers. The intensity of exercise was deducted by the equation for metabolic equivalent (MET) as follows: (total calories)/(kg body weight)/(hours of exercised performed). Activities with MET values below 3 were labelled as low intensity exercise, those with MET between 3 and 5.9 were classified as moderate intensity, and activities with MET values $\geq$ 6 were classified as vigorous intensity.

The subjects of the study were classified into the following groups:

1. Low intensity exercise group (LIG) and
2. Moderate intensity exercise group (MIG).

None of the study participants were subjected to vigorous intensity exercise. All the trainees were provided with a diet of 2400 to 2700 kcal/day. This study was approved by the Institutional Ethics Committee of SCB Medical College, Cuttack.

Statistical analysis

Normally distributed continuous variables were expressed as mean±standard deviation. Student's *t*-test for unpaired data

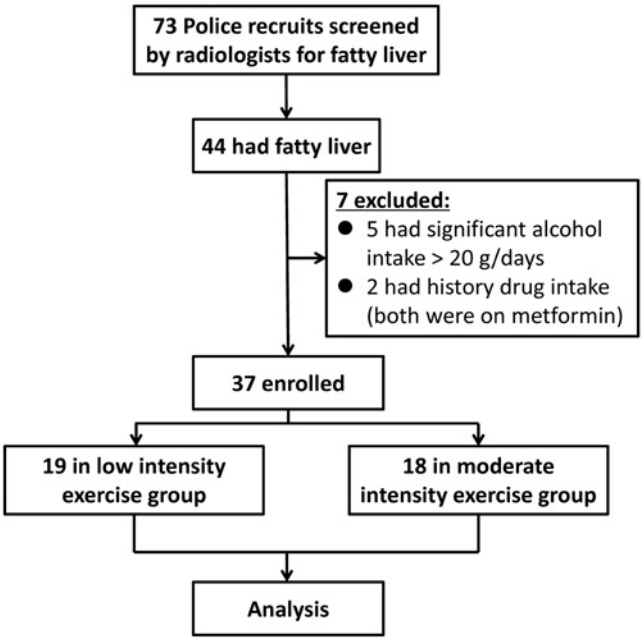

Fig. 1. Study consort diagram.

was used to compare the groups when the variables were normally distributed. Chi-square test was used to compare differences between categorical variables. All the anthropometric and biochemical parameters before and after 6 months of physical activity were compared by Student's *t*-test for paired data. All calculations were performed using the statistical software, SPSS version 16. A *p* value of less than 0.05 was taken as significant.

Results

Out of 62 police recruits, a total of 37 subjects were to found to have NAFLD, out of which 18 performed moderate intensity exercise and 19 performed low intensity exercise for 6 months (Fig. 1). Baseline comparison between the two groups is depicted in Table 1. The mean intensity of physical activity in the MIG was 3.6 MET (with mean total volume of exercise: 261.8 Kcalorie), whereas that of the LIG was 2.1 MET (with mean volume: 156.6 Kcalorie).

Patients in the LIG were older (53.3±3.7 years vs. 37.3 ±8.2 years; $p<0.001$), with significantly higher systolic blood pressure (156.6±18.9 mmHg vs. 132.2±13.2 mmHg; $p<0.001$), lower serum total cholesterol (181.4±34.8 vs. 211.2±30.0; $p=0.018$), lower low-density lipoprotein (LDL) cholesterol (102.5±29.9 vs. 30.5±22.0; $p=0.007$), lower serum aspartate transaminase (AST; 23.9±7.9 vs. 39.2 ±29.5; $p=0.039$) and lower serum alanine transaminase (ALT; 26.3±13.1 vs. 61.8±30.2; $p<0.001$). However, both of the groups had comparable BMI, diastolic blood pressure, fasting and 2-h post-glucose load plasma glucose, serum triglycerides, high-density lipoprotein, very low-density lipoprotein, bilirubin, alkaline phosphatase, gamma-glutamyl

Table 1. Baseline characteristics of NAFLD subjects before 6 months of physical training

Parameters	Low intensity exercise, *n*=19	Moderate intensity exercise, *n*=18	*p* value
Age (years)	53.3±3.7	37.3±8.2	<0.001
BMI (kg/m^2)	26.1±2.1	26.9±1.9	0.257
SBP (mm Hg)	156.6±18.9	132.2±13.2	<0.001
DBP (mm Hg)	90.7±10.1	87.4±8.3	0.339
FPG (mg/dL)	183.7±79.2	104±20.7	0.224
PGPG (mg/dL)	185.8±67.4	145.2±60.2	0.090
Triglycerides (mg/dL)	186.2±72.6	157.9±57.2	0.248
Cholesterol (mg/dL)	181.4±34.8	211.2±30.0	0.018
HDL (mg/dL)	42.1±8.9	46.0±4.8	0.159
LDL (mg/dL)	102.5±29.9	130.5±22.0	0.007
VLDL (mg/dL)	36.8±14.5	34.6±16.8	0.669
Bilirubin (mg/dL)	1.1±0.7	0.8±0.3	0.225
AST (U/L)	23.9±7.9	39.2±29.5	0.039
ALT (U/L)	26.3±13.1	61.8±30.2	<0.001
ALP (U/L)	189.6±37.4	202.7±51.3	0.409
GGT (U/L)	52.7±49.6	61.3±51.3	0.639
HOMA IR	3.75±5.27	2.56±2.55	0.458
Exercise duration (minutes)	57.21±4.22	56.57±6.87	0.728
Total calories burnt in exercise	153.58±29.10	261.81±37.18	**<0.001**
MET	2.11±0.27	3.61±0.42	**<0.001**

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; PGPG, post-glucose load plasma glucose (after 2-h glucose load); HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; HOMA-IR, homeostatic model assessment of insulin resistance; MET, metabolic equivalent.

transpeptidase (GGT), HOMA-IR, 24-h dietary intake, and total duration of physical activity. On the other hand, subjects with moderate intensity exercise had significantly higher total calories burnt in exercise (261.81±37.18 Kcalorie vs. 153.58 ±29.10 Kcalorie; $p<0.001$).

In the LIG, there was significant decrease in waist hip ratio and waist height ratio, whereas decrease in mean BMI was modest (0.2) and non-significant ($p=0.623$) (Table 2). Besides, there was a significant reduction in blood pressure, serum total, high-density lipoprotein, very low-density lipoprotein cholesterol, and serum GGT. However, changes in all other parameters, including serum transaminases (AST and ALT), were non-significant. Furthermore, only 5 out of 19 (26.3%) had resolution of fatty change in liver on ultrasonography.

Similarly, for the subjects who were subjected to moderate intensity exercise—although they showed modest improvement in BMI, waist hip ratio and waist height ratio, these changes were non-significant. However, there was significant reduction in blood pressure (Table 2). Further, there was significant improvement in lipid profile (except LDL cholesterol) and serum GGT. Unlike the LIG, subjects with moderate intensity exercise had elevated serum ALT at the baseline, and there were significant drops in serum levels of the transaminases (both AST and ALT) and decrease in HOMA-IR. Similarly, fasting glucose and triglycerides were normal/borderline normal at baseline in the MIG, which decreased significantly after intervention; whereas, in the LIG, these parameters were abnormal, with no significant change. Moreover, the fatty changes in liver disappeared in 12 out 18 (66.7%) subjects. The percentage of non-significant ($p>0.05$) decrease in weight was higher in those who achieved resolution of fatty change in liver (Table 3).

Univariate logistic regression analysis was performed to assess the risk factors for resolution of fatty change in liver after physical training (Table 4). Out of all demographic, anthropometric, biochemical parameters and exercise indices, serum triglycerides, very low-density lipoprotein, presence of metabolic syndrome and exercise intensity were found to be significant predictors for response (sonographic resolution of fatty change in liver) to physical exercise in NAFLD participants. A multivariate logistic regression analysis was performed including the above risk factors (Table 4) and only exercise intensity was found to be an independent factor for resolution of fatty changes in liver on ultrasound. The non-invasive scores of fibrosis [NAFLD fibrosis score, fibrosis-4 score, and AST to platelet ratio (commonly referred to as APRI)] as well as fatty liver index (FLI) were computed for all study subjects. Participants in the MIG showed significant improvement in APRI score ($p=0.005$) and FLI ($p<0.001$) (Table 5). However, in the subjects belonging to the LIG, no significant improvement was observed in any of the scores, except for reduction in FLI ($p=0.031$) after physical training (Table 5).

Table 2. Changes of parameters after 6 months of physical training in the low intensity exercise group and moderate intensity exercise group

	Low intensity exercise group (n=19)			Moderate intensity exercise group (n=18)		
Parameters	Before 6 months of physical training	After 6 months of physical training	p value	Before 6 months of physical training	After 6 months of physical training	p value
BMI (kg/m^2)	26.1±2.1	25.9±1.8	0.623	26.9±1.9	26.7±2.0	0.428
Waist hip ratio	1.01±0.35	0.93±0.22	< 0.001	0.97±0.04	0.96±0.04	0.262
Waist height ratio	0.59±0.04	0.57±0.4	< 0.001	0.56±0.04	0.55±0.04	0.168
SBP (mm Hg)	156.6±18.9	140.1±21.3	< 0.001	132.2±13.2	116.4±8.9	0.001
DBP (mm Hg)	90.7±10.1	85.8±11.1	0.018	87.4±8.3	77.8±8.6	0.005
FPG (mg/dL)	183.7±79.2	129.7±48.3	0.333	104±20.7	85.8±19.0	< 0.001
PGPG (mg/dL)	185.8±67.4	185.1±85.5	0.961	145.2±60.2	120.1±40.8	0.068
Triglycerides (mg/dL)	186.2±72.6	166.8±77.8	0.303	157.9±57.2	124.6±63.5	0.013
Cholesterol (mg/dL)	181.4±34.8	167.1±33.2	0.009	211.2±30.0	196.7±26.6	0.020
HDL (mg/dL)	42.1±8.9	37.8±6.7	0.001	46.0±4.8	41.5±6.8	0.006
LDL (mg/dL)	102.5±29.9	99.4±26.6	0.571	130.5±22.0	130.5±21.9	0.398
VLDL (mg/dL)	36.8±14.5	29.8±11.6	0.032	34.6±16.8	24.8±12.7	0.010
Bilirubin (mg/dL)	1.1±0.7	0.9±0.8	0.133	0.8±0.3	0.7 ±0.4	0.190
AST (U/L)	23.9±7.9	22.0±5.7	0.204	39.2±29.5	29.8±8.5	0.253
ALT (U/L)	26.3±13.1	22.6±6.6	0.205	61.8±30.2	29.1±9.8	0.002
ALP (U/L)	189.6±37.4	176.3±18.3	0.137	202.7±51.3	193.2±50.9	0.440
GGT (U/L)	52.7±49.6	36.7±25.7	0.033	61.3±51.3	37.4±24.1	0.024
HOMA-IR	3.75±5.27	2.71±2.60	0.466	2.56±2.55	1.69±2.59	0.005

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; PGPG, post-glucose load plasma glucose (after 2-h glucose load); HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; HOMA-IR, homeostatic model assessment of insulin resistance.

Table 3. Comparison of percentage of change in weight in the study groups

	Subjects with regression of fatty liver	Subjects without regression of fatty liver	*p* value
All subjects	-2.28%	-1.03%	0.305
Low intensity exercise group	-0.20%	-0.11%	0.946
Moderate intensity exercise group	-3.19%	-2.97%	0.916

Table 4. Logistic (univariate and multivariate) regression analysis of factors predicting resolution of fatty changes in liver in NAFLD subjects (*n*=37)

Factors	OR	95% CI	*p* value	AOR	95% CI	*p* value
Age (years)	1.02	0.93-1.12	0.673	1.07	0.92-1.26	0.383
BMI (kg/m^2)	1.02	0.73-1.43	0.908			
Waist circumference	0.94	0.84-1.06	0.302			
Waist hip ratio	0.002	0.0001-1546	0.443			
Waist height ratio	0.54	0.0001-73566	0.942			
SBP (mm Hg)	0.98	0.95-1.01	0.262			
DBP (mm Hg)	1.01	0.93-1.07	0.985			
FPG (mg/dL)	0.98	0.96-1.01	0.057			
PGPG (mg/dL)	0.99	0.98-1.00	0.106			
Triglycerides (mg/dL)	0.98	0.96-0.99	**0.009**	0.58	0.26-1.31	0.189
Cholesterol (mg/dL)	1.01	0.98-1.03	0.631			
HDL (mg/dL)	1.04	0.95-1.14	0.362			
LDL (mg/dL)	1.02	0.99-1.04	0.158			
VLDL (mg/dL)	0.90	0.83-0.98	**0.012**	13.84	0.24-80.79	0.205
Bilirubin (mg/dL)	0.26	0.02-3.51	0.311			
AST (U/L)	1.03	0.98-1.08	0.324			
ALT (U/L)	1.01	0.98-1.04	0.340			
ALP (U/L)	1.01	0.99-1.02	0.457			
GGT (U/L)	1.01	0.99-1.02	0.490			
Fasting insulin	0.87	0.69-1.07	0.191			
HOMA-IR	0.52	0.24-1.11	0.089			
Metabolic Syndrome	4.29	1.06-17.36	**0.041**	0.910	0.13-6.55	0.926
Total duration of exercise	1.01	0.89-1.29	0.978			
Total calories burnt during exercise	1.01	0.98-1.02	0.122			
Exercise intensity (low vs. moderate)	0.18	0.04-0.74	**0.017**	17.18	1.16-253.61	**0.038**
MET	2.35	1.01-5.47	**0.043**			

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; PGPG, post-glucose load plasma glucose (after 2-h glucose load); HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; HOMA-IR, homeostatic model assessment of insulin resistance; MET, metabolic equivalent; OR, odds ratio; AOR, adjusted odds ratio; 95% CI: 95% confidence interval.

Discussion

The MIG had an elevated ALT and borderline normal fasting glucose and serum triglycerides at baseline, with significant improvement in these parameters at the end of the 6-month period. However, the LIG had normal ALT and abnormal fasting glucose and serum triglycerides at baseline but no significant change after intervention. The basic principle underlying use of physical exercise in treatment of NAFLD is improvement in insulin sensitivity. Moderate to vigorous physical activity leads to reduction in total body fat, especially in visceral adipose tissue.[15] Decreased visceral adiposity leads to reduced fatty acid delivery to the liver, thereby decreasing hepatic steatosis and resultant necroinflammation.

In our study, moderate intensity aerobic exercise resulted in significant resolution of fatty change in liver and improvement in serum aminotransferases (AST and ALT). The role of physical activity in NAFLD has been investigated in various studies. Suzuki *et al.*[16] demonstrated that regular exercise was associated with ALT reduction. Further, for every 5% in

Table 5. Non-invasive fibrosis scores of NAFLD and FLI before and after exercise

Score	Moderate intensity group			Low intensity group		
	Before exercise	After exercise	*p* value	Before exercise	After exercise	*p* value
NFS	-1.35	-1.59	0.278	0.66	0.75	0.621
FIB-4	0.84	0.81	0.722	0.88	0.86	0.772
APRI	0.41	0.27	0.005	0.43	0.40	0.285
FLI	62.84	50.55	<0.001	66.08	60.43	0.031

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NFS, NAFLD fibrosis score; FIB-4, fibrosis-4 score; APRI, aspartate transaminase to platelet ratio index; FLI, fatty liver index.

weight loss, a 3.6 greater likelihood of ALT normalization was observed in their study.[16] Studies employing magnetic resonance spectroscopy (known as 1H-MRS) also confirm these reports.[17,18] In our study, although the degree of weight loss in both the groups was not significant, there was a significant fall in serum transaminases in the MIG. However, in the LIG, the reduction in transaminases was not significant.

The effect of exercise with or without hypocaloric diet on NAFLD patients has been studied in various clinical trials.[8,19–27] Most of these studies had smaller number of subjects, absence of measurement of volume (calorie) and intensity of exercise, and some lacked histological endpoints. A comparison of the results of eight studies, including the present study, is shown in Supplementary Table 1. All trials showed significant improvement in BMI, serum enzymes (AST/ALT), and degree of fatty liver. There are several studies that have demonstrated a dose-dependent improvement in liver histology[27] and intrahepatic triglycerides[26] dependent on the degree of weight loss achieved. However, the improvement in transaminases, HOMA-IR, FLI and resolution of ultrasonographic fatty change in liver was independent of the degree of weight loss in our study. Further studies are required, with longer duration and with more intense physical activities, to demonstrate changes in fibrosis score.

Recently, the intensity rather than duration of exercise and total calorie expenditure during physical activity has caught the attention of researchers. Kistler *et al.*[23] in a retrospective study examined the effect of exercise intensity on histological severity of NAFLD. In this study, the exercise volume and intensity was calculated by self-reported physical activity data from adult patients with biopsy-proven NAFLD enrolled in the Nonalcoholic Steatohepatitis Clinical Research Network, and the NAFLD patients were classified into moderate and vigorous exercise groups as per the federal recommendations. The study demonstrated an inverse relationship between the intensity of physical activity and severity of NAFLD. However, this study was limited by its cross-sectional nature, measurement limitations, and misclassification due to reporting and recall bias. On the contrary, in our study, we measured the exercise intensity prospectively with objective methods (by accelerometers).

Our study had several limitations. The two study groups were not entirely comparable as there were significant differences in age, baseline levels of serum AST and ALT, serum total cholesterol, and serum LDL cholesterol. Besides, the sample size was small and only male NAFLD subjects were included. Furthermore, the diagnosis of NAFLD was based on transabdominal ultrasound, which can miss fatty liver when the degree of steatosis is less than 30%.[28]

Conclusions

Moderate intensity of physical activities for a duration of 6 months helps in improvement in hepatic steatosis, serum transaminitis, glycemic and lipid profiles as well as IR, as compared with low intensity exercise. All individuals with NAFLD should perform moderately intense physical activities for maximum benefits. More rigorous, controlled studies, of longer duration and with defined histopathological end-points are the need of the hour for better evidence-based lifestyle modification guidelines.

Funding

This study was sponsored by the Kalinga Gastroenterology Foundation, Cuttack, Odisha (India).

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (PN, SPS), acquisition of data (PN, MKS, JN, RKS, AAP, SJ, AKP, AJ), analysis and interpretation of data (PN, MKP, SPS), drafting of the manuscript (PN, MKP), critical revision of the manuscript for important intellectual content (PN, MKP, JN, SPS), study supervision (SPS).

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Original Article

Approaches to Assessing Burden in Caregivers of Patients with Cirrhosis

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Abstract

Background and Aims: Being a caregiver for a patient with chronic liver disease (CLD) can be burdensome mentally, emotionally financially, and physically. The aim of this study was to systemically review the available tools and propose tools that can comprehensively evaluate caregiver burden for individuals caring for patients with CLD. ***Methods:*** We searched the PubMed database for all studies on the impact of patients with CLD on caregiver burden without timeframe restriction. Eligible studies included cohort studies, review studies, or cross-sectional studies. The number of patients and caregivers was isolated from each paper. Studies in the same categories were isolated and statistically compared. ***Results:*** A total of 13 studies meeting our inclusion criteria as stated in the methods sections were included. In total, 2528 caregivers were taking care of 2003 patients with CLD. Women made up the majority of caregivers at 78.2%, 95.7% of whom identified as the patient's spouse. Caregiver strain index is one of the most comprehensive tools; however, the questions are very general and do not fully elucidate financial strain. Beck depression and anxiety were correlated (p=0.0001), and both depression and anxiety were correlated with perceived caregiver burden (PCB) and Zarit Burden Interview (ZBI) (p=0.002). Depression scale correlated with Interpersonal Support Evaluation – Short Form, and Model for End-Stage Liver Disease score correlated with ZBI and PCB (total and in most domains; p=0.001). Patient's poorer cognitive performance correlated with higher ZBI and PCB (employed patients had higher cognitive performance and lower ZBI and PCB). ***Conclusions:*** Caregiver burden remains poorly understood due to the lack of uniformity in the assessment tools used to evaluate caregiver burden. None of the tools used to evaluate caregiver burden are comprehensive; however, most tools correlate statistically in the ability to identify caregiver burden. A comprehensive tool is lacking for identifying caregiver burden in patients with CLD.

Citation of this article: Yanny B, Pham NV, Saleh H, Saab S. Approaches to assessing burden in caregivers of patients with cirrhosis. J Clin Transl Hepatol 2020;8(2):127–134. doi: 10.14218/JCTH.2019.00054.

Introduction

Liver disease causes approximately 2 million deaths per year worldwide.[1,2] In the USA, liver disease is the twelfth most common cause of mortality.[1,2] Approximately 34,000 deaths are reported annually from liver cirrhosis.[3] Liver transplant (LT) has dramatically improved survival and quality of life (QOL) for patients with complicated liver disease.[1,2] Transplant is a life-altering change for the patient and their families. The process of LT evaluation is long and stressful. Patients' families provide care and support for their loved ones mentally, emotionally, medically, and financially. Research shows that family support is essential for a good LT outcome.[4]

The LT waitlist contains approximately 16,000–17,000 patients awaiting transplantation.[5] All patients on this waitlist undergo a social evaluation and should have family members who are willing to be fulltime caregivers during the transplant process and after LT, as required by most transplant centers in the USA. Caregivers play an important role in a transplant patient's health care during the whole process and particularly recovery. Having a responsible caregiver can strengthen the information relayed to the patients and effectively help them with treatment, ultimately improving LT outcomes and compliance.

Accordingly, caregivers for LT recipients play a critical role in the pre- and post-LT stages. Previous studies have shown that a poor caregiver QOL predicts that a LT recipient will receive low quality care by their caregiver.[1,2,6] Furthermore, caregivers with a heavy financial burden have a poorer QOL, which leads to less optimal care delivery for the LT recipient.[1,2] The focus during the LT evaluation process is often shifted to the individual receiving the transplant. The caregiver assessment often stops after ensuring that a dedicated caregiver and possibly a secondary caregiver will be present to help the LT recipient. Most transplant centers do not assess caregivers' QOL or other burdens such as mental, physical, psychological and financial despite their proven role in providing a good post-LT outcome to their loved ones who have received a LT.[1] Therefore, this study focuses on caregiver

Keywords: Caregiver burden in liver disease; Liver cirrhosis; Liver transplant.
Abbreviations: AUDIT, alcohol use disorders identification test; BDI-II, Beck depression inventory-II; CLD, chronic liver disease; HADS-A, hospital anxiety and depression scale-anxiety; HADS-D, hospital anxiety and depression scale-depression; HCC, hepatocellular carcinoma; HRQOL, health-related quality of life; ISEL-SF, interpersonal support evaluation – short form; LT, liver transplant; MCS, mental component summary; MCSDS, Marlowe Crowne social desirability scale; MELD, model for end-stage liver disease; NAFL, non-alcoholic fatty liver disease; PBC, primary biliary cirrhosis; PCB, perceived caregiver burden; PCRS, Picot caregiver reward scale; PCS, physical component summary; QOL, quality of life; SD, standard deviation; STAI, state trait anxiety information; ZBI, Zarit burden interview; ZBI-SF, Zarit burden interview – short form.
Received: 9 November 2019; Revised: 12 February 2020; Accepted: 15 March 2020
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burden, and tools available to the clinician and transplant center to evaluate the burden.

In this systemic review, we evaluated caregiver burden assessment tools available to the clinician by reviewing the available literature specifically for individuals with chronic liver disease (CLD), and compared the tools utilized. We identified the most appropriate tools in each category including mental, emotional, financial, and physical, and proposed a combination of tools that can comprehensively evaluate caregiver burden for individuals caring for patients with CLD.

Methods

Search strategy and identification of studies

We searched the MEDLINE database for all studies on the impact of patients with CLD on caregiver burden without timeframe restriction. We used a combination of keywords 'caregiver,' 'care giving,' 'informal care,' 'caretaker,' 'family,' 'spouse,' 'parents,' 'friends,' 'mother,' 'father,' 'liver cirrhosis,' 'liver,' 'liver disease,' 'cirrhosis,' 'chronic liver disease,' and 'PBC.' Bibliographies of all identified studies were searched for relevant articles for additional studies. We also searched additional electronic databases such as ProQuest.

Inclusion and exclusion criteria

We included all studies published in scientific journals that investigated burden experienced by individuals who are caregivers for patients with CLD or cirrhosis, or who are on the LT waitlist. As our study attempted to assess all available information on caregiver's burden as a result of caring for adult patient with liver disease, studies whose source populations were above 18 years of age and resided in or outside the USA, and studies published in English were included. We included studies that used a quantitative method of analysis to describe the burden of caregiving, mental health outcomes of caregivers, and their QOL. Studies that only used qualitative interview-based instruments were excluded from our review. The inclusion criteria were: all studies published in scientific journals that investigated burden experienced by caregivers of patients with CLD, cirrhosis, or who are on the LT waitlist; peer-reviewed articles in English and a full-text version of the study available; source populations residing in or outside the USA; source populations only including adult patients and caregivers (18 years old or above); patients diagnosed with CLD, cirrhosis of any etiologies, or who are on the LT waitlist; and studies that used a quantitative method of analysis to describe burden of caregiving or mental health (stress, distress, depression, anxiety) of caregivers or QOL or a combination of these outcomes. We excluded the following: experimental trial study design, systemic review, dissertations/theses, published abstracts, studies published in language other than English leading to unavailable full-text articles, patient population or caregiver population <18 years of age, patient population with primary diagnosis other than CLD, cirrhosis, or not on the LT waitlist such as hepatocellular carcinoma, non-alcoholic fatty liver disease, post-LT studies, studies that investigated caregivers impacted by their own liver disease, and studies that only utilized a qualitative interview-based instrument to assess caregiver's burden (qualitative methodology).

Caregiver's burden definition

The definition of caregiver's burden has been a topic of ongoing discussion. Caregiver's burden is defined as the impact of caregiving on caregiver's perceived emotion, physical health, social life, and finance over time.[7,8] There have been attempts to distinguish caregiver's burden into subjective and objective burdens.[9,10] Subjective burden refers to caregivers' reflection on their caregiving experience through their attitude, emotion, awareness, perception, and affective orientation.[9] Objective burden reflects the disruptions of caregivers' physical health, household, financial status, and other aspects of life that are the results of caregiving.

Caregiver assessment tools

Zarit burden interview – Short form (ZBI-SF): This 12-item self-reported instrument assesses caregiver's burden with a total score of 48. It is a shortened version of the 22-item Zarit Burden Scale. Higher score indicates higher caregiver burden. This tool focuses on time schedule, physical health, mental and psychosocial burden.[1,11,12]

Zarit burden scale: This 22-item self-reported instrument assesses caregiver's burden with each item on a 9-point rating scale. A rating of 9 for each item indicates a higher level of burden. The scale measures physical, emotional, and financial toll of providing care. This also focuses on time schedule, physical health, mental and psychosocial burden.[13]

Health-related QOL: This 136-item questionnaire used to assess patient's physical, psychosocial, and general health outcomes. It has two overall domains: physical and psychosocial; 12 subcategories: sleep and rest, eating, work, home management, recreation and pastimes, ambulation, mobility, body care and movement, social interaction, alertness behavior, emotional behavior, communication. Higher score indicates a poorer level of health.[11,14]

Perceived caregiver burden scale (PCB): This 31 item self-reported questionnaire assesses perceived caregiver's burden with five domains: impact on finances, impact on schedule, sense of abandonment, impact on health, sense of entrapment. A higher score indicates a higher level of perceived burden. Although brief, this tool asks questions regarding physical health, mental health, social situation, finance, and sleep.[15]

Caregiver strain index: This 13-item questionnaire to assess caregiver burden. Higher scores mean more strain. If caregiver answers "yes" to seven or more items, clinically significant caregiver strain is indicated. This briefly asks about time schedule, physical health, mental health, finance, and sleep. We suggest this tool be used as a pathway to determine the reason for the patient's caregiver's most serious concern, and based on the concern, another in depth tool may be utilized to further evaluate the burden.[16,17]

Caregiver benefit index: This index examines benefits perceived by transplant caregivers in 12 areas. Higher scores mean more benefits. Questions focus on benefit gained from helping patient, spending time with patients, personal growth, and interpersonal benefits.[16,18]

Results

A total of 13 studies meeting our inclusion criteria as stated in the Methods section were included (Fig. 1).[7–10,12–13,16,19–24]

A total of 2528 caregivers were taking care of 2003 patients with CLD. Women made up the majority of caregivers at 78.2%, of whom 95.7% identified as the patient's spouse. Overall caregiver burden fell on 73.5% of spouses, parents made up 12.7% of the caregiver cohort, 3.2% were children, and 10.5% fell into the 'other' relationship category. The cumulative mean (± standard deviation [SD]) age of the caregiver was 52.7 (± 7.2) years. Unemployed caregivers made up 65.2% of the caregiver cohort. Viral hepatitis contributed to 54.3% of patients being cared for by a caregiver, alcoholic liver disease made up 23%, non-alcoholic steatohepatitis was 12.7% and 10% contributed to other liver diseases.

Patients on the LT list made up 20.1% of the entire cohort included in this systemic review.[7–10,12–13,16,19–24] None of the tools were incorporated during the LT evaluation to aid in determining outcomes. Caregiver burden was highest in those taking care of patients on the LT list with a mean (± SD) Zarit burden assessment score of 14.8 (± 1.01). PCB and ZBI were correlated (p=0.0001).[7–10,12–13,16,19–24] Beck depression and anxiety were correlated (p=0.0001), depression and anxiety both correlated with PCB and ZBI (p=0.004), the depression scale correlated with the Interpersonal Support Evaluation – Short Form, and Model for End-Stage Liver Disease (MELD) score correlated with ZBI and PCB (total and in most domains; p=0.002). Patient's poorer cognitive performance correlated with higher ZBI and PCB (employed patients had a higher cognitive performance and lower ZBI and PCB).

The largest study evaluated seven caregiver assessment tools,[11] and the smallest two tools.[19] The number of unique tools identified and used in the different studies was 27. Select studies that validated and utilized the instruments are shown Table.[25–48] The mean (± SD) number of assessment tools used was 3.42 (±1.7). The most commonly used caregiver burden assessment tool used in the studies was the Zarit Burden score assessment, which was used in 77% of the studies looking into caregiver burden.[1,2,4,12,13] BDI-II: 21-item validated questionnaire assessing depression was used

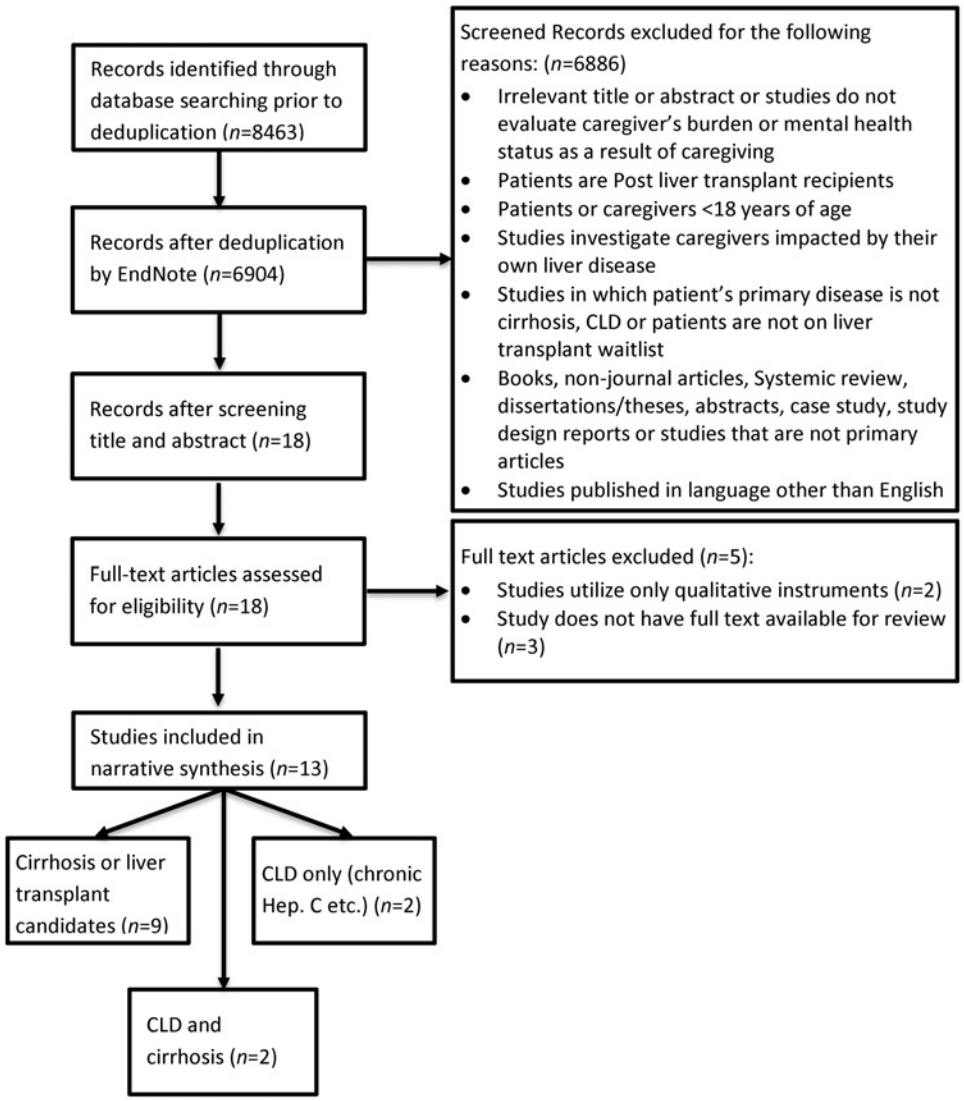

Fig. 1. Methods utilized to isolate the included articles.

Table 1. Tools studied in the evaluation of caregiver burden in patients with chronic liver disease

Instrument (validation studies)	Description (selected studies utilized the instrument)
Perceived caregiver burden scale[15]	31-item self-reported questionnaire assesses perceived caregiver's burden with 5 domains: impact on finances, impact on schedule, sense of abandonment, impact on health, sense of entrapment. A higher score indicates a higher level of perceived burden.[1,11]
Zarit burden interview – short form[12]	12-item self-reported instrument assesses caregiver's burden with total score of 48. It is a shortened version of the 22-item Zarit Burden Scale. Higher score indicates higher burden.[1,11]
Zarit Burden Scale[13]	22-item self-reported instrument assesses caregiver's burden with each item on a 9-point rating scale. A rating of 9 for each item indicates higher level of burden. The scale measures physical, emotional, and financial toll of providing care.[4,12]
Beck depression inventory (BDI-II)[23]	21-item validated instrument assesses depression (including attitude, depressive symptoms, and suicidal ideation). Each item is rated on a scale of 0 to 3. The cutoff scores are: <11, minimal depression; 12 to 19, mild to moderate depression; 20 to 35, moderate depression; and 36 to 63, severe depression.[1,11,14,16]
Beck Anxiety Inventory[24]	21-item validated instrument assesses anxiety. Score 0 to 21 indicates mild to very low anxiety; score 22 to 35 indicates moderate anxiety; score above 36 indicates severe anxiety.[1,11]
Interpersonal support evaluation list – short form inventory[28]	16-question validated questionnaire assesses level of social support perceived by caregiver. Each question has 2 answer options, "probably false" or "probably true." Higher score is worse.[1]
Pittsburgh sleep quality index[29]	24-item questionnaire assesses quality of sleep and sleep disturbances over 1 month. 19 questions are self-reported and 5 are rated by bed partner or roommate. There are 7 component scores: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, daytime dysfunction and a global score. Higher score indicates worse quality of sleep or higher sleep disturbance.[11]
Epworth sleepiness scale[30]	8-item questionnaires on a 4-point scale with score ranging from 0 to 24 assessing daytime sleepiness. The higher the score means the higher the person's daytime sleepiness.[11]
HRQOL: sickness impact profile[14]	136-item questionnaire used to assess patient's physical, psychosocial, and general health outcomes. It has 2 overall domains: physical and psychosocial; 12 subcategories: sleep and rest, eating, work, home management, recreation and pastimes, ambulation, mobility, body care and movement, social interaction, alertness behavior, emotional behavior, communication. Higher score indicates a poorer level of health.[11]
Medical outcomes Study SF-36[31]	36 questions assessing 8 domains of health including physical functioning, bodily pain, role limitations due to physical condition, role limitations due to emotional health, social functioning, energy/fatigue, emotional well-being, and general health perceptions. Mean score is compared to national norms.[4]
Center for epidemiological studies depression scale[32]	20-item scale used to measure extents of depressive symptoms experienced by caregivers. Score of 0 to 15 indicates no depressive symptoms; 16 to 20 indicates mild distress; 21 to 30 indicates moderate distress; 31 and higher indicates severe distress.[12,15]
Hamilton anxiety rating scale[33]	Self-report instrument with 14 items, each on a 5-point scale from 0 to 4, assess level of anxiety. A score of 18 indicates mild anxiety, a score of 25 indicates moderate anxiety, and a score of 30 is severe anxiety.[12]
Alcohol use disorders identification test[34]	10-item screening tool assesses alcohol intake, use frequency, dependency, and problems caused by drinking. The AUDIT distinguishes between at-risk users and alcohol-dependent users.[12]
Picot caregiver reward scale[35]	25-item self-report scale assesses caregiver's perceived rewards. The PCRS measures pleasures, satisfactions, good feelings, and positive consequences connected to caregiving responsibilities. Scores range from 0 to 64, with higher scores indicating greater perceived reward.[12]

(*continued*)

Table 1. (*continued*)

Instrument (validation studies)	Description (selected studies utilized the instrument)
Hospital anxiety and depression scale[36]	14-item measure assesses anxiety and depression. A 4-point severity scale is used for each item. The HADS has two subscales, anxiety (HADS–A) and depression (HADS–D). Scores higher than or equal to 11 on either scale indicate a definitive anxiety and/or depression.[13,17,18]
Caregiver burden scale (Brazilian version)[37]	22 questions used to assess caregiver's burden with 5 subscales: general strain, isolation, disappointment, emotional entanglement, environment. Higher scores indicate higher burden.
Inventario de sintomas de stress para adultos de Lipp[38]	Instrument used to assess stress based on a 4-phase model and the effects of stress in the somatic and cognitive domains. First phase is the alert phase; second phase is the resistance phase; third phase is almost-exhaustion phase and fourth phase is exhaustion phase.
Spielberger state trait anxiety inventory-state form[39]	20-item self-report measure assesses state-related anxiety. Participants rate descriptive statements on their emotion with a 4-point scale (not at all to very much). Scores range from 20-80. Higher score indicates elevated anxiety. Normative data are used to categorize clinically elevated anxiety (STAI >48).
Medical coping modes questionnaires[40]	20-item self-report questionnaire assesses coping mechanism of caregivers among 4 categories: resignation, avoidance, social support seeking, information seeking. A 5-point scale is used to rate each item. Higher scores indicate higher use of each coping mechanism.
Scale for caregiver burden[44]	20-item self-report questionnaire measures objective and subjective burden. 10 items measure objective burden which reflects the amount of practical caregiving based on severity of patient's condition and functional needs. 10 items measure subjective burden which reflects caregiver's perceived distress due to caregiving tasks and quantity of caregiving activities. Scores range from 0 to 40 for each subscale with higher scores indicating more burden.
Marlowe Crowne social desirability scale-short form[45]	13 scored items separated into 2 sets measure an individual's level of socially acceptable and/or unrealistic responses. The scale assesses the degree to which participants providing responses that are favored by others such as over reporting positive attributes or underreporting negative attributes. Score range from 0 to 13 with higher scores indicating higher level of socially desirable responding. Normative data is used to classify individuals who respond in often unrealistic, socially desirable way (MCSDS>7).
SD-36v2 health survey[46,47]	Measures Quality of Life (QOL) by assessing perceptions of health in eight domains: physical functioning, role functioning-physical, role functioning-emotional, vitality, pain, general health, social functioning, and mental health. Scores range from 0-100. Higher scores reflect higher QOL. SD-36 has 2 component scores – physical component summary (PCS) and mental component summary (MCS).[19]
Quality of life inventory[48]	32 statements on 16 life domains which reflect life satisfaction. 16 Life domains include health, self-esteem, goals-and-values, money, work, play, learning, creativity, helping, love relationship, friendships, relationship with children, with relatives, home, neighborhood, community. Higher scores indicate higher life satisfaction.[19]
Profile of mood states-short form[49]	Assesses mood disturbance. Caregivers read descriptive adjective and rate how they feel about them on a 5-point scale. Form provides total score and factor scores: tension-anxiety, depression-dejection, anger-hostility, vigor-activity, fatigue-inertia, confusion-bewilderment. Higher score means more mood disturbance.[19]
Caregiver strain index [50]	13-item questionnaire to assess caregiver burden. Higher scores mean more strain. If caregiver answers "yes" to 7 or more items, clinically significant caregiver strain is indicated.[19]
Caregiver benefit index[30,51]	Examines benefits perceived by transplant caregivers in 12 areas. Higher scores mean more benefits. Questions focus on benefit gained from helping patient, spending time with patients, personal growth, interpersonal benefits[19]
Miller social intimacy scale[52]	Assesses caregiver's perceived closeness to their spouse. It provides two intimacy subscales: Frequency and Intensity and culminated in a total intimacy score. Higher score indicates greater intimacy.[19]

Table 2. Instruments used to assess rewards and benefits perceived by caregivers of patients with chronic liver disease or cirrhosis.

Instrument (validation study)	Number of items	Subscale/Domain	Score report	Number of pre-transplant caregiver administered from selected studies
Caregiver benefit index[30]	12	4 benefit categories: helping the patient, time with patient, self/personal growth, relationship with others Assess: benefits from caring, spending time with patient, personal growth, interpersonal benefits	Composite score is reported 0 to 1 scale (yes or no response) used for each item	49 pre-transplant patients' caregivers
Picot caregiver reward scale[38]	25	2 subscales: external and Internal Reward Assess: perceived pleasures, satisfactions, good feelings, positive consequences	Composite score is reported 5- point scale ("Not at all" to "A Great Deal") used for each item	73 caregivers of patients with cirrhosis

in 23% of the studies; Beck Anxiety Inventory: 21-item validated questionnaire assessing anxiety was used in 23% of the studies; Pittsburgh Sleep Quality Index: self-rated questionnaire assessing sleep quality and disturbances over a 1-month interval was used in 15.3% of the studies; and Epworth Sleepiness Scale: 8-item questionnaire on a 4-point scale ranging from 0 to 24 assessing daytime sleepiness was used in 15.3% of the studies.[2,19–21,23,24,26,29,30] A higher score indicating a poorer level of health was used in 77% of the studies.[20,21,25] Perceived Caregiver Burden: 31-item questionnaire was used in 77% of the studies.[15,16,22,25,27]

Overall, only 7.6% of the studies included a comprehensive assessment that included mental, emotional, physical, financial, and psychological.[1,2,4,16,18–22,25,27,49–52] The study included a 20-patient caregiver dyad and used a total of seven assessment tools in order to be inclusive. Financial burden was considered in 38% of the studies and psychological in 77% of the studies, which included anxiety, depression, sleep heath, and feeling of entrapment.[2,16,19–22,25–27,49] QOL was assessed in 53.8% of the studies. Caregiver burden assessed with the Zarit Burden tool with a score of 15 (± 0.8) posed the poorest outcomes for patients with CLD.[1,2,4,11,19,20,21,26]

A number of other instruments were used in assessing caregiver burden in patients with CLD (Table 1), which measured different aspects of burden in caregivers. All studies are listed in Tables 1 and 2 with references. The studies evaluated caregiver burden both in the inpatient and outpatient settings. Inpatient questionnaires were answered in 38% of the studies, and 62% evaluated the caregivers in the outpatient setting. The assessment tools were administered to patient's primary and secondary caregivers who care for patients with CLD.

Discussion

Caregivers have a critical role in the outcome and disease progression of patients with CLD. Multiple studies have shown a better outcome in patients with a responsible caregiver.[1,2] The results of our systemic review revealed that there is no comprehensive way to evaluate caregiver burden via one tool. A comprehensive evaluation of caregiver burden is possible by combining multiple tools; however, this can be cumbersome as many tools have similar questions that can be redundant for the patient and family (Table 3). Although difficult, it is doable. Higher MELD score correlated with ZBI and PCB (total and in most domains). Patient's poorer cognitive performance correlated with higher ZBI and PCB (employed patients had higher cognitive performance and lower ZBI and PCB), and not a single tool was able to evaluate mental, physical, social, and financial burden all together.[2] Our study also noted that caregivers of those who are on the transplant list have the highest caregiver burden based on ZBI and PCB score, and higher caregiver burden correlates with poor patient outcomes. Patients with a caregiver who had a ZBI score of 15.7 or higher had the poorest outcomes with increased number of hospitalizations and higher number of missed appointments, which ultimately leads to increased morbidity and mortality. Financial burden seems to have the most impact on outcomes.

LT centers do not use a caregiver burden tool and there are no cutoff values to move forward with transplant evaluation.

Table 3. Components of caregiver burden assessed by different burden instrument utilized

	PCB	ZBI-22	ZBI-SF	Scale for caregiver burden	Caregiver strain index
Time/schedule	X	X	X		X
Physical health	X	X	X	X	X
Mental/psychosocial health	X	X	X	X	X
Finance	X	X		X	X
Sleep					X

Components of caregiver burden are selected based on established domains for each burden instrument or based on the items or questions asked.
Abbreviations: PCB, perceived caregiver burden; ZBI-22, 22-item Zarit burden interview; ZBI-SF, Zarit burden interview–short form.

The social evaluation prior to LT is a subjective evaluation in most cases. An objective measure is needed to evaluate the caregiver situation prior to moving forward with transplantation. Perhaps transplant centers should utilize caregiver burden tools more often as a standard practice in LT evaluation. However, those would be limited, as based on previous studies, in order to have a comprehensive assessment, one must use a total of seven assessment tools. This would most likely be cumbersome for the patient and family. Our study showed that the most commonly used tool for evaluating caregiver burden in patients with CLD is the Zarit caregiver burden tool.

Another suggestion is to use a general tool to evaluate what the patients' caregivers preserve as the highest burden then follow-up with a more in detail tool that correlates to the caregivers concern. A tool that is general and brief is the Caregiver Strain Index, which is a brief 13-question survey that evaluates time schedule, physical health, metal, social, finance, and sleep. Based on the answers, Table 1 may be used to hone in on a more specific tool that matches the caregivers' needs for evaluation. Table 3 includes components of caregiver burden assessed by the different burden instrument utilized.

This study is the only systemic review available with the greatest number of patients. It is limited as it is review, and there was no uniformity in the tools used by the research papers. This does not undermine the strength of the study due to number of patients included. In the future, work should be focused on developing a comprehensive tool to assess social, financial, physical, psychological burden with one tool, and incorporating the caregiver burden assessment tool into the LT evaluation process as an objective measure. The limitations of the study included the absence of an index assessment to use for comparison, which caused heterogeneity in the statistical analysis; this was balanced by the number of patients included. The abovementioned limitation does not undermine the strength of the study, which included the large number of studies and patients studied. Future research should focus on a more comprehensive tool to assess caregiver burden.

Conclusions

Caregiver burden remains poorly understood due to the lack of uniformity in the assessment tools used to evaluate caregiver burden. None of the tools used to evaluate caregiver burden are comprehensive; however, most tools correlate statistically with the ability to identify caregiver burden. A comprehensive tool is lacking for identifying caregiver burden in patients with CLD. Due to the correlation between caregiver burden and outcomes in patients with CLD, one should highly consider using a standard caregiver assessment tool in the LT evaluation process.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (SS, BY), acquisition of data (NP, HS), analysis and interpretation of data (SS, BY, NP), drafting of the manuscript (BY), critical revision of the manuscript for important intellectual content (SS, BY), statistical analysis (BY, PN), obtained funding (N/A), administrative, technical, or material support, study supervision (SS).

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Original Article

Incidence, Mortality and Predictors of Acute Kidney Injury in Patients with Cirrhosis: A Systematic Review and Meta-analysis

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Abstract

Background and Aims: Acute kidney injury (AKI) is common in patients with cirrhosis but the incidence is heterogeneous among studies. We performed a meta-analysis to describe the incidence of AKI and its impact on patient mortality in patients with cirrhosis. We also evaluated the admission variables predicting development of AKI. ***Methods:*** A systematic search of various databases was performed up to November 2018. Meta-analyses were performed using random effects models. ***Results:*** Of 18,474 patients with cirrhosis from 30 selected studies, 5,648 developed AKI, with a pooled incidence of 29% (95% confidence interval [CI]: 28-30%, I^2 of 99%). In-hospital mortality assessed in eight studies was six-fold higher among AKI patients, as compared to those without AKI (odds ratio [OR] 6.72, 95% CI: 3.47-13, $p<0.0001$, I^2 of 70%). Three studies on patients admitted to intensive care showed about six-fold higher mortality among AKI patients (OR 5.90, 95% CI: 3.21-10.85, $p>0.0001$). Mortality remained significantly high, at days 30 and 90 and even at 1-year follow up after development of AKI. Of 12 admission variables analyzed, model for end-stage liver disease score, Child-Pugh-Turcotte stage C, presence of ascites, and presence of sepsis/septic shock were statistically significant risk factors for AKI. ***Conclusions:*** AKI occurred in about 29% of patients with cirrhosis and is associated with a six-fold increased risk of in-hospital mortality. Mortality remained high even in long-term follow-up of 1 year. Patients at risk for AKI development can be recognized at admission. Prospective studies are needed to develop strategies for improving outcome of these patients.

Citation of this article: Tariq R, Hadi Y, Chahal K, Reddy S, Salameh H, Singal AK. Incidence, mortality and predictors of acute kidney injury in patients with cirrhosis: A systematic review and meta-analysis. J Clin Transl Hepatol 2020; 8(2):135–142. doi: 10.14218/JCTH.2019.00060.

Keywords: Acute kidney injury; Cirrhosis; Mortality; Outcomes.

Abbreviations: AKI, acute kidney injury; AKIN, AKI Acute Kidney Injury Network; CI, confidence interval; KDIGO, Kidney Disease Improving Global Outcomes; LFK, Luis Furuya-Kanamori; OR, odds ratio; RIFLE, risk injury and failure.

Received: 18 December 2019; Revised: 4 February 2020; Accepted: 25 February 2020

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Introduction

Acute kidney injury (AKI) is a common event in the natural history of patients with cirrhosis, with an incidence rate varying from 14% to 50%.[1–3] Furthermore, the diagnosis of AKI in patients with cirrhosis is confounded by fluid overload,[4] the effect of bilirubin on the creatinine assays, and reduced muscle mass in patients with cirrhosis.[5] Splanchnic pooling from portal hypertension in cirrhosis results in decreased effective circulating blood volume and renal blood flow, putting patients at risk for AKI and hepato-renal syndrome.[6]

The definition of AKI has changed over the last two decades, recognizing that an elevation in serum creatinine of ≥0.3 mg/dL from baseline negatively impacts survival. Many definitions have been introduced to define and stage AKI, such as the Risk Injury and Failure (commonly referred to as RIFLE),[7] AKI Network (commonly referred to as AKIN) criteria,[8] and Kidney Disease Improving Global Outcomes (commonly referred to as KDIGO).[9] Variations in the definitions of AKI are one of the most important factors resulting in heterogeneity in the reported incidence of AKI among patients with cirrhosis. That being said, the essence of all the definitions of AKI seem to be similar. Although many studies have examined the incidence and impact on outcomes of AKI in patients with cirrhosis, pooled data from these studies is scarce. We performed this meta-analysis to pool the data from observational studies to define the incidence and etiology of AKI in patients with cirrhosis and its impact on patient survival. We also aimed to examine variables at baseline that could identify patients with cirrhosis who are at risk of developing AKI.

Methods

Study selection criteria

The studies considered in this meta-analysis were case-control or prospective cohort studies of patients with cirrhosis, reporting on the incidence of AKI or/and comparing mortality among patients with versus those without AKI.

Studies reporting mortality at short to medium term (in-hospital, 30 days, and 90 days) or long-term (1 year) were included. Studies were excluded if they did not include incidence and/or mortality associated with AKI in cirrhotic patients or if there were insufficient data for analysis. Studies published only in English language and as full manuscripts were included in the analysis.

Data sources and search strategy

All procedures used in this meta-analysis were consistent with the PRISMA criteria for observational studies.[10] We conducted a comprehensive search of Ovid MEDLINE, Embase, Cochrane Central Register of Controlled Trials, Cochrane Database of Systematic Reviews, Web of Science and Scopus, from January 1990 to November 2018. The search strategy was designed and conducted by experienced library staff. MeSH terms used in the search were 'acute kidney injury' or 'AKI' AND 'cirrhosis' AND 'risk factors' or 'incidence' or 'mortality'.

Two authors (R.T. and Y.H.) independently reviewed the titles and abstracts of the searched literature to identify potential studies for analysis. The full texts of these studies were reviewed for final selection to be included in the meta-analysis. The reference lists of articles with information on the topic were also reviewed for additional pertinent studies. Any discrepancy between these two investigators was resolved by joint re-evaluation of the article in question and consensus among the authors. A flow diagram of included studies is shown in Fig. 1.

The Newcastle-Ottawa scale was used independently by two investigators (R.T. and H.S.) to assess the quality of each selected study for the analysis. In this scale, observational studies were scored across three categories using the following parameters: selection (four questions), comparability (two questions), and ascertainment of the outcome of interest (three questions). For each question, 1 point was given if the study met the criterion, except for comparability of study groups, in which 2 points were awarded if the study controlled for age, sex, or both, and other confounding factors (Supplementary Table 1). Studies with a cumulative score of 7 or more were considered high quality and those with score of ≤6 were considered of low quality. Any discrepancies were addressed by a joint re-evaluation of the article in question and consensus amongst the authors.

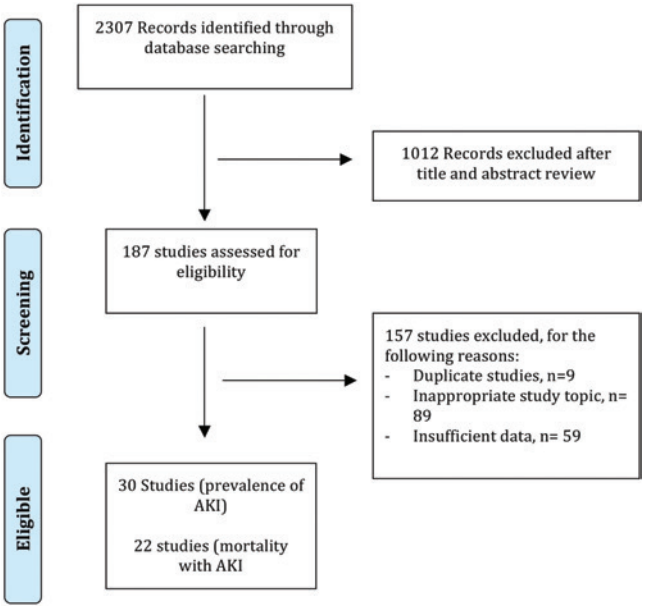

Fig. 1. Search strategy for included studies.

Outcomes

Our primary analysis focuses on the incidence and mortality associated with AKI in patients with cirrhosis. The secondary outcome was to evaluate the risk factors that predicted mortality in these patients

Data abstraction

Data were independently abstracted to a predetermined data collection Microsoft Excel spreadsheet by three investigators (R.T., Y.H and K.C.). For each study, data were collected for study design, location, year of publication, definition of AKI used, patient demographics, follow-up period, and outcomes. Conflicts on data abstraction were resolved by consensus amongst authors and referring to the original article.

Statistical analyses

The random-effects model described by DerSimonian and Laird[11] was used to calculate weighted incidence rate of AKI with corresponding 95% confidence interval (CI). Data were weighted based on sample size in each study. For mortality analysis at various time points, odds ratio (OR) with 95% CI were derived on the odds of dying among AKI patients compared to those without AKI. To identify variables at baseline predictive of AKI risk, ORs were determined for categorical variables and mean difference for continuous variables.

We assessed heterogeneity within groups with the I^2 statistic, which estimates the proportion of total variation across studies. I^2 value >50% suggested heterogeneity of the pooled data.[12] To address heterogeneity, subgroup analyses were performed on studies defining AKI using the AKIN criteria, high quality studies, and prospective studies. Publication bias was assessed by visual inspection of funnel plots and numerically using the Luis Furuya-Kanamori (LFK) estimate on a Doi plot. The scoring was: no asymmetry when the LFK index was within ±1; minor asymmetry when the LFK index exceeded ±1 but was within ±2; major asymmetry when the LFK index exceeded ±2. Publication bias was considered if the given analyses had major asymmetry on the inspection of funnel plots. If publication bias was found on funnel plot, we used the trim and fill for adjusting publication bias.[12,13] All p values were 2-tailed and considered statistically significant if <0.05. Review Manager (version 5.3; Cochrane Inc.) and MetaXL, version 5.1 (EpiGear International Pty Ltd) statistical software program were used to analyze the pooled data (www.epigear.com).

Results

Baseline characteristics of included studies

On the initial literature search, 2307 potentially relevant studies were identified. After screening titles and abstracts, 187 full-text articles were reviewed for study selection. Of

Table 1. Baseline characteristics of included studies

First author, year	Study type	Location of study	Total, *n*	With AKI, *n*	Mean age	% Males	Follow-up period	Definition of AKI
Angeli, 2014	P	Spain	510	98	55	64.9	90 days	AKIN
Bıyık, 2016	RT	Turkey	277	108	62.1	57.8	4 years	KDIGO criteria
Bucsics, 2015	RT	Austria	239	78	54.9	66.9	n/a	AKIN
Chen, 2011	RT	Taiwan	2,375	636	60.73	69.1	58 months	eGFR <60
Choi, 2014	RT	Korea	643	83	57.4	74.3		AKIN
Cholongitas, 2009	P	UK	312	128	49.3	NA	96 weeks	Serum creatinine ≥300 mmol/L
Cholongitas,2009b	RT	UK	412	205	49.3	59.2	17 years	RIFLE
de Araujo, 2014	RT	Brazil	46	20	56.94	63	13months	AKIN
du Cheyron, 2005	RT	France	186	73	56.4	69	5 years	ADQI definition
Fagundes, 2013	P	Spain	375	177	61	62	25 months	AKIN
Hampel, 2001	RT	New Mexico	93	23	57.5	NA	7 years	↑serum creatinine >1.0 mg/dl
Hseih, 2017	RT	Taiwan	117	46	61	72	6 weeks	ICA
Huelin, 2017	P	Spain and Italy	547	290	61	67	90 days	ICA
Hung, 2012	RT	Taiwan	2592	145	57.5	70.8	1 year	ICD-9-CM
Jaques, 2018	P	Switzerland	105	55	58.0	71.4	2 years	AKIN
Jindal, 2015	RT	India	241	55	46.12	85.47	33 months	Mild or moderate AKI with cut-off creatinine at 3 mg/dL
Maiwall, 2015	P	India	451	122	46	86	1 year	AKIN
Marciano, 2017	RT	Argentina	108	37	61.5	59.6	3 years	KDIGO
Nuthalapati, 2017	RT	USA	339	96	57.0	63	5 years	AKIN
Pan, 2016	P	Taiwan	242	152	58	75.7	2 years	AKIN and RIFLE
Piano, 2013	P	Italy	233	61	65.3	64.4	NA	AKIN & conventional criteria
Prakash, 2011	P	India	404	99	48.5	79	16 months	AKIN
Scott, 2013	P	UK	162	110	56.8	65.4	18 months	AKIN
Shi, 2016	RT	China	1167	308	NA	NA	1 year	KDIGO
Tandon, 2016	RT	Canada	4733	1850	60.4	64.3	10 years	KDIGO
Tsien, 2013	P	Canada	90	49	55.8	71.1	2 years	n/a
Warner, 2011	RT	USA	152	107	53	76%	2 years	AKIN
Wong, 2013	P	USA	337	166	55.91	56	30 days	ADQI definition
Wong, 2017	P	Multiple centers in North America	653	307	56.7	64	30 days	ICA
Zhou, 2017	RT	China	333	60	55.68	63.06	2 years	KDIGO
Summary			18,474	5,648	56.8	66.9	Median: 12 months	NA

Abbreviations: ADQI, Acute Dialysis Quality Initiative; AKI, acute kidney injury; AKIN, AKI Network; ICA, International Club of Ascites; KDIGO, Kidney Disease Improving Global Outcomes; RIFLE, Risk Injury and Failure; P, prospective; RT, retrospective.

these, 30 studies[14–43] met eligibility criteria and were included for analysis and the remaining 157 were excluded for different reasons (Fig. 1). Of the 30 studies (12 prospective and 18 retrospective) analyzed and including 18,474 patients with cirrhosis (median age 57 years and 67% males), 16 were from the Western world (10 from Europe and 6 from USA or Canada) and the remaining studies were from Asia (*n*=10), Middle East (*n*=1), or South America (*n*=3)

(Table 1). The median Newcastle-Ottawa quality score for the included studies was 8 (range: 6-9) (Supplementary Table 1). A total of 17 studies were high quality and 13 were low quality. Other details and a summary of the included studies are described in Supplementary Table 2. The percentage of patients with baseline kidney dysfunction was not discussed in most studies, as shown in Supplementary Table 3; although, a majority of the studies included patients with some degree of baseline renal dysfunction.

Incidence of AKI

Of the 18,474 patients with cirrhosis in the 30 selected studies, 5,648 had developed AKI, with a pooled incidence of 29% (95% CI: 28-30%). AKI was defined based on the AKIN in 11 studies and the definition of AKI was variable in the remaining studies (Table 1). The pooled data had significant heterogeneity, with an I^2 of 99% and $p<0.0001$ (Fig. 2). No publication bias was seen on visual inspection of forest plot, with minor asymmetry on Doi plot (LFK=1.45) (Supplementary Fig. 1). Heterogeneity remained high when pooled incidence was analyzed only for prospective studies (40%, 95% CI: 38-41%), for studies that used AKIN criteria (29%, 95% CI: 28-31%), and for studies with high quality (40%, 95% CI: 39-41%) (Supplementary Fig. 2A, 1B and 1C, respectively). One study was performed before 2005 and in order to ensure the universal definitions of AKI after 2005, subgroup analysis was performed after the exclusion of that study, which revealed the same incidence of AKI (29%, 95% CI: 28-30%) after exclusion of the above mentioned study.[26]

Mortality risk: comparing patients with AKI vs. no AKI

Of the 30 studies included, 22 reported patient mortality data for a median follow-up of 12 months (range: 30 days to 10 years) (Table 1). In-hospital mortality was assessed in eight studies. The rate of mortality among AKI patients was 215/620 (34.6%) vs. 61/624 (9.7%), which was six-fold higher among AKI patients compared to those without AKI (OR [95% CI]: 6.72 [3.47-13], $p<0.0001$). Separate analysis from three studies on patients admitted to intensive care also showed about six-fold mortality among AKI patients (277/353 (78%) vs. 154/387 (39.7%); OR [95% CI]: 5.90 [3.21-10.85], $p>0.0001$). Mortality at 30 days reported in seven studies was over three-fold higher with AKI (422/995 (42.4%) vs. no AKI 841/3973 (21.1%), OR [95% CI]: 3.37 [2.35-4.84], $p>0.0001$). Similarly, mortality remained higher at 90 days and at 1-year follow-up for those with compared to those without AKI (47.1% vs. 16.4%, OR [95% CI]: 4.43 [2.93-6.70], $p>0.00001$) and (68.3% vs. 45.1%, OR [95% CI]: 5.37 [2.45-11.79], $p>0.00001$). However, there was significant heterogeneity for all the analyses (Fig. 3 A-E). No publication bias was seen on visual inspection of forest plots (Supplementary Fig. 3 A-E).

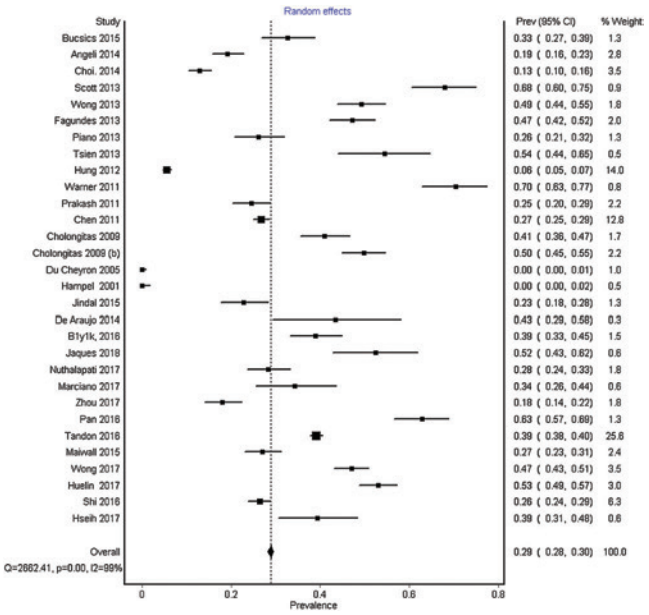

Fig. 2. Forest plot depicting pooled incidence of acute kidney injury in patients with cirrhosis.

Risk factors associated with development of AKI

A total of 12 variables at admission were analyzed among 22 studies as predictors for the development of AKI. Of these, four predicted the risk of AKI, given as OR (95% CI): model for end-stage liver disease score, 5.89 (5.17-6.62); Child-Pugh-Turcotte stage C, 2.51 (1.83-3.44); presence of ascites, 2.06 (1.25-3.41); and presence of sepsis/septic shock, 2.72 (1.05-7.06) (Fig. 4 A-D). Interestingly, history of variceal bleed was associated with a decreased risk of AKI, 0.69 (0.48-0.99) (Fig. 4E). Other factors, including etiology of cirrhosis (alcoholic and viral), encephalopathy, bacterial infection on admission, male sex, age, and diabetes mellitus were not associated with risk of AKI (Supplementary Fig. 3A-G).

Discussion

The main findings of this meta-analysis on pooled data from 30 studies of patients with cirrhosis are a high incidence of AKI (at 29%) and higher mortality during hospitalization and on follow-up to 1 year among patients who develop AKI when compared to those who do not. Further, patients at risk of development of AKI can be identified at presentation or hospitalization with higher model for end-stage liver disease or Child-Pugh-Turcotte score with ascites and/or sepsis/shock.

Portal hypertension with resultant splanchnic pooling of blood in patients with cirrhosis results in decreased effective circulating blood volume, setting the stage for development of AKI with decompensation of cirrhosis or introduction of any precipitant, such as volume loss, use of diuretics, administration of radio-contrast agents or nephrotoxic drugs, and onset of infections or sepsis.[44] Cirrhosis is the 12th leading cause of mortality in the general population, with over 40,000 annual deaths from this disease.[45] In one study, mortality rate among patients with cirrhosis was over 20% at 2 years.[46] Not only does AKI portend a worse prognosis in these patients but the mortality risk remains elevated in these patients at 1-year follow-up among those surviving the index hospitalization or development of event. Data in the current literature regarding renal recovery and its effect on mortality is scant, but a recent study shows a high mortality rate (of 15%) in cirrhosis patients who experienced complete renal recovery after an AKI episode, as observed in the current analysis.[15] Clearly, AKI represents a significant event in the natural course of these patients with cirrhosis, and this may be viewed as a sixth stage in the already five-stage

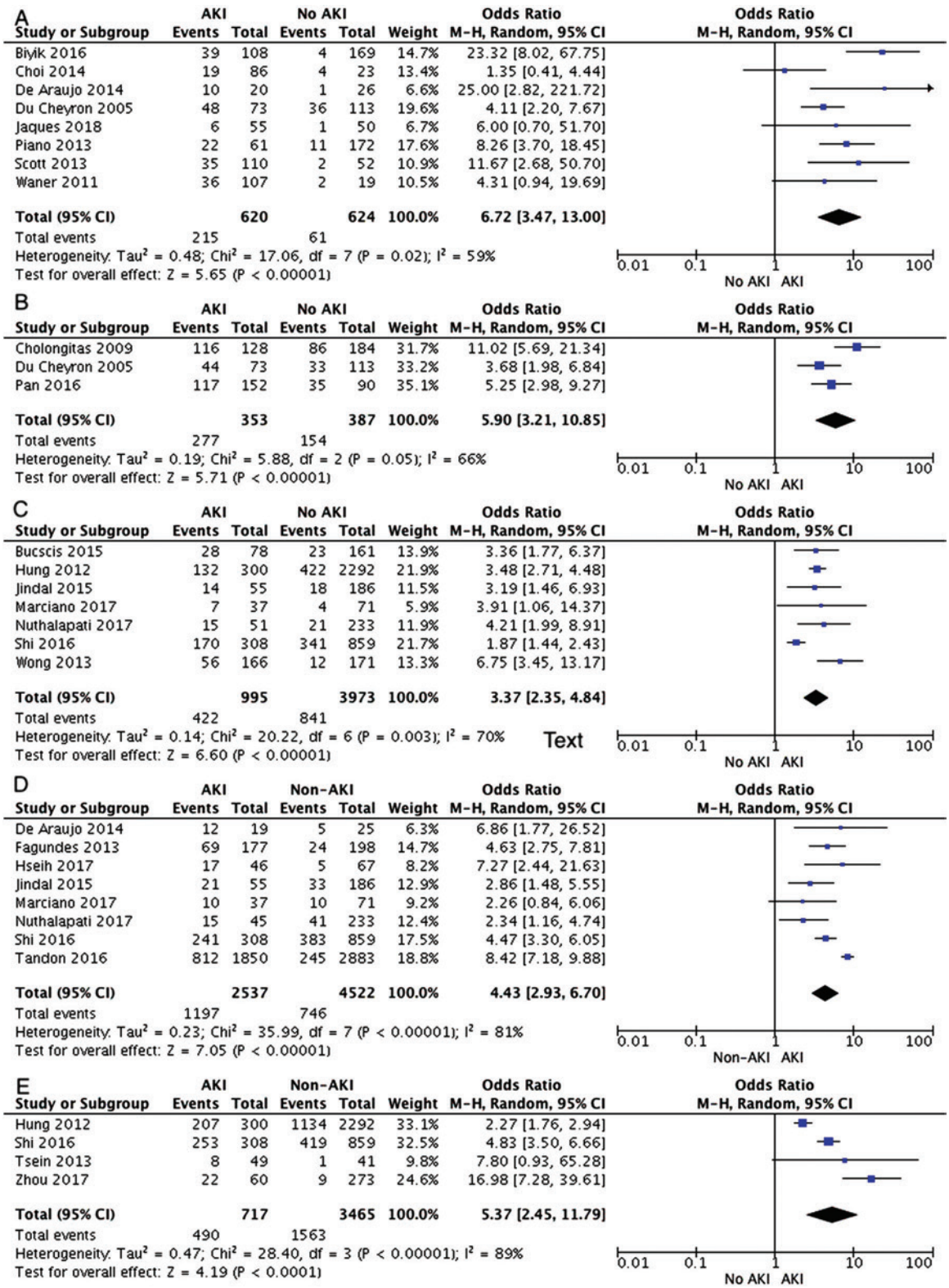

Fig. 3. Forest plots on mortality outcomes comparing cirrhosis patients with acute kidney injury vs. without acute kidney injury for A) overall in-hospital mortality, B) in-hospital mortality for intensive care patients, C) mortality at 30 days follow-up, D) mortality at 90 days follow-up, E) mortality at 1-year follow-up.

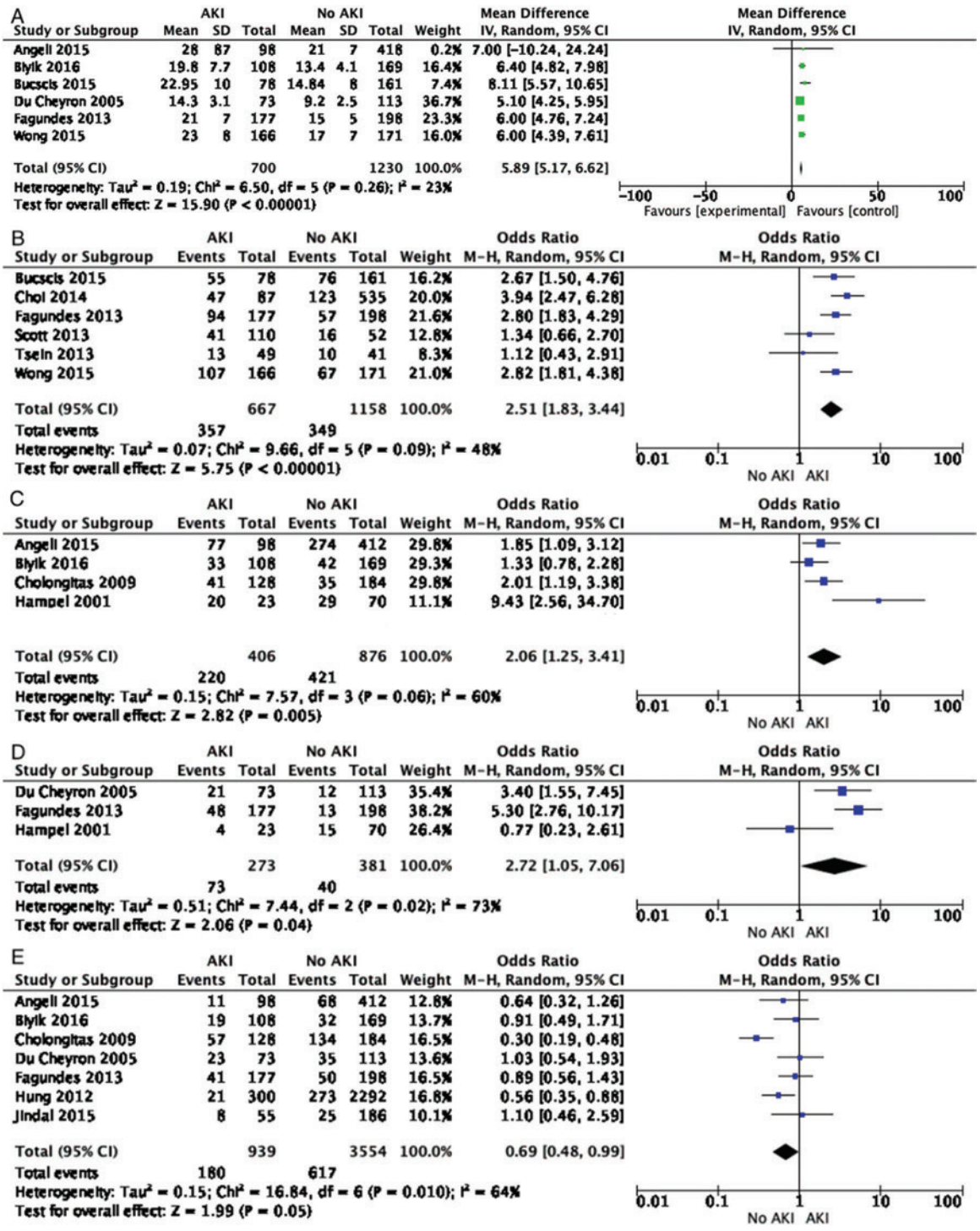

Fig. 4. Forest plots showing admission variables predicting acute kidney injury.

A) Model for end-stage liver disease score, B) Child-Pugh-Turcotte score, C) presence of ascites, and D) presence of sepsis/septic shock. Risk of acute kidney injury is reduced among patients with variceal bleeding (E).

model of cirrhosis, with linear increase in short-term and long-term mortality.[47] It has been shown in prospective studies that the index episode of AKI is a risk factor for subsequent episodes of AKI.[48] With each episode of AKI, the renal reserve declines due to the inability of kidneys to recover function completely to original baseline level and resulting in risk for development of chronic kidney disease and impacting the outcomes negatively.[31,33]

While patients with cirrhosis constitute a heterogeneous cohort, the subpopulations at an increased risk of developing AKI have not been sufficiently studied. In our pooled analysis, model for end-stage liver disease score, Child-Pugh stage, presence of ascites, and presence of severe sepsis/septic shock were associated with an elevated risk of developing an AKI. Severe sepsis/septic shock has been studied as independent risk factors for developing AKI regardless of cirrhosis. Also, association of AKI with model for end-stage liver disease score and Child-Pugh class found in our study are in line with the prior studies.[48–50] Model for end-stage liver disease score is the most frequently used score all over the world to estimate patient outcomes and survival among patients with cirrhosis. Renal function apart from serum bilirubin and coagulation status is an important component of the model for end-stage liver disease score. Use of diuretics, large volume paracenteses, and fear of physicians to give volume expansion are some speculated reasons explaining higher risk of AKI in patients with ascites.[51] Interestingly, presence of a history of or current admission with a variceal bleed was associated with a decreased risk of AKI. Patients with variceal bleeding receive antibiotics for spontaneous bacterial peritonitis prophylaxis, as recommended by guidelines from major societies; this use of spontaneous bacterial peritonitis prophylaxis may be the reason for lower incidence of AKI in this cohort.[52] Diabetes and the etiology of cirrhosis were not found to be associated with AKI.

Pooled data on a large patient population with cirrhosis is the strength of this meta-analysis. Furthermore, our study also identified the predictors of AKI apart from pooled incidence and risk of mortality. However, our study does have some limitations. Studies included in our meta-analysis varied on study design, patient population, and status of cirrhosis, resulting in significant heterogeneity. Pooled data using the individual patient data from these studies may potentially overcome this limitation and provide more homogeneous data on incidence, impact on outcomes, and variables predictive of AKI. Furthermore, due to the very limited data available in the included studies regarding the mortality rates among subgroups with different stages of AKI, we could not perform a pooled mortality analysis based on severity of AKI. To explore the heterogeneity, meta-regression was considered with various predictor variables including sex, viral cirrhosis, alcoholic cirrhosis, Child-Pugh score, concomitant diabetes, presence of ascites, variceal bleeding, encephalopathy, bacterial infection, septic shock/sepsis, mean difference in age and model for end-stage liver disease scores. The number of studies in each individual analysis was limited (all <10). Moreover, information for each predictor variable was also poorly present. At most, one predictor (sex) was present for three studies in one outcome (30-day mortality); the rest were present for one or two studies only. Hence, meta-regression was not performed based on poor information availability of predictor-variables.[53]

In conclusion, AKI is common in cirrhotic patients, and leads to increased mortality among patients admitted to hospital in the wards as well as in the ICU, which remained high even at long-term follow-up at 1 year. Multicenter prospective studies are also suggested using pre-defined criteria to define AKI, study outcomes, and risk factor variables as basis for development of homogeneous data.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Contributed to concept, data interpretation drafting and revision of manuscript (RT, AKS), data collection, drafting and revision of manuscript (YH), data collection and interpretation (KS), and data collection (SR, HS).

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Original Article

Performance of Non-invasive Blood Parameters for Ruling Out Significant Liver Fibrosis in Patients with Chronic Hepatitis B

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Abstract

Background and Aims: Evaluation of significant liver fibrosis is important for treatment decision and treatment response evaluation in patients with chronic hepatitis B. Since liver biopsy is invasive and transient elastography (TE) has limited availability, various non-invasive blood parameters need evaluation for their capabilities for detection of significant fibrosis. ***Methods:*** In this retrospective study, records of patients who had undergone liver biopsy for treatment-naïve chronic hepatitis B were evaluated to obtain various non-invasive blood parameters (aspartate aminotransferase-to-platelet ratio index [referred to as APRI], Fibrosis-4 score [referred to as FIB-4], gamma-glutamyl transpeptidase-to-platelet ratio [referred to as GPR], and gamma-glutamyl transpeptidase-to-albumin ratio [referred to as GAR]), in addition to TE, to assess significant liver fibrosis and compare these to fibrosis stage in liver biopsy. ***Results:*** A total of 113 patients were included in the study (median age 33 [interquartile range: 11-82 years], 74% males). Most (75%) patients were HBeAg-negative. The liver biopsy revealed significant fibrosis (Ishak ≥3) in 13% of the patients and nil or mild fibrosis (Ishak <3) in 87% of the patients. TE findings were available for 85 patients, APRI and FIB-4 for 95 patients, GPR for 79 patients, and GAR for 78 patients. The median values of all the parameters were significantly higher in patients with significant fibrosis, as compared to patients with non-significant fibrosis, and all the blood parameters as well as TE were able to identify patients with significant fibrosis significantly well (p<0.05). All non-invasive parameters had low positive predictive value but negative predictive value above 92%. Compared to TE, all the non-invasive blood parameters had similar area under the curve for detecting significant fibrosis, with excellent negative predictive value (≥93%). ***Conclusions:*** Non-invasive blood parameters (APRI, FIB-4, GPR, and GAR) with negative predictive values above 93% are excellent parameters for ruling-out significant fibrosis in patients with chronic hepatitis B. These can be used at bedside in place of TE.

Citation of this article: Khare S, Arora A, Sharma P, Dhawan S, Bansal N, Singla V, *et al.* Performance of non-invasive blood parameters for ruling out significant liver fibrosis in patients with chronic hepatitis B. J Clin Transl Hepatol 2020;8(2):143–149. doi: 10.14218/JCTH.2020.00002.

Introduction

Of approximately 2 billion people who have been infected with hepatitis B virus (HBV) worldwide, more than 248 million (5–7% of the world's population) suffer from chronic HBV infection (CHB) and about 1 million of these die per year.[1] India has over 40 million HBV carriers, accounting for 10-15% of the total HBV carriers in the world.[2]

HBV has a complex natural history, and the interaction between viral proteins and the immune system leads to a cycle of hepatocyte damage and tissue repair.[3] This repair leads to progressive liver fibrosis over time, which can be rapid, slow, or sporadic depending on disease state and the degree of active liver inflammation and injury. The assessment of liver fibrosis is vital to disease prognostication and to determining the need for treatment as well as the response to therapy. Studies in Asia and the USA have revealed that 20% to 30% of HBV carriers with persistently normal alanine aminotransferase (ALT) levels and HBV DNA levels >10000 copies/mL have grade ≥2 inflammation and stage ≥2 fibrosis on liver biopsy.[4,5] A fair proportion of patients with CHB infection with normal ALT have HBV DNA ≥5 log copies/mL and significant histologic fibrosis.[5] At present, the gold standard for assessment of liver fibrosis is liver histology using the Ishak[6] or METAVIR[7] systems. However, liver biopsy is prone to sampling error and substantial intra- and inter-observer variability, leading to over- or under-staging of fibrosis;[8] in addition, the procedure also has significant morbidity, including infections, major bleeding, and ascites leakage, and can lead to mortality.[9] Consequently, there is a need for non-invasive methods to accurately diagnose the presence of liver fibrosis and cirrhosis, especially while making a decision to start antiviral therapy.

Transient elastography (TE) has been shown to be an excellent non-invasive modality for assessment of fibrosis;[10,11] however, it has limited availability, especially in resource-poor countries. So various non-invasive blood parameters need evaluation to find the most useful parameter for ruling out significant fibrosis.

Keywords: Hepatitis B; Liver fibrosis; Cirrhosis; Transient elastography; APRI; FIB-4; GPR; GAR.

Abbreviations: ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; CHB, chronic hepatitis B; FIB-4, Fibrosis-4; GAR, gamma-glutamyl transpeptidase-to-albumin ratio; GGT, gamma-glutamyl transpeptidase; GPR, gamma-glutamyl transpeptidase-to-platelet ratio; HBV, hepatitis B virus; NPV, negative predictive value; PPV, positive predictive value; TE, transient elastography.

Received: 14 January 2020; Revised: 6 May 2020; Accepted: 10 May 2020

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A number of non-invasive models containing serum markers, such as serum aspartate aminotransferase (AST) to platelet ratio index (APRI),[12–14] Fibrosis 4 score (FIB-4),[15] gamma-glutamyl transferase (GGT)-to-platelet ratio (GPR),[16] and GGT-to-albumin ratio (GAR)[17] have been described in the literature. Among these markers, the FIB-4 and APRI[12,18] are widely used to assess patients with chronic hepatitis but their value for assessing patients who are chronically infected with HBV remains controversial.[19–22] Recently, GPR showed better performance than FIB-4 and APRI in detecting liver fibrosis in CHB West African patients; however, this was not true for French populations.[23]

There has been no published data from India evaluating the performance of these non-invasive blood parameters for ruling out significant fibrosis in patients with CHB. The aim of the present study was to evaluate and find out the most useful non-invasive blood parameter for ruling out significant fibrosis in CHB and to compare it with TE.

Methods

Patients

This was a retrospective study conducted in the Institute of Liver, Gastroenterology & Panceatico-Biliary Sciences of Sir Ganga Ram Hospital, New Delhi, India. Records of patients with CHB who had undergone liver biopsy between February 2009 and May 2017 were analyzed. The study included consecutive patients who fit the following inclusion criteria: treatment-naïve CHB; age between 10 and 70 years; and had undergone pre-treatment liver biopsy. The following patients were excluded from the study: with co-infection with hepatitis C virus, hepatitis A virus, hepatitis E virus or human immunodeficiency virus; with significant cardiac and/or pulmonary co-morbidities; renal dysfunction (serum creatinine >1.5 mg/dL); grade 3-4 hepatic encephalopathy; with hepatocellular carcinoma or other malignancies; with acute-on-chronic liver failure; or with acute flare of hepatitis (serum bilirubin >4 mg/dL, AST or ALT >300 U/L).

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The study being a retrospective analysis of data did not require Ethics Committee approval. Also, the retrospective analysis of data, without revealing any patient's identity, precluded requirement of informed consent from patients.

Liver biopsy

Liver tissue (1.5-2 cm) was obtained by percutaneous or transjugular biopsy by the Gastroenterologist and sent to the Histopathology Department, where it was stained with hematoxylin and eosin. Fibrosis staging was done according to the modified Ishak grading system.[6] Significant fibrosis was defined as Grade III or more by modified Ishak grading.

Liver stiffness measurement by TE

Liver stiffness measurement was performed using a FibroScan® device (Echosens, Paris, France), in accordance with the manufacturer's recommendations. Measurements were made on the right lobe of the liver through intercostal spaces, with the patient lying in a supine position with the right arm in maximal abduction. The tip of the transducer probe was covered with coupling gel and placed on the skin between the rib bones at the level of the right lobe of the liver. When the target area was located, the operator pressed the probe button to commence the measurements. The measurement depth was between 25 mm and 65 mm. Ten successful measurements were performed on each patient. The results were expressed in kPa. The median value was considered as the liver stiffness. Interquartile range/median <30% and success rate >60% were considered as good quality criteria for TE. Patients with significant ascites underwent large volume paracentesis before liver stiffness measurement. All liver stiffness measurements were performed by a single operator.

Laboratory tests

All blood samples were obtained within 1 day of liver biopsy. Blood biochemical parameters included bilirubin, ALT, AST, GGT, albumin, prothrombin time, and platelets. Virological parameters included HBV serological markers and HBV DNA. Non-invasive blood parameters were calculated as per the recommended formulae:[12,15–17]

- APRI = (AST/ [upper limit of normal]/platelet [109/L]) X 100
- FIB-4 = (age [year] X AST [U/L]) / {(platelet [109/L]) X (ALT [U/L]) 1/2}
- GPR = (GGT/upper limit of normal) X 100/platelet
- GAR = GGT/albumin

Statistical analysis

Quantitative data were expressed as median (interquartile range) and compared using the Mann-Whitney *U* test or Wilcoxon signed-rank test. Qualitative data were expressed as number (%) and compared using Fisher's exact test or Pearson's chi-square test. A p value of <0.05 was considered significant. Statistical analysis was conducted using the SPSS 17.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Patients

A total of 129 patients were enrolled in the study; however, 16 patients were excluded due to following reasons: co-infection with human immunodeficiency virus (n=1); renal dysfunction (serum creatinine >1.5 mg/dL) (n=3); acute-on-chronic liver failure (n=6); and acute flare of hepatitis (n=6). Hence, the remaining 113 patients were included in the study.

The demographic and biochemical parameters of the included patients is given in Table 1. The median age was 33 (interquartile range of 14) years, and 77% were males. The median HBV DNA was 2×10^3 (interquartile range of 1×10^5) IU/dL, and 25% of the patients were positive for hepatitis B e antigen. According to the modified Ishak grading system, 98 (87%) had non-significant fibrosis (Ishak stage <3), while 15 (13%) of patients had significant fibrosis (Ishak stage ≥3). Values of platelet count, GGT, albumin and proportion of patients with hepatitis B e antigen positivity were significantly different between patients with non-significant and significant fibrosis (Table 1).

Table 1. Demographic and biochemical parameters of the study population

Parameters	All patients, *n*=113	Patients with Ishak <3, *n*=98	Patients with Ishak ≥3, *n*=15	*p* value
Age in years	33 (14)	32 (12)	45 (24)	0.138
Sex **Males** **Females**	87 (77%) 26 (23%)	74 (76%) 24 (24%)	13 (87%) 2 (13%)	0.514
Hemoglobin in g/L	14.3 (2.1)	14.3 (2.2)	14.0 (3.7)	0.637
White blood cells as $\times 10^3$/L	7.0 (3.3)	7.0 (3.1)	5.9 (6.0)	0.992
Platelets as $\times 10^6$/L	184 (92)	187 (96)	160 (94)	0.013
Creatinine in mg/dL	0.8 (0.3)	0.8 (0.3)	0.8 (0.5)	0.159
Bilirubin in mg/dL	0.7 (0.4)	0.7 (0.4)	0.7 (0.4)	0.615
Aspartate aminotransferase in U/L	32 (22)	32 (19)	69 (114)	0.080
Alanine aminotransferase in U/L	38 (40)	36 (29)	85 (144)	0.064
Gamma-glutamyl transpeptidase in U/L	21 (23)	20 (20)	41 (46)	0.019
Serum alkaline phosphatase in U/L	90 (45)	88 (44)	98 (48)	0.089
Albumin in g/L	4.3 (0.5)	4.3 (0.6)	4.2 (1.3)	0.041
International normalized ratio	1.1 (0.2)	1.1 (0.1)	1.1 (0.2)	0.178
HBV DNA in IU/dL	2×10^3 (1×10^5)	2×10^3 (5×10^4)	2×10^4 (5×10^7)	0.512
Hepatitis B e antigen-positive	25%	17%	53%	0.006
Ishak fibrosis stage **0** **1** **2** **3** **4** **5** **6**	 69 (61%) 25 (22%) 4 (4%) 4 (4%) 2 (2%) 5 (4%) 4 (4%)	 69 (70%) 25 (26%) 4 (4%)	 4 (27%) 2 (13%) 5 (33%) 4 (27%)	-

All values are median (interquartile range) or number (%).

Performance of TE in detecting significant fibrosis

TE findings were available for 85 patients, APRI and FIB-4 for 95 patients, GPR for 79 patients, and GAR for 78 patients. All of the five parameters were available for 60 patients. Table 2 shows the comparison of median values of TE, APRI, FIB-4, GPR, and GAR in patients with non-significant fibrosis to those with significant fibrosis. The median values of all the parameters were significantly higher in patients with significant fibrosis, as compared to patients with non-significant fibrosis.

TE had an area under the curve of 0.793 (95% confidence interval of 0.665, 0.921) in the receiver operating characteristic curve for detecting significant fibrosis (Fig. 1). The area under the curve values of APRI, FIB-4, GPR and GAR ranged between 0.723 and 0.764 (Fig. 2), and these values were not significantly different from the area under the curve of TE. Thus APRI, FIB-4, GPR, or GAR can be used in place of TE with similar accuracy.

Table 3 shows the best cut-off values along with positive predictive values (PPVs) and negative predictive values (NPVs) for all the non-invasive parameters for detecting significant fibrosis. All parameters had NPV above 93%. TE had the highest NPV (100%) at a cut-off of <5.35 kPa. Among the blood parameters, GPR had highest NPV (95%) at a cut-off of <0.444. The PPV of all the parameters were low; thus, all these non-invasive tests can be best utilized for ruling out significant fibrosis, rather than ruling in.

Table 4 shows a sub-group analysis of only those patients which had data on all the 5 non-invasive parameters. There were a total of 60 patients, and the AUC of all the parameters was still above 0.690.

Discussion

In this study, we compared the diagnostic value of non-invasive blood parameters (APRI, FIB-4, GPR, and GAR) for assessing liver fibrosis in a patients with CHB and found that these blood parameters have NPVs above 93% and are excellent parameters for ruling-out significant fibrosis. These data indicate that these parameters can be used at bedside in place of TE, especially if the latter is not available.

Assessment of significant fibrosis is an important step for decision-making of antiviral treatment in chronically HBV-infected patients.[24] The Indian National Asssociation for the Study of the Liver (INASL) guidelines recommend that in patients with hepatitis B e antigen-negative states, if ALT is <80 U/L (i.e. <2×upper limit of normal), HBV DNA is 2,000-20,000 IU/mL, and if non-invasive or invasive assessment of liver fibrosis does not show significant fibrosis, antiviral treatment need not be started and these patients may be kept under observation.[2] Similarly, in patients with hepatitis B e

Table 2. Comparison of non-invasive tests between patients with and without significant fibrosis

	Patients with Ishak <3, *n*=98	Patients with Ishak ≥3, *n*=15	*p* value
Transient elastography, *n*=85	5.4 (2.8)	12.0 (12.6)	0.004
APRI, *n*=95	0.45 (0.35)	0.79 (2.34)	0.013
FIB-4, *n*=95	0.94 (0.68)	1.92 (3.00)	0.003
GPR, *n*=79	0.24 (0.22)	0.46 (1.03)	0.009
GAR, *n*=78	4.87 (4.64)	17.98 (21.44)	0.013

All values are median (IQR).

Abbreviations: APRI, aspartate aminotransferase-to-platelet ratio index; FIB-4, Fibrosis-4; GAR, gamma-glutamyl transpeptidase-to-albumin ratio; GPR, gamma-glutamyl transpeptidase-to-platelet ratio.

antigen-positive state, if ALT is 40-80 U/L, HBV DNA is >20,000 IU/mL, and if non-invasive or invasive assessment of liver fibrosis does not show significant fibrosis, antiviral treatment need not be started as these patients are considered to be in the immune-tolerant phase.[2]

In the past (over one decade), TE has gained importance as one of the best non-invasive tests to assess liver fibrosis. In our study, TE had the best area under the receiver operating characteristic curve (0.793) compared to the blood parameters. In addition, our cut-off of 5.35 kPa for TE for significant fibrosis was similar to that in a previous French study on 1307 patients which gave a cut-off of 5.2 kPa[25] and another study from India which gave a cut-off of 6 kPa.[26] We found TE to have the best NPV of 100% when using this cut-off. However, TE has many disadvantages. It is not universally available, especially in resource-poor settings; its applicability is approximately 80%, which is lower than that of serum biomarkers, especially when used in the presence of ascites, obesity, and limited operator experience. It can also lead to false positive values in the case of acute hepatitis, extra-hepatic cholestasis and liver congestion. Finally, it is unable to discriminate between intermediate stages of fibrosis, it requires a dedicated device, and it does not allow for a region of interest to be chosen.[27] In contrast, non-invasive serum biomarkers have many advantages: They do not require extra cost and are widely available, can be assessed both in in-patient and out-patient settings, have good reproducibility and high applicability, and most are well validated.[27] However, with the multitude of blood parameters, with varying sensitivities and specificities, the best parameter for detection of or for ruling-out significant fibrosis needed evaluation. Hence, in this study, we included commonly used parameters which are available even in most resource-poor settings.

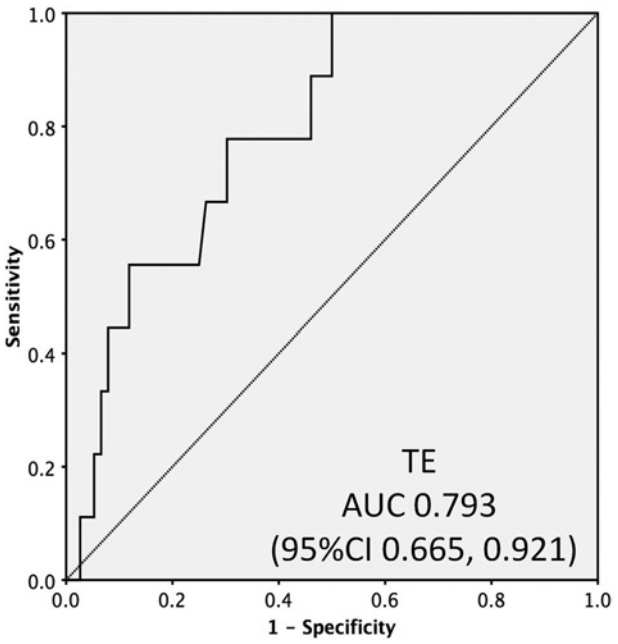

Fig. 1. Receiver operating characteristic curve for TE for detecting significant fibrosis.

Abbreviations: AUC, area under the curve; CI, confidence interval; TE, transient elastography.

We found that the NPVs of all non-invasive blood parameters were nearly similar and ≥93%. So, all these parameters were found to have similar and excellent performance in ruling-out significant fibrosis in CHB patients (in comparison to liver biopsy). The best cut-off values of GPR, APRI, FIB-4 and GAR, especially for ruling-out of significant fibrosis, were 0.935, 2.324, 0.444 and 17.848. However, the ruling-in performance of these parameters was low, with PPVs of GPR, APRI, FIB-4 and GAR at 28%, 33%, 37% and 35% respectively. GPR was found to have slight superiority because of the highest NPV of 95%, while the NPVs of APRI, FIB-4 and GAR were 93%, 93% and 92% respectively.

APRI is the oldest and probably the most widely used non-invasive parameter to assess liver fibrosis,[12,22,28] and even portal hypertension.[13,14] In our study, we found the area under the receiver operating characteristic curve of APRI for significant fibrosis was 0.723, and 0.935 was the best cut-off. Our results are similar to a meta-analysis of 17 studies[29] (*n*=3,573) that assessed APRI, and found the area under the summary receiver operating characteristic curve to be 0.77, which is almost similar to the area under the receiver operating characteristic curve in our study of 0.723. Another meta-analysis of five studies found that a cut-off of 0.5 for APRI gave a specificity of 41%, while a cut-off of 1.5 of APRI gave a specificity of 84% for detection of significant fibrosis.[28]

After APRI, the next non-invasive parameter which became popular was FIB-4.[15] In a meta-analysis[29] of 10 studies assessing the FIB-4 for the prediction of significant fibrosis (*n*=1,996), the area under the summary receiver operating characteristic curve was 0.75, which is similar to the area under the receiver operating characteristic curve of 0.764 in our study.

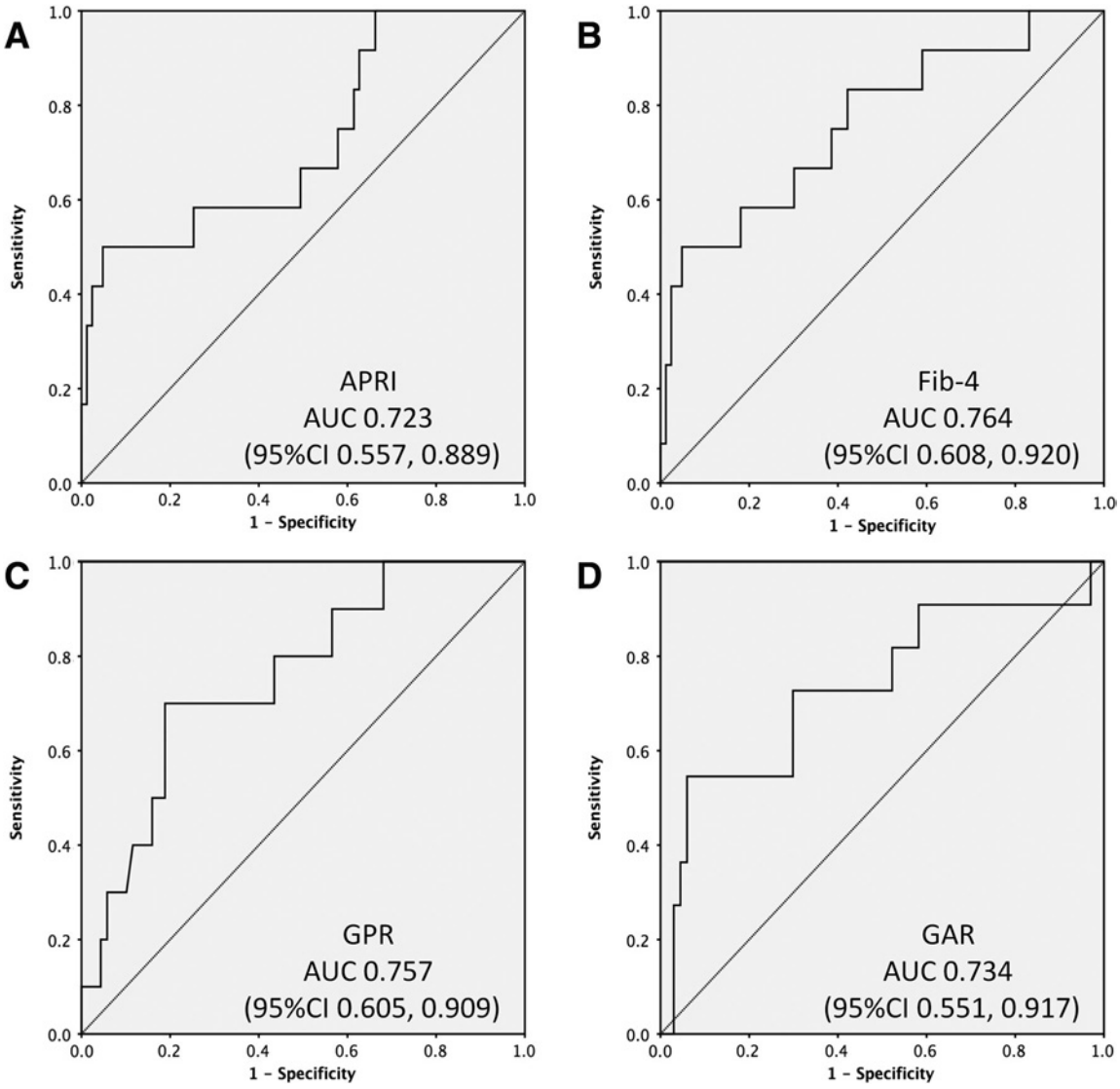

Fig. 2. Receiver operating characteristic curves for detecting significant fibrosis. (A) APRI, (B) FIB-4, (C) GPR, and (D) GAR.

Abbreviations: APRI, aspartate aminotransferase-to-platelet ratio index; FIB-4, Fibrosis-4; GAR, gamma-glutamyl transpeptidase-to-albumin ratio; GPR, gamma-glutamyl transpeptidase-to-platelet ratio.

APRI and FIB-4 have been compared in many previous studies and meta-analyses and FIB-4 was found to be slightly superior. In a study Lin *et al.*,[30] FIB-4 and APRI were compared to evaluate their diagnostic values in identifying significant fibrosis and cirrhosis among 631 CHB patients. FIB-4 had a significantly higher area under the receiver operating characteristic curve than APRI to identify significant fibrosis and cirrhosis. Using FIB-4 outside the 0.87-3.40 range, significant fibrosis could be excluded in 69.2% of patients and cirrhosis could be diagnosed in 84.4%.[30] Another meta-analysis of 39 studies found that the mean area under the summary receiver operating characteristic curve value of FIB-4 was higher than that of APRI (0.76 vs. 0.72) for predicting significant fibrosis.[21] Similar results were shown by Houot *et al.*[31] in their meta-analysis, where FIB-4 had better performance than APRI. A recent large study of almost 4000 patients (the SONIC-B study aimed at ruling-out cirrhosis) also found FIB-4 performing better than APRI.[22] In contrast to these studies, a small Indian study found APRI to be superior to FIB-4 and Forn's index. The study found NPV of APRI to be 95% for excluding significant liver fibrosis, while FIB-4 with a PPV of 61% showed fair correlation with significant fibrosis.[32] Moreover, the World Health Organization recommend the use of APRI for estimating liver fibrosis in patients with CHB, where limited availability of resources was an issue.[24]

The next non-invasive parameter was GPR, which was developed in France and Western Africa to evaluate fibrosis in subjects with HBV, particularly in low-resource settings. The investigators had compared GPR with APRI and FIB-4 and found that the area under the receiver operating characteristic curve value of GPR was significantly superior to APRI and FIB-4 at identifying ≥F2 and ≥F3 in the African training and

Table 3. Performance of transient elastography and non-invasive blood parameters in detecting significant liver fibrosis

Parameter	Formula	*n*	AUROC	95%CI	*p* value	Best cut-off	PPV	NPV
Transient elastography	-	85	0.793	0.665, 0.921	0.004	5.35	19%	100%
APRI	$\frac{(\frac{AST}{ULN}) \times 100}{Plt}$	95	0.723	0.557, 0.889	0.013	0.935	60%	93%
FIB-4	$\frac{Age \times AST}{Plt \times \sqrt{ALT}}$	95	0.764	0.608, 0.920	0.003	2.324	60%	93%
GPR	$\frac{(\frac{GGT}{ULN}) \times 100}{Plt}$	79	0.757	0.605, 0.909	0.009	0.444	35%	95%
GAR	$\frac{GGT}{Albumin}$	78	0.734	0.551, 0.917	0.013	16.918	60%	93%

Abbreviations: APRI, aspartate aminotransferase-to-platelet ratio index; FIB-4, Fibrosis-4; GAR, gamma-glutamyl transpeptidase-to-albumin ratio; GPR, gamma-glutamyl transpeptidase-to-platelet ratio; NPV, negative predictive value; Plt, platelet; PPV, positive predictive value; ULN, upper limit of normal.

validation cohorts.[23] Another comparative evaluation of GPR versus APRI and FIB-4 in predicting different levels of liver fibrosis of CHB also found that GPR had the best performance among the three. Using a cut-off of GPR >0.50 as standard, the sensitivities and specificities of GPR in predicting significant fibrosis in hepatitis B e antigen-positive patients were 59.6% and 81.2%, and those of hepatitis B e antigen-negative patients were 60.3% and 78.3% respectively. The authors suggested that this cut-off is almost similar to our cut-off of 0.444 for ruling-out significant fibrosis.[33]

The most recent of the non-invasive blood parameters assessed in our study was GAR, which was developed by Li *et al.*[17] in 2017. The investigators had compared GAR to APRI and FIB-4 and had found GAR to have the highest area under the receiver operating characteristic curve for ≥F2, ≥F3, and ≥F4 fibrosis. The area under the receiver operating characteristic curve for GAR in our study was 0.734.

There are several limitations to our study. First, the performance of these non-invasive methods was assessed in a low fibrosis setting (13%), which is assumed to be reflective of the HBV population in India. Performance in higher fibrosis settings could be different from our results. Second, our results may not apply to patients in the immune-tolerant phase. Since this was a retrospective study conducted on patients who had undergone pre-treatment liver biopsy most patients in the immune-tolerant phase, who do not merit treatment, were excluded. Third, many confounding variables, such as coexisting obesity, metabolic syndrome and metabolic associated fatty liver disease, could have influenced the results. Fourth, GPR and GAR, both of which use GGT, can be affected by biliary tract disease and by some types of drugs, and this had not been evaluated in the reported studies. As such, our results of GPR and GAR need further evaluation in prospective studies.

Table 4. Subgroup analysis of those patients which had data on all the five non-invasive parameters

Parameter	*N*	AUROC	95% CI
TE	60	0.786	0.624, 0.948
APRI	60	0.692	0.469, 0.916
FIB-4	60	0.757	0.577, 0.938
GPR	60	0.779	0.584, 0.974
GAR	60	0.690	0.427, 0.953

Abbreviations: APRI, aspartate aminotransferase-to-platelet ratio index; AUROC, area under the receiver operating characteristic; CI, confidence interval; FIB-4, Fibrosis-4; GAR, gamma-glutamyl transpeptidase-to-albumin ratio; GPR, gamma-glutamyl transpeptidase-to-platelet ratio; TE, transient elastography.

In conclusion, we found that non-invasive blood parameters such as GPR, APRI, FIB-4 and GAR could be a useful parameters for screening of CHB patients who are at risk for developing liver fibrosis, especially in resource-poor settings and when TE is not available. Despite significant advances in developing non-invasive biomarkers that will help in evaluating hepatic fibrosis in patients with CHB, further large, prospective studies remain essential to validate accuracy, particularly for patients with mild hepatic fibrosis.[34] In addition, a combination of these non-invasive biomarkers with or without TE may help to establish an algorithm to increase diagnostic accuracy of non-invasive assessment of liver fibrosis.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Collected the data and wrote the first draft of the manuscript (SK), provided the fibrosis scores of the liver biopsies (SD), performed the statistical analysis (AK), conceived of and supervised the study (AA). All the authors edited the manuscript and provided intellectual input.

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Review Article

Prevention of HBV Recurrence after Liver Transplant: A Review

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Abstract

Globally, hepatitis B virus (HBV) infection is recognized as a major risk factor for the development of hepatocellular carcinoma, and HBV-induced liver failure is one of the leading indications for liver transplantation. Until about two decades ago, liver transplantation in patients with chronic HBV infection was a relative contraindication, due to high risk of viral replication with the use of immunosuppressants which could result in graft infection. In the 1990s, hepatitis B immunoglobulin (HBIg) use significantly reduced the risk of graft infection, improving outcomes of liver transplant in patients with chronic HBV infection. However, very high costs, especially with the need for long-term use, became a major concern. With the advent of nucleos(t)ide analogs (NAs), there was less need for high-dose, long-term HBIg use to prevent HBV recurrence. Lamivudine was initially used but resistance soon became a major issue. This was followed by more potent NAs, such as entecavir and tenofovir, emerging as the more preferred agents. Additionally, the use of these antiviral agents (HBIg and/or NAs) have made it possible to use the grafts from donors with positivity for hepatitis B core antibody, allowing for expansion of the donor pool. Nevertheless, there is no consensus on management protocols, which vary significantly amongst centers. In this review, we appraise studies on management strategies used and the role of active vaccination in the prevention of HBV recurrence in post-liver transplant patients.
Citation of this article: Nasir M, Wu GY. Prevention of HBV recurrence after liver transplant: A review. J Clin Transl Hepatol 2020;8(2):150–160. doi: 10.14218/JCTH.2020.00003.

Introduction

In the USA, hepatocellular carcinoma (HCC) and liver failure due to hepatitis C are the most common indications for liver transplant (LT).[1] However, worldwide, hepatitis B virus (HBV) infection is the major risk factor for development of HCC, and has remained the leading indication for LT in Asian countries.[2] Until the 1990s, HBV infection was considered a relative contraindication to LT, due to high risk of graft infection and subsequent liver failure as a result of post-transplant immunosuppression.[3] Positivity for hepatitis B surface antigen (HBsAg) and presence of HBV DNA in liver biopsies after transplantation of HBV-naïve donor liver was considered to be diagnostic for recurrence of HBV infection post-LT and was associated with poor long-term outcomes of those transplants.[4]

Over the past two decades, with the use of hepatitis B immunoglobulin (HBIg) and oral antivirals, a significant reduction in post-transplant recurrence of HBV infection has been noted, allowing for successful LT in patients with chronic hepatitis B.[5] The goal of antiviral therapy is the suppression of HBV DNA and preferably achievement of sustained virologic response (SVR). HBIg can be used to neutralize viral particles by binding to HBsAg, while nucleos(t)ide analogs (NAs) can be used to inhibit viral reverse transcriptase with consequent inhibition of HBV DNA replication. Combination of HBIg and NAs can also be used. However, there are no standardized protocols for the prevention of HBV recurrence after LT.[5] In addition, the high cost of long-term HBIg use and resistance to certain NAs can limit their use, requiring alternate management strategies. Additionally, factors, such as presence of hepatitis B core antibody (HBcAb),[6] HBsAg or HBV DNA at the time of LT introduce varying degrees of risk of recurrence post-LT.

In this article, we review various regimens used for prevention of recurrence of HBV in post-LT patients.

Definition of HBV recurrence

The studies included in this article defined HBV recurrence as reappearance of HBsAg in patients on anti-HBV treatment who initially had clearance of this marker, unless specified otherwise.

Clinicopathological features of HBV recurrence post-LT

Lerut *et al.*[7] reported time to HBV recurrence after LT ranges between 15 and 2615 days (median of 145 days). In this study, 3/16 patients with recurrence developed fibrosing cholestatic hepatitis and died within a year of LT.

HBV recurrence can result in varying degrees of pathological damage, including mild self-limited hepatitis, chronic active hepatitis, fulminant hepatitis, and fibrosing cholestatic hepatitis.[8]

Zhang *et al.*[8] enrolled 184 patients who had received LT for HBV-related liver disease in a study, out of which 11 patients

Keywords: Hepatitis B; Recurrence; Liver transplant; Vaccination.
Abbreviations: ADV, adefovir dipivoxil; cccDNA, covalently closed circular DNA; ETV, entecavir; FTC, emtricitabine; HBcAb, hepatitis B core antibody; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBIg, hepatitis B immunoglobulin; HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LAM, lamivudine; LT, liver transplant; NA, nucleos(t)ide analog; SVR, sustained virologic response; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate.
Received: 17 January 2020; Revised: 16 April 2020; Accepted: 27 April 2020
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developed HBV recurrence. In the early stages, hepatocyte swelling, ballooning degeneration, small necrosis, periportal inflammatory cell infiltration was seen on tissue. Five patients died, while the remaining six received adefovir dipivoxil (ADV) and entecavir (ETV), resulting in improvement in histology that had manifested by a decrease in the number of liver cells showing positivity for HBsAg and hepatitis B core antigen (HBcAg), fewer nuclei with detectable HBV DNA, inconspicuous fibrous tissue proliferation, and decreased inflammation and hepatocyte swelling. Of the five patients who died, four received lamivudine (LAM) monotherapy. They developed fibrosing cholestatic hepatitis characterized by fibrous tissue development in periportal areas, bile duct hyperplasia and extensive cellular and canalicular cholestasis.

In a study including 45 patients with HBsAg-positive status who received LT and HBV treatment with HBIg or recombinant alpha interferon, Demetris *et al.*[9] demonstrated recurrent HBV infection in 33 patients. Out of these 33, 11 died due to multi-organ failure as a complication of HBV recurrence, 3 died due to recurrence of HCC, and 1 died due to intracerebral hemorrhage.

Studies including a larger patient population who experienced HBV recurrence post-LT are required to further investigate the clinical and pathological implications of HBV recurrence after LT.

Mechanism of HBV recurrence after LT

Covalently closed circular DNA (cccDNA) is a template for transcription for hepatitis B viral RNA (Fig. 1). When grafts from donors with history of HBV infection are used, the graft hepatocytes may contain cccDNA, accompanied by its replicative potential.[10] No currently approved drugs target the elimination of cccDNA, resulting in risk of HBV recurrence.

It is proposed, however, that certain genotypes of HBV may have higher risk of causing recurrence. Devarbhavi *et al.*[11] demonstrated such potential to be present in genotype D, with a higher mortality risk as well, when compared with genotype A.

Jiang *et al.*[12] analyzed the genomic DNAs of LT recipients who suffered from HBV-related liver disease and found that recipients with CTLA-4 +49 GG genotype had a lower risk of recurrence than those without the genotype (p=0.032). This finding suggests that the genetic variations of recipients may be associated with the risk of recurrence.

Occult HBV infection is defined as HBsAg-negative status with detectable HBV DNA in serum or liver specimen (Fig. 2). Ferrari *et al.*[13] found 4.4% of patients with cirrhosis undergoing LT to have occult HBV infection, according to results from a nested polymerase chain reaction assay. However, this study was conducted in Brazil, where the prevalence of HBV infection is low, limiting its applicability to other parts of the world with higher HBV prevalence. Nevertheless, this study manifests the risk of HBV recurrence post-LT due to occult infection.

LT patients often undergo immunosuppressive therapy, which may lead to increased viral replication. *In vitro* studies have shown direct stimulation of HBV replication by immunosuppressants, especially by steroids which can act on the corticosteroid response element in the HBV DNA, resulting in increased transcription of the HBV DNA.[14,15] Immunosuppressive effect on the host innate and adaptive immune cells may also result in unopposed viral replication, followed by an aggressive immune response after the immunosuppressants are withdrawn, ultimately resulting in liver injury.[14]

Prevention regimens

HBIG monotherapy

The proposed mechanism of action of HBIg includes binding to the viral particles and HBsAg, resulting in neutralization and

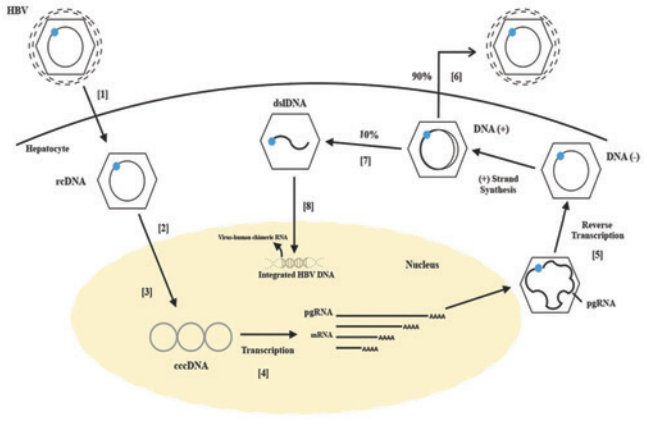

Fig. 1. Simplified illustration of HBV cell cycle.[16]

The viral envelope proteins, such as HBsAg, bind to host cell surface receptors, resulting in endocytosis of the hepatitis B virion into the host cell.[1] The nucleocapsid releases relaxed circular HBV DNA (rcDNA) into the nucleus,2 which is converted into covalently closed circular double stranded DNA (cccDNA).[3] The cccDNA is a template for transcription of viral RNA.[4] The transcribed pregenomic RNA (pgRNA) undergoes reverse transcription,[5] forming rcDNA. At the endoplasmic reticulum, virions assemble (not shown in diagram)[6] and the mature virions are excreted from the host cell via budding. Occasionally (10%), double-stranded linear DNA (dslDNA) is produced,[7] which can be integrated into the host genome.[8]

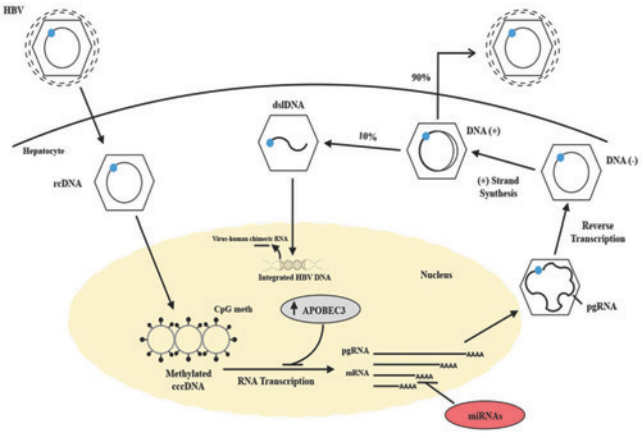

Fig. 2. Selected proposed mechanisms involved in occult HBV infection.[17,18]

Methylation of covalently closed circular DNA (cccDNA) correlates with decreased HBV replication. Chronic HBV infection can upregulate the production of *APOBEC* genes, which is associated with hyperedited HBV genome and decreased HBV replication. MicroRNAs are small, non-coding molecules found in viruses that function in RNA silencing and post-transcriptional mechanisms which may be involved in decreasing viral replication. These mechanisms do not result in complete viral suppression and low-level replication may persist. These mechanisms may be reversible, resulting in overt infection.

thereby preventing viral attachment to the hepatocytes. Infected hepatocytes express HBsAg, which the HBIg binds to, resulting in cell-mediated cytotoxicity.[19] Monoclonal HBIg has been shown to decrease secretion of wild-type HBsAg but not of mutant HBsAg from infected cells, suggesting that HBIg may be internalized in hepatocytes.[20] With the use of HBIg, McGory *et al.*[21] found a significant improvement in patient survival at an average follow up period of 22.7 months, with the prevention of HBV recurrence in >82% of patients post-transplant, regardless of the presence or absence of hepatitis B e antigen (HBeAg) or HBV DNA pre-transplant. The dose of HBIg required varied amongst the patients and was individualized. Patients who were HBeAg-positive were noted to require higher doses to maintain the serum anti-HBs titer at a desired level. High doses and long-term HBIg were used in many patients due to risk of late recurrence, with one out of twenty-seven patients developing reappearance of HBsAg at 1.5 years after LT. Though this is a single-center study, including a small population, larger studies have also shown favorable outcomes with the use of HBIg. However, HBV DNA detectability is a major risk factor for recurrence, but this information was only available for seven out of the twenty-seven patients in this study.

Samuel *et al.*[22] conducted a multicenter study including 372 patients. They reported a recurrence of HBV infection in about 75% of the patients who received no or only short-term (2 months post-LT) HBIg. With the use of long-term (6 months or more) HBIg, they showed a 3-year actuarial risk of recurrence of about 56%, compared with about 78% for patients receiving no immunoprophylaxis. Though this is a retrospective analysis, it includes a large patient population from 17 European centers.

The mechanism of recurrence may include saturation of HBIg by high viral count or mutations in the HBsAg due to emergence of antibody-induced escape HBV mutants resulting in inadequate treatment.[23–26] Mutations mostly occur at codon 145 of HBsAg, leading to a glycine-to-arginine substitution, which has been seen in post-LT patients receiving HBIg and HBV vaccine recipients.[25,27]

In patients with renal failure, the risk of worsening of the kidney function appeared to be a major concern with ADV and tenofovir disoproxil fumarate (TDF). Additionally, HBIg was administered intravenously, which can be quite inconvenient for long-term treatment. The safety and efficacy of subcutaneous HBIg monotherapy was investigated in a prospective study, showing 100% success rate (no HBV recurrence) after a mean follow-up period of 36±5 months, without worsening of kidney function; this suggested that HBIg monotherapy was not only highly effective in preventing recurrence but also not associated with deleterious renal effects, which is a risk with the use of certain NAs.[28] Target antibody levels for patients at high risk of recurrence (such as those positive for HBV DNA pre-LT) was ≥200 IU/L, and those at lower risk was ≥150 IU/L. However, it is important to note that this study included only 43 patients, all Caucasian in origin, all HBsAg- and HBV DNA-negative at inclusion. Most of these patients received a combination of HBIg (intravenous) and NA initially after LT, which was switched to HBIg IV monotherapy after 1 year, followed by a switch to subcutaneous HBIg. Randomized controlled trials, with larger patient populations, are required to support the findings in this study. Moreover, it is important to note that renal failure has now become less of a concern with the advent of tenofovir alafenamide (TAF).

Though the use of HBIg has been reported to prevent recurrence by neutralizing HBsAg, its failure may be attributed to its inability to inhibit viral replication,[29] which argues against the use of HBIg alone. Antibody-induced escape HBV mutants can arise, resulting in failure of HBIg treatment. The high cost of HBIg is also a major concern, especially with long-term, high dosage use. Additionally, it has been reported to cause mercury toxicity,[23,30] due to thimerosal in the HBIg treatment, when long-term and high-dose HBIg is used. HBIg is administered parenterally and is associated with severe back pain, anaphylactic reactions, tremors and hypotension. Given these concerns and the fact that there was still a risk of HBV recurrence, albeit significantly lower than without any prophylaxis, alternative management strategies, including the use of NAs, have increased in favor.

NAs with or without HBIg

Nucleoside analogs

LAM/ADV plus HBIg: LAM is a nucleoside analog which inhibits the reverse transcriptase of HBV, thereby inhibiting viral replication. Mutimer *et al.*[31] used LAM monotherapy to treat HBV prior to LT and continued it as prophylaxis against recurrence. Ten patients were included and started on LAM at least 4 weeks prior to LT. Recurrence of graft infection with LAM-resistant virus was observed in 50% of the patients. Recurrence was mainly seen in patients with high viral replication and resistant viremia even prior to LAM exposure. This study suggests LAM monoprophylaxis was inadequate and highlighted the importance of LAM resistance. The use of HBIg in combination was proposed to neutralize the LAM-resistant species. However, this was a small study, including only 10 patients.

A larger, multicenter USA-Canadian trial of LAM demonstrated that 60% of the patients remained HBsAg-negative at 12 or more weeks post-transplant with the use of LAM alone.[32] In this study, 47 HBsAg-positive patients were included. LAM was started pre-transplant and was continued for 5 years with the first test for recurrence or HBsAg-positive status at 12 weeks post-LT. An important finding in this study was that 80% of HBV DNA-positive patients at baseline had recurrence at the 156th week of treatment, as compared with 0% of patients who were HBV DNA-negative at baseline. Though this is a large study with a long follow-up period, the long-term consequences of LAM resistance resulting in HBV recurrence and other clinical outcomes were not investigated.

Other, smaller studies on combination of HBIg with LAM showed significant reductions in the recurrence, to 3-4%[33–35] (Table 1). Lower doses of HBIg were used,[36] possibly reducing cost compared with HBIg monotherapy and prophylaxis. It is important to note that in one study with 0% recurrence, only six patients were included, all of who were HBV DNA-negative at the time of LT.[34] In the other two studies with higher rates of recurrence, patients with positive HBV DNA at the time of LT were included.[33,35] This raises the concern that the HBIg and LAM combination may only be safe to use in patients with low risk of HBV recurrence, limiting its use.

Beckebaum *et al.*[37] recruited 371 patients in a study to evaluate the recurrence of HBV infection post-LT with long-term HBIg use. Prior to LT, 217 patients received an NA, whereas 347 received an NA post-LT. LAM was the most frequently used NA. The population of all patients who received HBIg included 299/371 who received intravenous HBIgB, 236 of which were switched to subcutaneous HBIg, and 136 patients who received another HBIg product. The total

Table 1. Main results of studies using nucleoside or nucleotide analogs with hepatitis B immunoglobulin

Reference	Patients, *n*	Median follow-up, months	NA	Patients with detectable HBV DNA at LT, *n*	HBIg use	HBV recurrence
Yao, *et al.*[33]	10	15.6	LAM	2	45 mL (10,000 U) IV HBIg daily for 7 days, then 5 mg IM HBIg for weekly for 4 weeks, then every 3 weeks	10%
Yoshida, *et al.*[34]	6	44.3	LAM	0	2170 IU IM intraop. and daily for 14 days, then twice weekly, then every 2-4 weeks. By 1 year post-LT	0%
Marzano, *et al.*[35]	25	31	LAM	7	46,500 IU in first mo. Post-LT, then 5000 IU/ month.	4%
Beckebaum, *et al.*[37]	371	78[a]	LAM or LAM/ ADV	101/239[b]	IV HBIgB: 238 IU daily Sc HBIg: 71 IU daily Other HBIg: 71 IU daily	4.3%
Darweesh, *et al.*[42]	44 (18 in ETV+HBIg group, 14 in other NA +ETV+HBIg group, 10 in other NA+HBIG group and 2 in ETV+other NA group)	~96	ETV Other NA (TDF or ADV)	38	2000 IU IM in anhepatic phase, then 1600 IU daily till negative HbsAg after LT and HBsAb >500 IU/L, then 800 IU/week with subsequent decreasing HBsAb titer goal over 12 months	ETV+HBIg: 0% ETV+other NA+HBIg: 0% Other NA +HBIg: 30%
Shen, *et al.*[43]	5333 total Group A: *n*=4684, received HBIg +LAM Group B: *n*=491, received HBIg+ETV Group C: *n*=158, received HBIg and ADV	~42.1	LAM, ETV and ADV	Group A: 1024 Group B: 40 Group C: 17	2000 IU in anhepatic phase, followed by 800 IU daily for next 6 days, then weekly for 3 weeks, then monthly	At 5 years Group A: 4.7% Group B: 1.5% Group C: 4.4%

[a]Mean follow up period

[b]239 patients out of 371 had HBV DNA serologies available.

Abbreviations: ADV, adefovir dipivoxil; ETV, entecavir; HBIg, hepatitis B immunoglobulin; HBsAb, hepatitis B antibody; HBV, hepatitis B virus; IM, intramuscular; intraop., intraoperative; IV, intravenous; LAM, lamivudine; NA, nucleos(t)ide analog; sc, subcutaneous.

durations of treatment were 8993, 8379 and 5392 months respectively. The mean follow-up time was 6.8±3.5 years. Recurrence was noted in 4.3% of the patients (16/371), out of which 5/16 had discontinued HBIg and 7/16 had anti-HBs levels of less than 100 IU/L.

Though this study had an adequate patient population with a long follow-up period, its retrospective design is a limitation. Since it is a non-interventional study, anti-HBs levels were not routinely documented.

ADV had a better resistance profile compared to LAM.[38] In a systematic review, Cholongitas *et al.*[39] showed recurrence rates of as low as 2% in patients who received ADV and HBIg combination regimen with or without LAM, significantly lower than with LAM alone (p=0.024). They also demonstrated ADV prophylaxis, without HBIg, with or without use of LAM (0% recurrence), to be superior to LAM monoprophylaxis (recurrence of 25.4%). However, the patient population receiving ADV prophylaxis without HBIg was small (47 patients) and the post-LT follow-up period was short (median of 16 months). Larger studies with longer follow-up periods are required to support these findings. Nevertheless, high cost with risk for development of viral resistance and nephrotoxicity has limited the use of ADV.[40]

LAM/ADV versus ETV and/or tenofovir: According to the AASLD 2018 guidelines, ETV, TDF and TAF are preferred over LAM and ADV due to their higher potency and lower rates of drug resistance.[41] In a recent retrospective study including 44 patients (86% with positivity for HBV DNA at the time of LT), none of the 34 patients receiving the combination of ETV with HBIg tested positive for HBsAg in the 8 year follow-up period.[42] However, four out of the fourteen patients on ETV plus another NA (TDF/ LAM) with HBIg developed recurrence with positive HBsAg, which eventually converted to HBsAb towards the end of the 8 year follow-up period. There was no evidence of clinically significant hepatitis or presence of HBV DNA in these patients.

Shen *et al.*[43] used a national database to demonstrate better efficacy of ETV/HBIg prophylaxis compared to LAM/HBIg use with the 1-year, 3-year and 5-year recurrence rates of HBV of 0.5%, 1.5% and 1.5% with ETV/HBIg, respectively compared to 1.7%, 3.5% and 4.7% with LAM/HBIg (p=0.023). Before LT, patients with positive HBV DNA received one NA daily, which was then continued post-LT.

Although both the studies mentioned above were retrospective, they included adequate population size with long follow-up periods, both showing very low recurrence rates.

LAM/ADV with HBIg withdrawal: Studies have shown that the use of NAs has made it possible to eventually withdraw HBIg in patients who were initially started on a combination regimen (Table 2). Now, the American Association for the Study of Liver Diseases, the European Association for the Study of the Liver and the Asian Pacific Association for the Study of the Liver guidelines include an option of HBIg withdrawal post-LT in patients with low risk of recurrence, such as those with low or undetectable HBV DNA levels prior to transplant or without resistant HBV.[44–46]

Until 2010, the standard prophylactic regimen included indefinite HBIg and LAM. In 2011, Saab *et al.*[47] maintained 61 patients on a combination of HBIg and LAM for 12 months post-LT and then withdrew HBIg. Three months before withdrawing HBIg, a NA (TDF or ETV) was added to the regimen. Within a mean follow-up period of around 15 months after conversion to oral antivirals only, two patients (3.3%) presented HBV recurrence, which was similar to the recurrence rate when using LAM and long-term HBIg.[48] In addition, this regimen was noted to be more cost effective than the standard regimen using lifelong HBIg. However, for dual nucleoside and nucleotide analog combinations renal toxicity was a major concern. In one of the two cases with the recurrence in this study, dose of oral antivirals had to be decreased due to renal injury which may have led to the recurrence. Moreover, the study population only included patients with low risk of recurrence, such as undetectable HBV DNA at the time of transplant and no viral co-infection.

ETV/TDF/TAF with HBIg withdrawal: Lee *et al.*[5] administered 10,000 IU during the anhepatic phase and during surgery followed by 2000 IU daily for a week postoperatively. Two hundred and thirty-two patients were divided into groups labeled Q and S (those with quick decline of anti-HBs titers (<200 IU/mL; 1-month post-operation) and those with slow decline of the titers (>200 IU/mL 1-month post-operation) respectively. From postoperative day 1, NA (ETV or TDF) was started and continued indefinitely. Patients in the Q group received HBIg boosters to maintain HBIg titers. HBV recurrence was found to be 18.9% in group Q and 7.3% in group S. This study suggests using long-term HBIg in patients with quick decline of HBIg levels. Group Q had patients with higher MELD scores and higher HBV viral loads compared with group S, representing important confounders. However, multivariate analysis was done which did not show these variables to be significantly different.

In a single-center, retrospective study, LAM, ETV, TDF, and TAF were used alone or as two in combination to study the impact of these drugs on the renal function.[49] HBIg was used for a mean of 633 days (standard deviation (± 552 days) in 79 patients with the NA(s) after which HBIg was stopped and the NA(s) were continued. Patients were followed up for a mean of 1723 days (standard deviation (±1164) after HBIg withdrawal. There was no significant change in the serum creatinine or glomerular filtration rate compared before and after using TAF but there was an increase of 0.55 mg/dL of serum creatinine in patients who were never on TAF (p<0.05). Up to 6% of patients on TAF experienced an increase in chronic kidney disease stage compared with 23% of the patients who received NA(s) other than TAF. However, there was no standard immunosuppression protocol used in the patient population included in this study. Immunosuppressants are known to cause renal dysfunction, which could have been a significant confounding factor regarding observed changes in renal function. This factor was not adjusted for in this study.

NA without HBIg

A prospective, multicenter study used the combination of ADV and LAM and showed prevention of HBV recurrence post-LT in all patients after a median follow-up period of 5 years, with the median time of HBsAg undetectability being 7 days. HBV recurrence in this study was specified as reappearance of both HBsAg and HBV DNA. Of the 20 patients, 13 tested positive for HBV DNA at the time of LT (1 patient was not tested). HBIg was given during the anhepatic phase and daily for only 7 days after LT, whereas LAM and ADV were continued long term. Of note is the fact that >50% of the patients were at high risk of recurrence (i.e. having detectable HBV DNA at the time of transplant).[50] However, this study included a small study population without a randomized control group and the risk of viral resistance with LAM and ADV is still a concern.

Stravitz *et al.*[51] demonstrated successful substitution of HBIg/LAM combination with TDF/emtricitabine (FTC) combination, preventing post-LT HBsAg recurrence in 18/21 patients and HBV DNA recurrence in 20/21 patients. In the latter case, recurrence occurred in the patient who was non-compliant, and after resuming TDF/FTC, HBsAg and HBV DNA became undetectable. This allowed for cost savings of about $12,500 per year, as compared to HBIg/LAM regimen. However, it must be noted that the 13/21 had negative HBeAg and 8/18 had undetectable HBV DNA at the time of LT, making the population included in this study largely at low risk for recurrence.

Fung *et al.*[52] studied the long-term outcome of ETV monotherapy post-LT in a larger cohort of 265 patients, with >60% of patients with detectable HBV DNA at the time of LT, and demonstrated HBsAg clearance rate of 92% and HBV DNA undetectable rate of 100% at 8 years post-LT. Although this study suggests favorable outcomes with ETV monotherapy, six patients did receive an addition of tenofovir in addition or were switched to tenofovir due to concern for no or delated virologic response. In a large meta-analysis including 17 studies with a total of 7274 patients, ETV monoprophylaxis, when compared with LAM (odds ratio of 4.62), TDF (odds ratio of 1.11), ADV (odds ratio of 3.78), LAM+TDF (odds ratio of 2.00) and LAM+ADV (odds ratio of 2.83), was found to have the lowest probability of HBV recurrence, making it the most preferred oral agent for prophylaxis.[53] However, a major limitation in this analysis is the lack of information on reappearance of HBV DNA after treatment in the studies included in this meta-analysis. Other sources of bias include different HBIg and antiviral protocols used in the studies.

In all studies mentioned above, NAs were continued indefinitely.

Withdrawal of both HBIg and NA

Recipients who have negativity for HBeAg and undetectable HBV DNA have been historically noted to have lower rates of

Table 2. Main results of studies using HBIg/NA combination therapy with eventual withdrawal of HBIg

Reference	Patients, *n*	Median follow-up, months	NAs	HBIg protocol	HBV recurrence
Lee, *et al.* [5]	232	42.2	Either LAM or ETV	10,000 IU at anhepatic phase and during surgery. 2000 IU daily for a week post-LT	12.1% (18.9% in group Q and 7.3% in group S), $p = 0.013$
Vasudevan, *et al.*[80]	18	60	LAM started at 48 h after LT (100 mg daily). At 12 months, HBIg substituted by TDF.	800 IU at anhepatic phase, 800 IU daily for a week post-LT, then 800 IU twice weekly for weeks 2-4 post-LT, then 800 IU monthly till 12 months post LT	11%
Saab, *et al.*[47]	61	15.0 (±6.1)	LAM or ETV and ADV or TDF	Standard protocol[1a] with at least 12 months of IM HBIg	3.3%
Sabela, *et al.*[81]	338	72	LAM	5000-10,000 IU on post-LT day 0, then 5000-10,000 IU/day for 1 week, then 1000 IU/week for 1 month, followed by 1000 IU at different intervals to maintain target HBsAb target	11%
Manini, *et al.*[82]	77	69 (group A – HBV monoinfected), 61 (group B – HBV/HDV co-infection)	TDF or ETV	Post-1998, 5000 IU IV during anhepatic phase, then 5000 IU right after LT, then 5000 IU on alternate days during week 1 post-LT, then 5000 IU to keep HBsAb ≥500 till discharge, 1-4000 IU to keep HBsAb ≥ 250 1-6 months post-LT and 1-4000 IU to keep HBsAb ≥ 100 >6 months post-LT	Group A: 9% Group B: 0%
Teegan, *et al.*[83]	352	Retrospective analysis	LAM, ETV or TDF	10,000 IU at anhepatic phase followed by different modes of prophylaxis[2b]	33.8% in patients positive for HBsAg at LT 10.0% in patients negative for HBsAg at LT
Radhakrishnan, *et al.*[84]	42	Retrospective study	TDF or ETV or TDF/FTC	5000 IU in anhepatic phase and daily for 5 days only	Cumulative recurrence at 1, 3 and 5 years was 2.9%
Saab, *et al.*[49]	79	Retrospective study	LAM, ETV, TDF, TAF and ADV used alone or LAM + TDF or ADV, ETV + TDF or ADV	Mean number of days from LT to HBIg withdrawal: 633 days (SD ±552)	13.9%

[a]After 1998, IV HBIg 10,000 IU during anhepatic phase, 2000 IU daily postoperative days 2-7 and 2000 to 10,000 IU on postoperative day 20 to keep HBsAb titers >500 IU/mL, followed by 1560 IU every 2-4 weeks to keep titers >500IU/mL at 0-6 months, >250 IU/mL at 6-12 months and >100 IU/mL after 12 months.
[b]12 patients received no long-term prophylaxis, 97 received HBIg monoprophylaxis, 221 received HBIg+LAM, 22 received HBIg+ETV or TDF. Abbreviations: ADV, adefovir dipivoxil; ETV, entecavir; HBIg, hepatitis B immunoglobulin; HBsAb, hepatitis B surface antibody; HBV, hepatitis B virus; HDV, hepatitis D virus; IM, intramuscular; IV, intravenous; LAM, lamivudine; LT, liver transplant; NA, nucleos(t)ide analog; SD, standard deviation; TDF, tenofovir.

recurrence compared with recipients with positivity for viral markers.[54,55] In fact, the risk of recurrence has been noted to be directly proportional to viral replication pre-LT.[56] A retrospective study reported on 10 post-LT patients who initially received HBIg and NA but completely stopped it after a mean time from LT to withdrawal of around 24 months due to

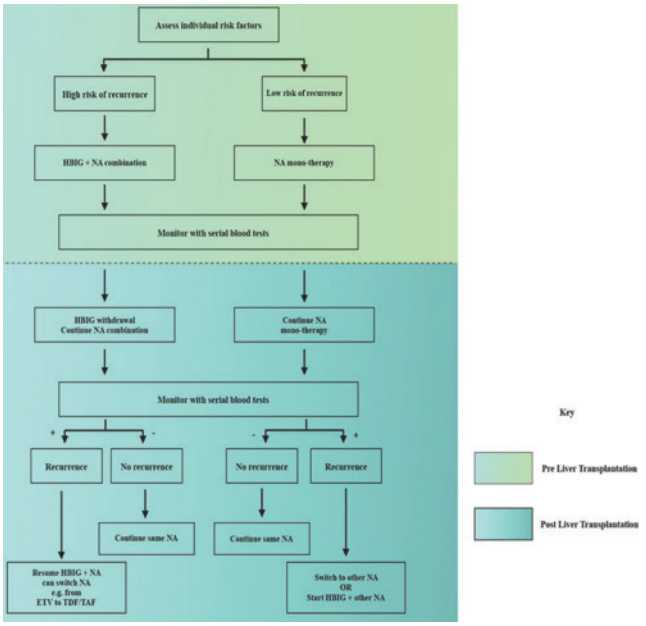

Fig. 3. An algorithm for suggested approach towards prevention and management of HBV recurrence in post-liver transplant patients.

Serial blood tests include those for hepatitis s antigen (HBsAg) and HBV DNA. + refers to positive test result or detectable markers. – refers to negative test result or undetectable markers.

Abbreviations: DNA, deoxyribonucleic acid; ETV, entecavir; HBIG, hepatitis B immune globulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NA, nucleoside analog; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate.

non-adherence. They were followed for a mean of 52 months after withdrawal with monthly tests for HBV markers, including HBV DNA and liver function biomarkers. Out of 10 patients, 9 did not develop recurrence.[56] It is important to note, however, that these nine patients tested negative for HBeAg and undetectable HBV DNA prior to LT and are considered 'low risk' for recurrence. They also maintained low tacrolimus levels (<3 ng/mL).

Tian *et al.*[57] reported a case of complete prophylaxis withdrawal in a patient at high risk of recurrence, having HBeAg positivity and detectable HBV DNA at the time of liver transplantation. This patient received initial treatment with HBIg and ETV and then was maintained on this regimen as prophylaxis for recurrence. The patient stopped taking the prophylaxis for economic reasons after 3 years following the LT and was followed up with monthly tests for viral markers (HBsAg, HBeAg, HBV DNA) for 4 years; all remained negative and HBsAb remained positive. Interestingly, this patient also maintained low levels of tacrolimus (1-2 ng/mL). However, this data was published in a case report and larger studies are required to investigate the HBV recurrence rates with complete withdrawal of treatment.

Whereas the observations in the aforementioned studies are results of patient non-adherence, Lenci *et al.*[58] employed a protocol with stepwise withdrawal of HBIg and NA. Thirty patients with positivity for HBsAg, negativity for HBV DNA, normal liver function and undetectable covalently closed circular DNA (cccDNA) in liver tissue, who received HBIg and NA since LT, were included. HBIg was withdrawn 6 months after the beginning of the screening phase and NA was withdrawn 6 months after withdrawal of the HBIg. Recurrence was noted in six (20%) of the patients, five of which had an early recurrence (between 2-4 months of HBIg withdrawal). Out of these five patients, three did not require treatment due to spontaneous seroconversion to HBsAb within 4 months of recurrence and HBV DNA levels remained negative throughout. However, with extended follow-up of 6 years post-withdrawal, 60% of the patients had experienced seroconversion to HBsAg but only 10% required treatment and 100% were HBV DNA negative. Though this withdrawal strategy saved about $20,000 per patient per year, it only applies to patients with low risk of recurrence. Larger studies are required to assess the safety and efficacy of withdrawal strategies.

Role of active vaccination

A single-center prospective study to investigate the efficacy and safety of active vaccination in patients was conducted, in which each patient was given double doses of the intramuscular vaccine at 0, 1, 2, 6 and 12 months of enrollment, with a follow up period of 6 months after completion of the vaccine protocol.[59] Out of 27 men included in this study, 9 were responders (33.3%). All the patients in this study were at least 1-year post-LT and were HBsAg- and HBV DNA-negative, with normal liver functions. They were all receiving HBIg and NA, and HBIg was stopped after the vaccination protocol was complete at 12 months. They were followed for 6 months after completion of the vaccination protocol and HBIg withdrawal. Throughout the follow-up period, the patients maintained their HBsAg-negative status. Although this study suggests that active immunization in patients who receive LT due to HBV-related disease is feasible and allows for HBIg withdrawal, the low vaccine response rates, mainly because of immunosuppressed states of the post-LT hosts, has limited its use in this patient population.[60,61]

Ishigami *et al.*[62] showed that frequent active vaccinations in post-LT patients can lead to production of escape mutants. This study included 18 HBV carriers and 7 non-HBV carriers who were recipients of grafts from HBcAb-positive donors. Of the 18 HBV carriers, 4 had detectable HBV DNA pre-LT. All patients received HBIg and NA and active vaccination was administered 1-year post-LT. Two of the HBV carriers and six of the non-HBV carriers were responders. In these patients, NAs were stopped after a successful vaccine response was obtained, and booster vaccinations were administered as needed. At a median of 12 months, two HBV carriers and two non-HBV carriers had detectable HBV DNA. Univariate analysis was done to investigate factors associated with viremia and frequent vaccination was found to be a significant risk factor. Moreover, amino acid sequencing showed several mutations, including the a-determinant in the HBV loop which plays an important role in the recognition of HBsAb proteins. Though this is a small study that only allowed for a univariate analysis, it cautions against NA withdrawal in HBV carriers and frequent vaccinations in post-LT patients. Larger prospective studies are required to establish the safety and efficacy of withdrawing HBIg and/or NAs in patients with prior HBV-related disease. Additionally, efforts should be made to improve the response rate of the vaccine.

Management of fulminant liver failure due to HBV recurrence Post-LT

With the advent of newer NAs, the risk of fulminant liver failure due to recurrence of HBV after LT has been significantly reduced.

In the study by Zhang *et al.*,[8] all 11 patients with HBV recurrence were on LAM, out of which 5 died. Four out of these five patients developed fulminant liver failure with jaundice and deteriorating liver function. The remaining six who survived were switched from LAM to either ADV or ADV/ETV combination in the early phases of recurrence, which resulted in improvement in their graft histology, including decreases in inflammation and hepatocyte swelling.

Roche *et al.*[63] reported successful re-transplantation in five patients who experienced failure of LT due to HBV recurrence with the use of ganciclovir and HBIg combination. However, though this study demonstrates retransplantation as a feasible option in fulminant hepatic failure post-LT due to HBV recurrence, the antiviral regimen used in this study is only of historical value now with the advent of newer NAs, such as TDF/TAF and ETV.

Prophylaxis of *De Novo* HBV infection from HBcAb-positive donors

Studies have defined *de novo* HBV infection according to positive viral markers (HBsAg and detectable HBV DNA) after transplant in recipients who were negative for these markers pre-transplant.[64,65] In the absence of prophylaxis, there was a high rate of HBV transmission from HBcAb-positive donors to HBsAg-negative recipients.[6] It is presumed that livers from HBcAb positive donors may contain cccDNA and pregenomic RNA in the hepatocyte nucleus which may result in *de novo* infection.[66] However, given the scarcity of suitable liver grafts and the significant end-stage liver disease burden, HBcAb-positive donors have been used to expand the donor pool.

A cohort study in Italy suggested that transplant using HBcAb-positive donors have comparatively favorable outcomes when the recipients were HBsAg-positive, as opposed to HBsAg-negative, with the latter resulting in suboptimal graft quality.[65] However, there are some important confounders that might explain this finding. The HBsAg-positive recipient group in this study had lower MELD scores, fewer recipients with concomitant HCV infection compared to the HBsAg-positive group, and the HBsAg-negative group received less rigorous prophylaxis.

On the contrary, a recent study conducted in China reported similar short-term and long-term outcomes using HBcAb-positive donors, irrespective of the HBsAg status of the recipients.[64] All the patients received HBIg. Patients who were treated with HBIg monotherapy had a higher rate of *de novo* infection as compared with HBIg and NA combination. Multivariable adjustment and propensity-score matching was performed to equilibrate selection bias and potential confounders between study groups (HBcAb-positive and HBcAb-negative recipients). Nevertheless, this is a retrospective study and is based in a single center, being subject to confounders and biases.

Wong *et al.*[67] reported *de novo* infection in 4.7% of their studied patients who received LAM monotherapy while all patients receiving ETV monotherapy remained free of infection, likely due to a high resistance barrier with the latter. Recipients in the HBcAb-positive donor group had a graft survival of ~77% versus ~78% in the HBcAb-negative donor group, with almost no difference in patient survival between the two groups. They also proposed active immunization as a therapeutic form of management, which may render the need for prophylaxis unnecessary. Though this is a retrospective, single-center study, it involved a large cohort with a long follow-up time (median of 7.8 years).

Active immunization appears to be a promising strategy towards preventing *de novo* hepatitis B infection after LT from HBcAb-positive donors. Ohno *et al.*[68] used HBIg in the peri- and post-transplant period with multiple administrations of active HBV vaccination, with a target to maintain HBsAb levels >300 mIU/mL for 1 year and >100 mIU/mL subsequently. When the target was achieved without HBIg, active immunization was achieved. No patient tested positive for HBsAg or HBV DNA at 112 months after achieving active immunization. However, most of the fast responders in this

Table 3. Future drug targets for treatment of hepatitis B infection

Therapies in Clinical Development		
Drug Class	Drug name	Mechanism of action
Entry inhibitors	Bulevirtide	Inhibit viral entry by inhibiting NTCP, an HBV receptor[75]
RNA interference/siRNA	ARC-520 AB-729	Bind to complementary mRNA, resulting in its elimination[76]
Core protein inhibitors	AB-506 RO7049389	Bind to hydrophobic pocket at dimer-dimer interface, resulting in allosteric conformational changes in core protein with inability of nascent capsids to encapsidate viral RNA[77]
TLR agonists	GS-9620 (TLR 7 agonist) GS-9688 (TLR 8 agonist)	Trigger TLRs that result in production of antiviral cytokines (interferon-α and -γ) and activation of natural killer and T cells[78,79]
Future drug targets (not enrolled in clinical trials yet)		
PAPD5/7 inhibitors	Inhibit catalytic domains of PAPD5 and PAPD7 enzymes that result in destabilization of HBV mRNA[72]	
Direct cccDNA targeting	Use of zinc finger nucleases can directly edit DNA[73,74]	

Abbreviations: cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; siRNA, small interfering ribonucleic acid; TLR, Toll-like receptor.

study were in the pediatric population and the slow responders were mainly adults.

Management of HBV infection from HBsAg-positive donors

Wei *et al.*[69] recruited 518 patients with HBV infection and divided them into two groups consisting of 259 patients each: one group received HBsAg-positive donor organ (observational) and one received HBsAg-negative donor organ (control). After LT, LAM, telbivudine, ETV and/or ADV were used for HBV treatment in both groups. The HBV recurrence rates at 1-year, 3-years and 5-years post-LT in the observational group versus the control group were 5.85% versus 1.97%, 11.63% versus 4.46% and 17.94% versus 4.46%, respectively (p=0.016). However, when early stage complications (within 30 days post-LT; such as pleural effusion, postoperative infection and transplant graft dysfunction), long-term complications (more than 30 days post-LT; such as postoperative infection, rejection and vascular complications), and patient survival at 1-year, 3-years and 5-years post-LT were compared between the two groups, no significant difference was found. This study suggests that HBsAg-positive donors can be used with appropriate NA use. However, this was a retrospective study, including data from 2007 to 2012, during which time at least half the patients used LAM, which limits its applicability to current time with the advent of newer NAs. Prospective studies using newer antivirals are required to evaluate the HBV recurrence rate in patients receiving transplants from HBsAg-positive donors.

Jeng *et al.*[70] recruited 14 patients with HBV (HBsAg positivity) who received LT from HBsAg-positive donors. All patients received ETV, to be continued indefinitely. In the follow-up period (median of 46 months), two died in the 13th and 33rd month respectively due to extrahepatic recurrence of HCC but both had undetectable HBV DNA levels at year 1. The rest of the patients maintained undetectable HBV DNA levels throughout the follow-up period. However, six of the fourteen recipients had undetectable HBV DNA levels prior to LT and all patients continued to have HBsAg positivity despite undetectable HBV DNA levels in the follow-up period. Larger, randomized studies with control groups are required to validate the efficacy of use of ETV in managing HBV infection in LT recipients from HBsAg-positive donors.

Emerging drug targets and future directions

The studies discussed above demonstrate NAs to be highly effective in preventing or managing HBV recurrence post-LT. However, their inability to inhibit cccDNA means that the replicative capacity is still present in the host nuclei. For the management of HBV, new drug targets, such as cccDNA, small interfering RNA-targeting viral transcripts, capsid assembly modulators and secretion of viral envelop proteins, are being proposed.[71] Some are undergoing clinical development while others are being explored and have not entered trials yet (Table 3). However, it is important to note that these drugs are being assessed for the treatment of HBV and have yet to be tested for prevention of HBV recurrence post-LT.

Other drug targets are currently being explored that have not entered clinical trials yet. Mueller *et al.*[72] found an RNA polymerase associated domain containing two proteins, PAPD5 and PAPD7 which are required for cellular RNA homeostasis. When RG7834, a potent HBV inhibitor belonging to the dihydroquinolizinone class, was made to interact with the two enzymes PAPD5 and PAPD7, destabilization and degradation of viral mRNA was seen. Technologies, such as CRISPR-Cas9 are now emerging that can directly edit DNA and can target cccDNA directly.[73,74] However, since these are non-clinical studies, it is unclear how potential drugs that directly target cccDNA would have access to all infected cells. It is also unclear if these potential drugs may have capacity to cause mutations in the host DNA, which can possibly result in carcinogenesis.

Clinical trials are required to study the efficacy and safety of using these drugs for HBV management, including in patients who received LT.

Conclusions

The mainstay of management of hepatitis B infection with prevention of its recurrence post-LT has been a combination of HBIg and NAs with high potency, such as TDF/TAF and ETV. However, other alternatives, such as combination therapy with HBIg withdrawal, HBIg monotherapy and NA monotherapy, have also been used with success. LT with anti-HBcAb-positive donors is now possible with the use of HBIg and NAs.

We suggest an individualized approach which takes patient risk factors, medication factors, cost and convenience into account (Fig. 3). For patients with high risk of recurrence, such as those with detectable HBV DNA levels at time of LT or known infection with resistant viral species pre-LT should receive HBIg for 6 months with a combination of NAs such as ETV and TDF/TAF. NAs may need to be continued indefinitely, especially if the patient has human immunodeficiency virus or hepatitis D virus co-infections. For patients at low risk of recurrence, such as those with undetectable HBV DNA levels at the time of LT, HBIg-free prophylaxis with NA monotherapy can be used. If ETV monotherapy fails, it can be switched to TDF monotherapy.

Further studies with larger patient populations are required to allow for better individualization of prophylactic protocols, which will allow for safe and cost-effective management of post-LT patients. Additionally, as noted in many studies mentioned in this review, many patients with HBsAg reappearance did not have detectable HBV DNA or deranged liver function or aminotransferases. This raises the question of the clinical significance of HBsAg positivity as a definition of HBV recurrence, raising the possible need to redefine HBV recurrence post-LT.

Acknowledgements

The support of the Herman Lopata Chair in Hepatitis is gratefully acknowledged.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Wrote and revised the review article (MN), edited the review article (GYW).

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Review Article

Body Building and Aminotransferase Elevations: A Review

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Abstract

In addition to liver injury, elevation of aminotransferases can be caused by strenuous exercise and use of muscle-building and weight-loss supplements. The purpose of this review is to discuss the various mechanisms of elevation of aminotransferases related to body building. A literature review was performed on clinical trials and case reports involving exercise or supplement use and their effects on aminotransferases. Normal aminotransferase levels varied according to gender, age, body mass index, and comorbidities. Strenuous exercise and weight lifting, especially in the unaccustomed, can cause elevated aminotransferases in the absence of liver damage. Supplements such as anabolic steroids, ephedra, and LipoKinetix, amongst others, have also been associated with aminotransferase elevations. The pattern of elevation of aminotransferases is not helpful in distinguishing liver from muscle injury. Other associated muscle enzymes can be useful in making that distinction. To prevent aminotransferase elevations, subjects not accustomed to moderate-high intensity workouts, are recommended to undertake gradual increase in intensity. When causes of liver injury have been ruled out, investigation into bodybuilding, extreme exercise, and supplement use is warranted.

Citation of this article: Villavicencio Kim J, Wu GY. Body building and aminotransferase elevations: A review. J Clin Transl Hepatol 2020;8(2):161–167. doi: 10.14218/JCTH.2020.00005.

Introduction

Aminotransferases are commonly elevated with liver injury, and therefore often used as serum markers of liver pathology. Although they are often called "liver enzymes", they are not found in liver exclusively. Therefore, conditions other than liver disease should be considered as causes of elevations. Because of the increase in popularity of bodybuilding, muscle or liver injury due to strenuous exercise as well as related to use of weight loss and muscle-building supplements is increasing in frequency. The purpose of this review is to discuss the relationship between exercise and bodybuilding and elevations of aminotransferases.

Keywords: Aminotransferases; Exercise; Weight-loss supplements; Muscle-building supplements.

Abbreviations: AAS, androgenic anabolic steroids; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase.

Received: 20 January 2020; Revised: 23 April 2020; Accepted: 8 May 2020

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Alanine and aspartate aminotransferases

Aminotransferases are enzymes that catalyze the transfer of an amino group from amino acids to oxoacids, a process known as transamination. Aspartate aminotransferase (AST; formerly known as glutamate oxaloacetate transaminase) and alanine aminotransferase (ALT; formerly termed as glutamate pyruvate transaminase) are the two aminotransferases with greatest clinical significance. Measurement of these is performed routinely for detection of hepatic disease.[1]

Organ distribution

In decreasing order of concentration, AST can be found in liver, heart, skeletal muscle, kidney, brain, pancreas, lungs, leukocytes, and erythrocytes.[1] Up to 20% of measured AST comes from the cytosol, while 80% comes from the mitochondria.[2] Cytosolic AST has a half-life of 17 h, while mitochondrial AST has a half-life of 87 h; although, most laboratories do not differentiate between them.[2] Clearance from plasma is performed by hepatocytes, sinusoidal cells, endothelial cells, and Kupffer cells.[3] Zone 3 of the hepatic acinus has higher concentrations of AST, so that damage to this zone by ischemia or toxins may result in greater levels of AST than ALT.[1]

Skeletal muscle and kidney contain lower concentrations of ALT than liver, and therefore, ALT elevation is more specific for liver damage.[4] ALT differs from AST in that it is solely present in the cellular cytoplasm.[1] Its half-life is around 47-48 h.[1,3] It is also cleared by hepatocytes and nonparenchymal cells, such as Kupffer cells and endothelial cells.[3]

General factors affecting normal aminotransferase levels

Mera *et al.*[5] compared, by age, females and males with normal levels of bilirubin and aminotransferases, and found significantly lower levels of AST and ALT in females compared to males in all decades of life except the 10th and 11th ($p < 0.05$) (Fig. 1). The median AST level was 24 U/L in females and 26 U/L in males. The median ALT was 26 U/L in females and 32 U/L in males. In the 10th and 11th decade, serum AST and ALT were higher in females compared to males, but this finding was not statistically significant.[5] Although the patients had no known history of liver disease, other confounding factors such as comorbidities, weight and social history were not stated, leaving questions about the validity of their conclusions on gender differences.

However, in a prospective study, Bussler *et al.*[6] also found higher levels of AST and ALT in boys compared to girls in a large sample size. In contrast with the Mera *et al.* study,[5]

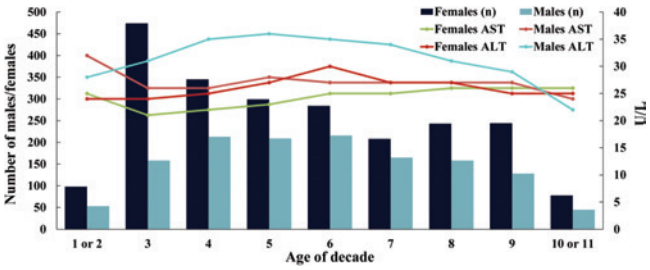

Fig. 1. A comparison of alanine aminotransferase and aspartate aminotransferase levels between females and males, stratified by decade of life.

Adapted from Mera *et al.*[5]

these subjects were healthy, not taking any hepatotoxic medications and were neither overweight nor underweight, making these results more convincing. A peak in ALT was found corresponding to puberty in both genders.[6]

In a study done in healthy subjects without prior liver disease who were hospitalized for experimental reasons,[2] AST and ALT levels were observed to increase 5% and 17.5% respectively above the upper limit of normal.[7] This was thought to be due to restricted physical activity in combination with hospital diet. Thus, in apparently healthy patients, determining the cause of elevated aminotransferases can be a difficult diagnostic problem.

Patients who engage in bodybuilding are at risk for elevated aminotransferases due to one of several potential mechanisms, including the physical activity itself or use of supplements that induce muscle and/or liver damage.

Potential mechanisms of aminotransferase elevations related to exercise/body building

Exercise-induced rhabdomyolysis

Exercise-induced rhabdomyolysis is a common consequence of strenuous exercise.[8,9] The degree of rhabdomyolysis depends on exercise experience, level of training, intensity, duration and type of workout.[9] It has been found to be more common in people with less exercise experience or who were less trained.[9] Significantly lower levels of creatine kinase (CK) and myoglobin have been found in highly experienced weightlifters compared to less experienced.[9] Other factors that play a role are: hot environments, electrolyte imbalances, nutritional deficiencies, creatine supplements, alcohol, and gender.[9]

Pal *et al.*[10] studied sedentary teenage girls and boys with normal pre-exercise AST, ALT, and CK levels who undertook an exercise regimen. Subjects taking medications or with any underlying condition were excluded. They found that CK levels were significantly higher in boys at 24 and 48 h post-exercise, with a percentage change in CK activity at 48 h of 84% in males and of 35% in females.[10] However, there was no difference in percentage of change in AST or ALT pre- and post-exercise, at 24 or 48 h between genders (Table 1).[10]

Fallon *et al.*[11] studied 7 male and 2 female subjects who had completed an ultra-marathon. They tested CK, AST, ALT pre- and post-race with follow-up tests on days 4 and 11. Before the race, all had normal transaminases and CK levels. The mean value of AST, ALT and CK were above the normal range after the race on days 4 and 11.[11] Although a small study, the results clearly showed that extreme exercise can elevate aminotransferases due to substantial muscle injury. Elevations in CK supported muscle injury. Co-existence of hepatocellular liver injury could not be entirely ruled out, although normal levels of alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) made that unlikely. The enzyme levels were still elevated at day 11, and normalization was not documented. Lactate dehydrogenase (LDH) iso-enzymes may have some value in detecting liver and muscle injury. However, although hepatocytes almost exclusively produce the M-isoform which comprises LDH-5, the latter is found in skeletal muscle as well as liver, which limits the value of LDH iso-enzymes in distinguishing liver from muscle injury.[12]

Apple *et al.*[13] studied 22 male and 8 female marathon runners, testing serum markers and gastrocnemius muscle biopsies before and after the race. There was a significant increase in serum ALT levels after the race compared to normal levels prior. However, there was no elevation in ALT in gastrocnemius biopsies (which were done on three occasions), suggesting the liver was the source of serum elevations.[13] The unknown sensitivities of the assays, as well as small sample sizes make this conclusion uncertain. Furthermore, other tests (i.e. ALP, CK, AST, GGT and LDH) were not

Table 1. CK, AST and ALT values before and after exercise in girls and boys[10]

	Mean values for CK, AST, ALT					
Variable	Gender (Group)	Before exercise (T1)	After exercise (T2)	24 h after exercise (T3)	48 h after exercise (T4)	% Change (T1-T4)
CK	Boys	139.65	141.18	253.79	257.4	84%
	Girls	126.98	128.59	162.47	168.68	35%
AST	Boys	23.95	25.27	29.2	30.72	28%
	Girls	18.48	19.5	23.72	25.51	38%
ALT	Boys	20.26	20.88	23.95	25.45	26%
	Girls	19.35	19.7	23.72	25.22	30%

Percentage of change of AST and ALT (pre- and 48 h post-exercise) between genders (n=44).
Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; CK, creatine kinase.
Adapted from Pal *et al.*[10]

done in this study, making it difficult to differentiate between damage of liver, muscle, or both.

Unfortunately, many of the studies did not present data on follow up of participants to the point of enzyme normalization. However, Pavletic *et al.*, Malinoski *et al.* and Delicata *et al.*[8,14,15] (amongst other authors) have presented case reports on patients with elevated ALT, AST and CK levels thought to be due to exercise. In those reports, normalization in enzyme levels was reported after discontinuation of physical activity.

Pathophysiology of exertional rhabdomyolysis

Acute high intensity exercise can induce oxidative stress and muscle damage especially in combination with other extrinsic factors, such as temperature, humidity, and medication use.[7,16] Disruption of the sarcolemma can release intramuscular proteins into serum, including CK, LDH, myoglobin, aldolase, AST and ALT.[16] Under resting conditions, ATP-dependent ion channels keep intracellular calcium and sodium at low levels and potassium at high levels. Any insult that damages the ion channels or depletes ATP can cause an imbalance of electrolyte concentrations, increasing intracellular sodium and calcium.[7,16] Likewise, with intense exercise, ATP is depleted and calcium concentration increases. These electrolyte imbalances can lead to cellular edema and activation of calcium-dependent proteases and phospholipases that ultimately result in functional degradation of cell signaling systems and decomposition of cell membrane, with release of enzymes into the extracellular space and eventually into the blood stream.[7,16]

Based on several case reports, it is thought that the risk of exertional rhabdomyolysis is higher with eccentric muscle training and high-intense work-outs, which may include low-weight high-repetition workouts in the unaccustomed.[17] As described by Armstrong *et al.*,[18] rhabdomyolysis can occur faster with exercise in the setting of heat strokes. High temperature increases muscle membrane permeability and is, therefore, a risk factor for rhabdomyolysis.[18]

Pattern of aminotransferase elevations in rhabdomyolysis

A study conducted on healthy men with normal baseline laboratory tests who engaged in moderate physical activity (but not weightlifting) found elevated ALT, AST, LDH, CK and myoglobin levels at 1 h after heavy weightlifting.[19] AST was noted to increase first, followed by ALT, with an AST/ALT ratio >1 at 1 week. At 10-12 days, the mean value for ALT was higher compared to AST.[19] Bilirubin, GGT and ALP remained within normal limits.[19] This was expected, as those enzymes are not present in muscle. Pettersson *et al.*[19] demonstrated that weightlifting could cause muscle damage, even in subjects who were accustomed to moderate physical activity.

Weibrecht *et al.*[20] also retrospectively studied 215 cases with rhabdomyolysis having CK greater than 1000 U/L. AST was greater than 40 U/L in 93% of patients, while an abnormal ALT was only found in 75%. CK and AST levels decreased in parallel, while ALT lagged. The authors excluded patients with chronically elevated aminotransferases, patients with myocardial infarction, on statin therapy, with viral hepatitis and acetaminophen toxicity.[20] However, other factors such as weight, diet and medical conditions could have contributed to elevations in this retrospective study and were not ruled out.

Muscle building supplements

Anabolic steroids

Many supplements for muscle building contain androgenic anabolic steroids (AAS), whether disclosed or not. AAS are synthetic derivatives of testosterone that promote muscle growth. These can cause cholestatic liver injury, peliosis hepatis, hepatic adenoma, and hepatocellular carcinoma.[21] Despite increasing efforts of the USA Food and Drug Administration, some bodybuilding supplements can still be contaminated with AAS, and the incidence of liver injury related to AAS use has been increasing.[21]

Anabolic steroids can cause elevation of aminotransferases up to 2-3 times the upper limit of normal.[22] However, most athletes who take anabolic steroids follow an intense training regimen, so that it is often difficult to determine whether aminotransferases are elevated due to rhabdomyolysis or liver damage. With liver damage, usually GGT is elevated as well but bilirubin and CK levels are normal.[22]

In a prospective study, Stolz *et al.*[23] followed 44 patients who were taking bodybuilding supplements and had elevated aminotransferases, ALP and/or bilirubin (Fig. 2). The investigators measured the medium and peak values and the percentage of increase of each laboratory test. The Drug Injury Liver Injury Network did an assessment of causality between liver injury and supplement taken based on available clinical, biochemical, radiological and histological findings at the 6-month follow-up visit. All cases were classified as 'highly likely' or 'definite', while none were deemed 'probable'. Imaging studies and additional laboratory tests (including hepatitis viral panel and autoantibodies) ruled out other liver diseases. Twenty-six patients underwent liver biopsy, of which 77% had a mixed hepatocellular and cholestatic injury and 18% had acute cholestasis. The investigators tested most of the supplements taken by the patients, but not all supplements were available, and anabolic steroids were not identified by chemical analysis among all supplements available.

From the pathology results and elimination of other causes, an association appears to have been established between certain bodybuilding supplements and cholestatic liver injury. However, this study had several limitations. It is difficult to establish a dose response to injury, and not all supplements were available for analysis. Also, other unidentified components could have contributed to liver injury. In addition, some patients were lost to follow-up, so resolution of laboratory abnormalities was not documented.

Creatine supplements

Creatine is a peptide that improves weight, strength, and muscle mass gain. It has been linked to liver damage, but the findings were not unequivocal. Whitt *et al.*[24] described a case of acute cholestatic liver injury in a 27 year-old healthy man who was taking a combination of whey protein and creatine supplements. Liver biopsy showed marked cholestasis with duct proliferation.[24] Other causes, such as exposure to solvents, recreational drug use, alcohol use, viral hepatitis and autoimmune liver disease, were ruled out. Moreover, the use of anabolic steroids was ruled out. The patient showed improvement after discontinuation of supplements, but normalization was not documented.

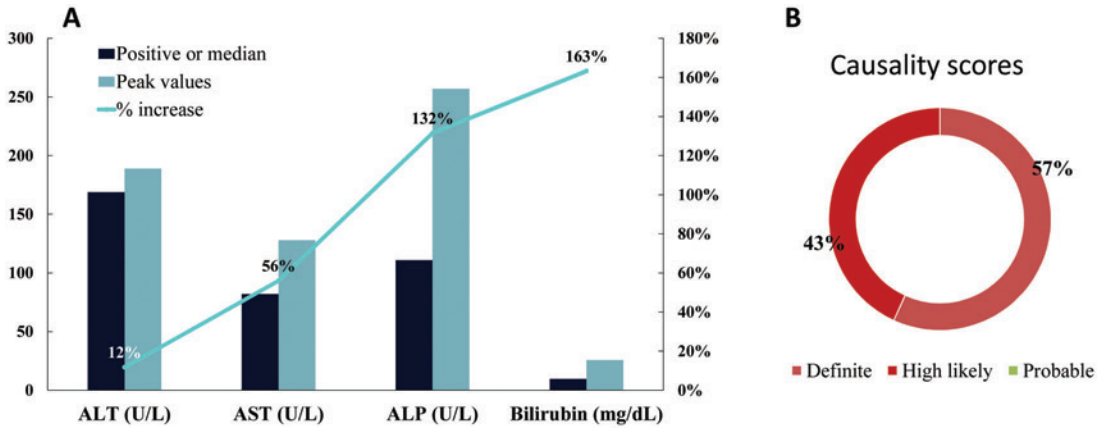

Fig. 2. Data of 44 patients who were taking bodybuilding supplements and had elevated aminotransferases, alkaline phosphatase and/or bilirubin.

(A) Alanine aminotransferase and aspartate aminotransferase, alkaline phosphatase and bilirubin levels in patients taking bodybuilding supplements. (B) Causality scores. Adapted from Stolz *et al.*[23]

It is difficult to draw conclusions from the case reports, especially since the quantitation of creatine ingestion was generally not available. Patients usually take more than one supplement, and testing for other hepatotoxic components is usually not done. No clinical studies on the adverse effects of creatine ingested alone have been performed, making an association difficult to demonstrate. Duarte *et al.* and Tarnopolsky *et al.*[25,26] found increased protein deposition and architectural changes in liver of mice supplemented with creatine. However these studies lack general applicability to a human population. Therefore, due to lack of evidence, creatine is still generally viewed as safe when taken in recommended amounts.

Herbal and dietary supplements

Stickel *et al.*[27] reviewed cases of liver damage related to Herbalife products (Los Angeles, CA, USA). This is a brand of supplements for weight-loss and sports performance. Hepatocellular, cholestatic and mixed patterns of liver damage were described. Elinav *et al.*[28] studied acute hepatitis of unknown cause in Israel. Twelve cases were identified with a common denominator of Herbalife product use. Infectious, autoimmune, metabolic and toxic causes of liver damage were investigated, and all patients denied illicit drug or alcohol abuse. Based on the World Health Organization criteria causality assessment, three cases were ruled as 'certain', six as 'probable' and three as 'possible'. The 'certain' cases were based on positive rechallenge, with development of a second episode of liver injury with reinitiation of supplements, and resolution with discontinuation of products. Similar results were found in a study from Switzerland.[29]

Despite this association, a direct causal relationship has not been drawn between Herbalife products and hepatic toxicity. All of the patients were taking more than one product, some of which could have been contaminated, possibly explaining the limited geographic distribution. Some of these patients tested positive for hepatitis B virus, antinuclear antibody, antimitochondrial antibody with biopsy-proven primary biliary cholangitis, and antismooth muscle antibody at 1:160 that became negative after recovery. Thus, there were possible confounding factors. Furthermore, accurate information regarding the ingestion of other medications was lacking.

LipoKinetix (used for weight loss; Syntrax Innovations Inc., Chaffee, MO, USA) has been associated with a hepatocellular pattern of liver injury and significant elevations of aminotransferases.[30] LipoKinetix contains usnic acid, which uncouples the respiratory chain.[30] These agents were withdrawn from the market after several cases of hepatitis and hepatic failure were reported to the USA Food and Drug Administration. Favreau *et al.*[30] found seven cases of patients with hepatotoxicity after use of LipoKinetix. Three of them were taking only this supplement at time of presentation. All seven patients were healthy, with normal body mass index, taking the supplement in recommended doses, not on any other medications, and tested negative for infectious and autoimmune causes of hepatitis. Additionally, all reported cases had spontaneous recovery after discontinuation of product.[30] Even though causality is challenging to prove based on case reports, these results are somewhat convincing given the common denominator. The USA Food and Drug Administration tested three of the products from different lots and ruled out contaminants, pointing towards an idiosyncratic reaction as the mechanism of injury.

Similarly, Hydroxycut products (Iovate Health Sciences, Oakville, Ontario, Canada) used for weight loss, were removed from the marked after 23 reports of acute hepatic failure, some requiring liver transplantation.[27] Kaswala *et al.*[31] reported one case of a patient using Hydroxycut, with biopsy-proven acute fulminant hepatitis. Autoimmune causes were ruled out in this case, but there was no mention of whether other causes, such as viral hepatitis, were checked.[31] The patient improved after stopping supplement use.[31] Although it is possible the presentation was due to Hydroxycut, once again, a causal relationship was not proven.

Several other products have been associated with drug-induced liver injury in case reports (Table 2). Patients usually underreport use of dietary supplements or take several supplements at once, making it challenging to pinpoint the causal agent of liver injury.[1]

Vitamins

DeKlotz *et al.*[32] retrospectively examined adolescents taking isotretinoin for acne, and reported those who developed aminotransferase elevations. All of them admitted to use of

Table 2. Bodybuilding products associated with drug-induced liver injury[27]

Product	Type of liver injury	Mechanism
LipoKinetix	Acute hepatitis	Possibly uncoupling of the respiratory chain
Anabolic steroids	Cholestasis, benign/malignant tumors	Dysfunction of biliary transport
Noni juice	Acute hepatitis, liver failure	Unknown
Senna	Acute hepatitis, granulomatous hepatitis, cirrhosis	Possibly drug idiosyncrasy or uncoupling of the respiratory chain
Green tea	Acute hepatitis	Possibly oxidative stress from epigallocatechin gallate
Ephedra	Acute hepatitis, liver failure	Unknown

Adapted from Stickel *et al.*[27]

herbal, protein or creatine supplementation, and some of them had initiated vitamin A therapy at the time. There were many confounding factors in this study, so it is difficult to determine if aminotransferase elevations were due to liver damage or muscle damage. There was no specific pattern in elevation of AST, ALT or supporting laboratory testing (such as CK). Nevertheless, because vitamin A alone is known to cause hepatic injury,[33] it is certainly possible that in combination with an underlying medical condition, alcohol use, medications or genetic predisposition, its use resulted in an increased risk of liver damage.

Potential causes of rhabdomyolysis with exercise/ body building

Ischemia

Conditions with generalized ischemia and hypoxemia can cause insufficient ATP production and sarcolemma dysfunction.[34] Causes include but are not limited to: shock, arterial thrombosis, air emboli, sickle cell disease, and status asthmaticus.[34] Compartment syndrome can be a cause or complication of rhabdomyolysis due to impaired blood flow.[34] Prolonged immobilization causes tissue compression and muscle ischemia as well.[34] Severe dehydration, especially in the setting of heat stroke and exercise can also cause rhabdomyolysis.[34]

Muscle building supplements

Creatine supplements can result in rapid weight gain due to intracellular and extracellular fluid retention.[35] Robinson *et al.*[35] speculated that increased intracellular water retention caused greater skeletal muscle compartment pressures, which increased risk of cellular wall breakdown. The first association of creatine supplementation and rhabdomyolysis was made in 1997 after three wrestlers died while on creatine supplements.[35] However, they were also using ephedra supplements for weight loss. Several studies have reported no effect of high-dose short-term or low-dose long-term creatine use in physically unstressed subjects or power athletes on high-dose creatine.[35–37]

Despite these studies, there are few cases reported of rhabdomyolysis in the setting of creatine supplement use. The majority were involved in extreme exercise regimens. Some subjects also ingested ephedrine or herbal supplements. In the setting of extreme, unaccustomed exercise and usage of other supplements, it is difficult to prove creatine as the culprit.

Weight loss supplements

The sympathomimetic amine ephedra was banned in 2004 after numerous reports of cardiovascular and neurologic adverse effects.[38] Synephrine became a popular alternative, due to its structural similarity to ephedrine. It is thought to increase the risk of rhabdomyolysis through vasoconstriction and vasospasm, causing ischemia, direct toxicity, and impairment of calcium homeostasis or myocyte thermoregulatory function.[38]

Burke *et al.*[38] reported a case of a male subject who engaged in vigorous exercise and ingested a weight loss supplement containing synephrine and caffeine. He developed rhabdomyolysis with elevated CK and aminotransferases on two different occasions. During his first hospitalization, he was not queried regarding use of supplements and continued to use Lipo 6 twice daily after discharge until his second presentation. Even though he had several predisposing risk factors, such as sickle cell trait, a previous episode of rhabdomyolysis, and exercise in warm climate, it is important to note that prior to use of supplements, there was no rhabdomyolysis in spite of the use of same exercise regimen. Although this may represent direct muscle injury by synephrine, the association has not been proven.

A 40-year-old man was reported to have developed rhabdomyolysis after taking Garcinia cambogia.[39] Also known as Malabar tamarind, this tropical fruit is a popular weight loss inducer. The patient denied use of prescription medications, rigorous exercising, other supplements or dehydration. This is the only case report where a single-ingredient supplement with Garcinia cambogia was associated with rhabdomyolysis. However, although suggestive, there is insufficient evidence to establish a causal relationship. Other factors might have predisposed the patient to muscle damage.

Preventative measures

Hill *et al.*[40] found that the strongest risk factors for rhabdomyolysis in army soldiers were prior heat stroke, black race and length of stay of less than 90 days. Although confounding factors such as hydration, temperature and humidity (which are known to increase risk for rhabdomyolysis)[7,40] were not considered, it seemed new recruits had double the likelihood

compared to soldiers who were there for more than 90 days.[40] Those with length of stay greater than 1 year had an odds ratio of developing rhabdomyolysis below 1.00.[40] This suggests that subjects who are unaccustomed to exercise have higher risk of muscle damage. Similarly, Oh *et al.*[41] described athletes who developed rhabdomyolysis 1 day after starting an intense exercise regimen in a football camp. Questionnaires were handed to athletes and those who were voted for as 'hardest working' had a relative risk of 2.1 compared to the group that did less effort.[41] The athletes denied drugs or medications and their aerobic and resistance exercises months prior were similar. Given that these athletes performed the same exercises under the same environmental conditions, the study suggests that intense training for first-timers or after a training hiatus could potentially increase the risk for rhabdomyolysis. It is reasonable to recommend a slow buildup to intensity of exercise desired in subjects who are not used to moderate-high intensity workouts. Since temperature is a risk factor, avoiding hot climates and wearing adequate clothing that aid heat dissipation might be protective.

Measures such as warm-ups, sufficient water intake and ingestion of protein in combination with carbohydrates can help prevent rhabdomyolysis.[7,40,42,43] Baty *et al.*[44] gave carbohydrate-protein supplements to a group of athletes and compared them to another group which received a placebo (electrolytes and artificial sweetener). They measured performance and muscle damage and found CK and myoglobin levels were significantly higher in the placebo group 24 h after exercise.[44] A health history was obtained from each participant, those taking enhancing supplements were excluded and they started performing the same training sessions weeks prior to the start of the study.[44] Because they were all under the same environment, and even had the same diet prior to start of exercise, these results seem convincing.

The type of exercise that should be done in order to prevent rhabdomyolysis is unclear, although it is known that eccentric contraction may cause more rhabdomyolysis than concentric contraction.[42,45] Stretching and warm-ups are also generally thought to decrease the incidence and likelihood of muscle injuries due to increase in flexibility and range of motion.[46] Small *et al.*[46] performed a systematic review to assess efficacy of static stretching as part of warm-up to prevent exertional rhabdomyolysis. They found that all randomized clinical trials and two out of three controlled clinical trials did not find a significant difference in all-injuries risk between control and intervention group. However, the hazard ratios from five of the seven studies would indicate that stretching reduces risk of muscular strains and ligament sprains. It might be reasonable to perform these activities prior to a more intense work-out, but there is no convincing evidence that it would prevent rhabdomyolysis.

Conclusions

Aminotransferases are commonly associated with liver disease, but can also be elevated secondary to exercise and supplement use in athletes and non-athletes. A history of new or recently intensified exercise regimen should prompt a search for muscle injury. The coexistence of elevated cholestatic serum markers, such as ALP, GGT, and 5'-nucleotidease, can be useful in diagnosing liver damage. Elevated levels of markers of muscle injury, including CK, can be helpful in diagnosing muscle injury. The pattern of elevation of aminotransferases is not valuable in distinguishing muscle from liver injury as it can vary depending on the number of days after injury when testing is done. Therefore, when causes of liver injury have been ruled out, investigation into muscle injury associated with bodybuilding, and supplement use is warranted.

Acknowledgements

The support of the Herman Lopata Chair in Hepatitis is gratefully acknowledged.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Wrote the manuscript and prepared figures (JVK), proposed the idea for the review and revised the manuscript with critical revisions (GYW).

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Review Article

Management and Treatment of Hepatocellular Carcinoma with Immunotherapy: A Review of Current and Future Options

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Abstract

With mortality rates of liver cancer doubling in the last 20 years, this disease is on the rise and has become the fifth most common cancer in men and the seventh most common cancer in women. Hepatocellular carcinoma (HCC) represents approximately 90% of all primary liver cancers and is a major global health concern. Patients with HCC can be managed curatively with surgical resection or with liver transplantation, if they are diagnosed at an early stage. Unfortunately, most patients with HCC present with advanced stages of the disease and have underlying liver dysfunction, which allows only 15% of patients to be eligible for curative treatment. Several different treatment modalities are available, including locoregional therapy radiofrequency ablation, microwave ablation, percutaneous ethanol injection, trans-arterial chemoembolization, transarterial radio-embolization, cryoablation, radiation therapy, stereotactic radiotherapy, systemic chemotherapy, molecularly targeted therapies, and immunotherapy. Immunotherapy has recently become a promising method for inhibiting HCC tumor progression, recurrence, and metastasis. The term "Immunotherapy" is a catch-all, encompassing a wide range of applications and targets, including HCC vaccines, adoptive cell therapy, immune checkpoint inhibitors, and use of oncolytic viruses to treat HCC. Immunotherapy in HCC is a relatively safe option for treating patients with advanced disease in the USA who are either unable to receive or failed sorafenib/lenvatinib therapy and thus may offer an additional survival benefit for these patients. The purpose of this review is to elaborate on some of the most recent advancements in immunotherapy.

Citation of this article: Ghavimi S, Apfel T, Azimi H, Persaud A, Pyrsopoulos NT. Management and treatment of hepatocellular carcinoma with immunotherapy: A review of current and future options. J Clin Transl Hepatol 2020;8 (2):168–176. doi: 10.14218/JCTH.2020.00001.

Keywords: Carcinoma; Hepatocellular; Immunotherapy; Immunotherapy; Adoptive; Nivolumab.

Abbreviations: ACT, adoptive cell therapy; CAR, chimeric antigen receptor; CTLA-4, cytotoxic T lymphocyte-associated protein 4; HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; TACE, trans-arterial chemoembolization; VEGF, vascular endothelial growth factor.

Received: 6 January 2020; Revised: 13 May 2020; Accepted: 16 May 2020

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Introduction

With mortality rates of liver cancer doubling in the last 20 years, this disease is on the rise and has become the fifth most common cancer in men and the seventh most common cancer in women.[1] Currently, hepatocellular carcinoma (HCC) represents approximately 90% of primary liver cancers and is a major global health concern.[2] There are many risk factors responsible for the development of HCC, such as viral infections, hereditary hemochromatosis, non-alcoholic fatty liver disease, increased alcohol-related liver disease, and cirrhosis, among others.[3] HCC development is a complex process involving multiple factors and pathways that lead to changes in gene expression, immune interactions, and changes in the tumor microenvironment that ultimately cause hepatocarcinogenesis.[4]

Screening for HCC in high-risk populations has become the standard of care, requiring imaging with ultrasound, computerized tomography, or magnetic resonance imaging every 6-12 months.[5] Laboratory tests, including mainly those for alpha feto-protein, the lectin Lens culinaris agglutinin-bound fraction of alpha feto-protein-3, and des-gamma-carboxy prothrombin, are used in conjunction with imaging to establish the diagnosis.[6,7] Multiphasic imaging modalities, such as computerized tomography and magnetic resonance imaging, are used for detection and diagnosis of HCC. Liver biopsy is not necessary once the Liver Imaging Reporting and Data System (known as 'Li-Rads') has been deemed to be category 5, which is diagnostic for HCC.[8]

If a lesion is deemed to be HCC, the available options for management are varied and depend on multiple factors, including the number of lesions, their size, the presence of extrahepatic spread, and the severity of the patient's underlying liver disease.[9] The recommendation from The European Association for the Study of Liver panel of experts is to consider the following four related aspects to determine treatment options: tumor stage, degree of liver function impairment, general condition of the patient, and treatment efficacy.[10,11] Fig. 1 depicts the Barcelona Clinic Liver Cancer system, which is one of the most commonly used algorithms to assist in determining treatment options based on the aforementioned factors.[11–14] To summarize, patients diagnosed as stage 0, with very early HCC, are ideal candidates for ablation or resection.[11] Patients who are deemed to be stage A, with early HCC, are candidates for radical therapies, including hepatoma resection, liver transplantation, or interventional radiology procedures.[10,11] Patients at stage B, with intermediate HCC, may benefit from chemoembolization.[10,11] Patients at stage C, who already have advanced HCC, are only candidates for systemic therapy if their performance status is acceptable; otherwise, they are managed with the best

supportive care available.[11] Approximately 85% of patients with HCC are diagnosed at later stages or have underlying liver dysfunction, limiting their treatment options.[9] These patients usually have a very poor prognosis, with survival of less than 1 year.[15] In patients treated with resection or ablation, tumor recurrence (both true recurrence due to dissemination and *de novo* tumors) is unfortunately common and is seen in up to 70% of patients 3-5 years after treatment.[16]

Liver transplantation is an important treatment modality for patients who meet Milan criteria (a single HCC nodule of 2-5 cm or 3 HCC nodules each ≤3 cm in diameter) or who undergo down-staging of their tumors to be within the Milan criteria.[17–20] Studies have shown that patients who met Milan criteria and received a liver transplant had survival rates exceeding 70% at 5 years, with recurrence in less than 15% of patients.[21] Approximately 30-40% of patients on the liver transplantation waitlist are patients who have received model for end-stage liver disease (MELD) exception points for HCC.[22] They receive these points 6 months after listing and then receive an incremental increase in their MELD points every 3 months until the maximum MELD exception point allowance is reached (that being 34).[23,24] MELD exception points give patients an increased chance of receiving a liver but they do not guarantee a liver to all listed patients. Therefore, additional treatments for HCC are greatly needed.

In 1891, the surgeon William Coley injected streptococcal organisms into a patient with inoperable osteosarcoma, successfully stimulating the immune system and leading to tumor regression and thus fathering the field of immunotherapy.[25] Since then, there have been many achievements in use of immunotherapy to fight cancer and in the development of a broad range of therapeutic applications, including the use of gene therapy, oncolytic viruses, cytokines, adoptive cell therapy, vaccines, and immune checkpoint inhibitors to fight cancer.[25]

Immunotherapy has recently become a new promising method for inhibiting HCC tumor progression, recurrence, and metastasis.[26,27] "Immunotherapy" is a catch-all term, encompassing a wide range of applications and targets, including HCC vaccines, adoptive cell therapy (ACT), immune checkpoint inhibitors, and use of oncolytic viruses to treat HCC. These approaches have often shown initial success in treating other types of cancers, with potential to be similarly successful in treating HCC. In this review, we will discuss some of the most recent advancements in immunotherapy for HCC.

Tumor immunology

Research has shown that cancer cells are able to escape from immunological surveillance and suppress the activation of immunocompetent cells (immune suppression), thereby allowing for their continued growth.[27] Cancer immunoediting is a proposed mechanism to explain how tumors evade the

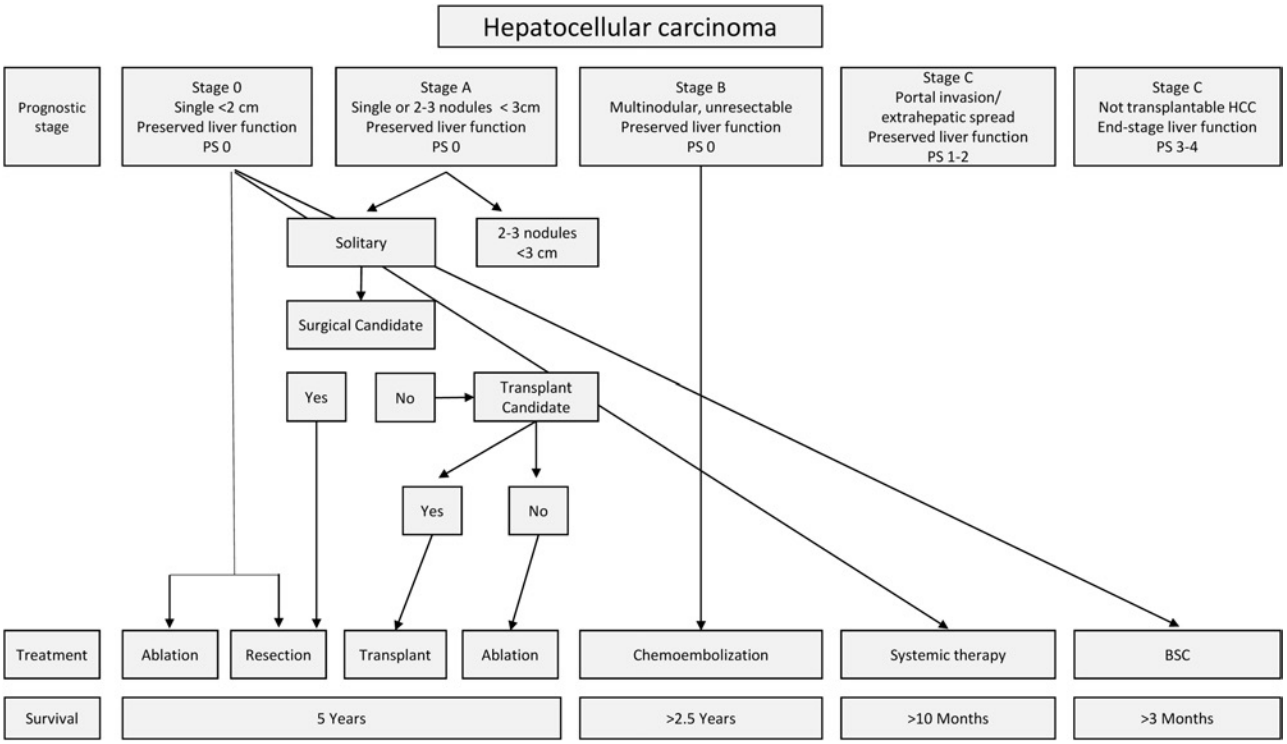

Fig. 1. Hepatocellular carcinoma treatment in patients diagnosed with hepatocellular carcinoma.

Modified Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy: The BCLC system recommends pathways for treatment based on prognostic stages. The stage is determined by the number of lesions and their size, evidence of extrahepatic spread/portal invasion, performance status (ps), preserved liver function, and evidence of decompensated liver disease (usually determined by the Child-Pugh classification or the model for end-stage liver disease score). As noted, there are multiple treatment options, including resection, transplantation, chemoembolization, ablation, systemic therapy or best support care, which is essentially palliative care. Survival is predicted based on what initial therapy was chosen.[11]

immune system, consisting of three sequential phases: elimination, equilibrium, and escape.[28] In the elimination phase, innate and adaptive immunity work together to destroy developing HCC long before it becomes clinically apparent. If this phase is not successful, the cancer cell variant may then enter the equilibrium phase, in which its growth is prevented by immunologic mechanisms.[28] Important players from the adaptive immune system, T cells, interleukin-12, and interferon-α suppress the growth of cancer cells during this phase.[28] The equilibrium state may represent an end stage of the cancer immunoediting process, creating occult cancers that do not grow any larger and are clinically insignificant during the lifetime of the host.[28] However, because of this constant immune selection pressure placed on "genetically unstable" tumor cells, new cell variants can become (1) unrecognizable to the adaptive immune system, due to antigen loss and defects in antigen processing or presentation, (2) insensitive to immune effector mechanisms, or (3) able to induce an immunosuppressive state within the tumor microenvironment.[28] These tumor cells may then enter the escape phase, in which their growth is no longer controlled by the immune system and clinically significant disease develops.[28]

An important escape mechanism allows tumor cells to upregulate their own expression of immune checkpoint molecules, including the programmed cell death-1 (PD-1) protein that binds to the programmed death-ligand 1 (PD-L1) and stimulates peripheral T cell depletion.[29] Another important checkpoint molecule, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), is found on the surface of T cells and can be activated by tumor cells, leading to down-regulation of T cells.[27] Interestingly, initial studies done by Duffy *et al.*[30] in 2017 showed that in liver biopsies of patients treated with tremeliomumab, an anti-CTLA-4 antibody, there was an increase in cytotoxic T cells, demonstrating that treatment with such molecules increased activity of the immune system. Monoclonal antibodies to PD-1, PD-L1, and CTLA-4 are called immune checkpoint inhibitors and have become an important part of immunotherapy treatments for many cancers, including melanoma, non-small cell lung cancer, and colorectal cancer, and are now emerging as valuable treatments in HCC.[29,31]

Checkpoint inhibitors

Checkpoint inhibitors are currently the most successful immunotherapy treatment for HCC.[27] As noted in Tables 1 and 2, and illustrated in Fig. 2, there are multiple novel treatments available and many active clinical trials investigating further checkpoint inhibitors.

Nivolumab

Promising results were reported in 2017 from the Checkmate 040 phase I/II trial which looked into survival rates for nivolumab, an anti-PD-1 antibody, used in the treatment of advanced HCC.[32] A dose-escalation and expansion phase was implemented in patients who met strict inclusion criteria, with clinically less severe underlying liver dysfunction.[32] Treatment in 262 patients yielded an acceptable safety profile and promising efficacy, and based on these results, the Food and Drug Administration fast-tracked the approval of nivolumab for the treatment of patients diagnosed with HCC who had been previously treated with sorafenib.[27] Another small phase II trial investigating nivolumab alone versus nivolumab and ipilimumab is currently underway, with preliminary results demonstrating a good safety profile and that treatment does cause delay to surgical resection.[33]

Table 1. Results of selected studies testing immune checkpoint inhibitors in hepatocellular carcinoma. Adapted from Pinter and Peck-Radosavljevic.[25]

Trial and year	Treatment (number of patients)	Target IT	Prior sorafenib treatment, %	ORR/ DCR, %	TTP/PFS in months	OS in months	Reference
Sangro 2013	Tremelimumab (21)	Anti-CTLA-4	23.8	17.6/76.4	6.48/NR	8.2	49
Duffy 2017	Tremelimumab + subtotal ablation (32)	Anti-CTLA-4	65.6	26.3/NR	7.4/NR	12.3	30
El-Khoueiry 2017	Nivolumab (80)	Anti-PD-1	0	22.5/62.5	NR/NR	28.6	32
El-Khoueiry 2017	Nivolumab (182)	Anti-PD-1	100	18.7/62.6	NR/NR	15.6	32
Wainberg 2017	Durvalumab (40)	Anti-PD-L1	92.5	10/32.5	NR/2.7	13.2	73
Kelley 2017	Durvalumab + tremelimumab (40)	Anti-PD-L1 + Anti-CTLA-4	75	25/57.5	NR/NR	NR	74
Zhu 2018	Pembrolizumab (104)	Anti-PD-1	100	17.3/61.5	NR/4.9	12.9	37
Ikeda 2018	Pembrolizumab + lenvatinib (30)	Anti-PD-1	13.3	42.3/100	NR/9.7	NR	42
Stein 2018	Atezolizumab + bevacizumab (43)	Anti-PD-1 + anti-VEGF	0	65/96%	NR/NR	NR	51

Abbreviations: CTLA–4, cytotoxic T lymphocyte–associated protein 4; DCR, disease control rate; IT, immunotherapy; NR, not reported; ORR, overall response rate; OS, overall survival; PD–1, programmed cell death-1; PD–L1, programmed cell death 1-ligand 1; PFS, progression–free survival; TTP, time to progression.

Table 2. Ongoing phase III trials testing immune checkpoint inhibitors in hepatocellular carcinoma

Drug	Target of IT	Setting	ClinicalTrials Identifier	Status	Primary completion
Nivolumab vs. placebo	Anti-PD-1	Curative adjuvant	NCT03383458	Recruiting	Q1 2022
Nivolumab + TACE	Anti-PD-1	Curative adjuvant	NCT03143270	Recruiting	Q1 2019
Nivolumab + TACE	Anti-PD-1	Curative, adjuvant	NCT03572582	Recruiting	Q3 2022
Nivolumab vs. sorafenib	Anti-PD-1	Palliative, 1st-line	NCT02576509	Recruiting	Q3 2017
Durvalumab ± tremelimumab vs. sorafenib	Anti-PD-L1 + Anti-CTLA-4	Palliative, 1st-line	NCT03298451	Recruiting	Q1 2020
Atezolizumab + bevacizumab vs. sorafenib	Anti-PD-L1	Palliative, 1st-line	NCT03434379	Recruiting	Q2 2021
Pembrolizumab vs. placebo	Anti-PD-1	Palliative, 2nd-line	NCT02702401	Active, not recruiting	Q1 2019
Pembrolizumab + TACE	Anti-PD-1	Curative, 2nd-line	NCT03397654	Recruiting	Q4 2019
Pembrolizumab vs. placebo	Anti-PD-1	Palliative, 2nd-line	NCT03062358	Recruiting	Q4 2019
Pembrolizumab vs. best supportive care	Anti-PD-1	Palliative, 2nd-line	NCT02702401	Active, not recruiting	Q4 2020
Tislelizumab vs. sorafenib	Anti-PD-1	Curative, 1st-line	NCT03412773	Recruiting	Q4 2022
Atezolizumab + bevacizumab	Anti-PD-L1 + anti-VEGF	Curative, 1st-line	NCT03434379	Recruiting	Q2 2022
Ipilimumab + nivolumab	Anti-CTLA-4 + Anti-PD-1	Curative, adjuvant	NCT03682276	Recruiting	Q4 2022
Nivolumab + yttrium-90	Anti-PD-1	Curative, adjuvant	NCT03033446	Recruiting	Q4 2019
Pembrolizumab + talimogene laherparepvec	Anti-PD-1 + Oncolytic Viral	Curative, adjuvant	NCT02509507	Recruiting	Q3 2021
durvalumab + tremelimumab + radiotherapy	Anti-PD-L1 + Anti-CTLA-4	Curative, adjuvant	NCT03482102	Recruiting	Q4 2025

Abbreviations: CTLA–4, cytotoxic T lymphocyte–associated protein 4; IT, immunotherapy; PD–1, programmed cell death-1; PD–L1, programmed cell death 1-ligand 1.

Checkpoint 459 is a phase III study comparing nivolumab and sorafenib as first-line treatment for HCC.[34] Preliminary data was released in June 2019, which showed that the overall survival failed to meet statistical significance (hazard ratio of 0.85; 95% confidence interval, 0.72-1.02; p=0.0752). The data reportedly did show a trend towards an overall survival improvement with nivolumab versus sorafenib.[34] At the time of this publication, Bristol-Myers Squibb has not yet released the full data.[34] There are also currently other ongoing trials investigating nivolumab as a single agent in CheckMate-9DX and also in combination with ipilimumab for previously-treated patients with HCC.[26,35,36]

Pembrolizumab

While nivolumab was investigated as a first-line treatment option for HCC, another anti-PD-1 antibody, pembrolizumab, is being developed as a second-line treatment after initial treatment with tyrosine kinase inhibitors have failed or were not tolerable.[27] In a phase II trial, patients with advanced liver cancer who were sorafenib-refractory, sorafenib-intolerant, or sorafenib-naïve received one standard dose of pembrolizumab.[37] Interval results showed an 18% response rate and a 12.9 month median survival period.[37] In November of 2018, the Food and Drug Administration granted an accelerated approval for pembrolizumab to be used in treatment of patients with HCC who have been previously treated with sorafenib.[37] However, in the follow-up, phase III KEYNOTE-240 trial, pembrolizumab failed to meet the primary endpoints for both overall survival and progression-free survival when pembrolizumab was compared to placebo and best supportive care in HCC patients that had already failed systemic therapy.[38] While pembrolizumab compared with placebo did show improvement in overall survival and progression-free survival, the improvement was not deemed statistically significant (overall survival:hazard ratio, 0.78; 95% confidence interval, 0.611-0.998; p=0.0238; progression-free survival:hazard ratio, 0.78; 95% confidence interval, 0.61-0.99; p=0.0219).[38] Although disappointing, this has not deterred other investigations of pembrolizumab in HCC patients. In the UK, a phase II/III study is underway investigating pembrolizumab as an adjunctive treatment to trans-arterial chemoembolization (TACE) using doxorubicin and gelatin sponges.[27]

An additional phase III study, KEYNOTE-394 (NCT03062358), is currently evaluating and recruiting patients and is using the same inclusion criteria as set in the Keynote-240 trial in hopes of better outcomes in an Asian population.[39]

Tislelizumab

Tislelizumab, another anti-PD-1 antibody, is currently under development by BeiGene.[40] The safety of tislelizumab was

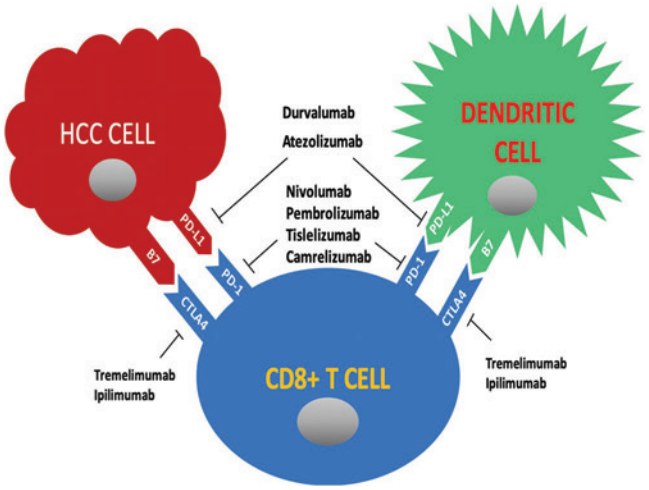

Fig. 2. Mechanism of action of checkpoint inhibitors under investigation for hepatocellular carcinoma treatment.

Hepatocellular carcinoma (HCC) tumor cells can up-regulate expression of programmed cell death-1, PD-1, which binds the programmed death-ligand 1, PD-L1, and stimulates peripheral T cell depletion.[29] They can also activate cytotoxic T lymphocyte-associated protein-4 (CTLA-4), found on the surface of T cells and leading to down-regulation of T cells.[27] Ipilimumab and tremelimumab bind to and inactivate CTLA-4, preventing its activation. PD-1-PD-L1 binding may be prevented by therapeutically blocking either PD-1 (nivolumab, pembrolizumab, tislelizumab, and camrelizumab) or PD-L1 (durvalumab and atezolizumab).

established in an earlier phase I trial, with multiple different solid cancers, including HCC.[41] Currently, there is a multicenter global phase III trial looking at tislelizumab versus sorafenib as first-line treatment for unreachable HCC that started recruitment in 2017.[27,40,42] This trial set survival rate as the principal endpoint and was designed to validate that non-inferiority of tislelizumab compared to sorafenib (Table 2). No interim data are currently available.

Camrelizumab

Camrelizumab is an anti-PD-1 antibody, for which a phase II/III trial is currently underway in China, looking at patients who failed to respond to systemic treatments or were intolerant to previous systemic treatments.[27] Provisional results from the phase II part was presented in 2018 at a meeting of the European Society for Medical Oncology, demonstrated a response rate of 13.8% (30/217) with a 6-month overall survival rate of 74.7%.[27] It is notable that only two patients (0.9%) experienced grade 5 treatment-related adverse events, which in turn showed camrelizumab to have a suitable toxicity profile.[27] No interim date is available from the phase III part. Currently, there is an ongoing phase II trial in China looking at camrelizumab plus the FOLFOX4 regimen (consisting of 5-fluorouracil, leucovorin, and oxaliplatin) for treatment of advanced HCC and biliary tract cancers in patients who failed systemic treatment.[43] Interim results from October 2018 were promising, with median progression-free survival of 5.5 months; however, over 85% of patients had severe treatment related side-effects.[43]

Durvalumab

Durvalumab is the only anti-PD-L1 antibody for HCC under investigation currently.[27] Cancer cells can avoid immune surveillance by overexpressing PD-L1 and activating PD-L1/PD-1 signaling, which is observed in HCC tissues (Fig. 2).[44,45] A basic science study recently showed that inhibition of PD-L1 and DNA methyltransferase 1 (commonly known as DNMT1) significantly suppressed the growth of sorafenib-resistant HCC cells *in vitro*.[46,47] This points to a possible novel treatment option for sorafenib-resistant HCC.[26,48] A phase I/II trial looking at the safety of durvalumab monotherapy in treating solid tumors showed durvalumab to have an acceptable safety profile and to be promising, with a 10% response rate and a median survival time of 13.2 months for the HCC cohort.[26] A phase III trial that started in 2017 is currently underway looking at durvalumab plus tremelimumab combination therapy as a first-line treatment for patients with advanced HCC; however, currently there are no interim results available.[27]

Tremelimumab

Tremelimumab is another anti-CTLA-4 antibody under investigation for HCC.[27] To date, remelimumab monotherapy has been investigated for patients with HCC and chronic hepatitis C.[49] Results from the trial were promising, and showed that of the 21 patients enrolled, there was a partial response rate of 17.6% and a median time to progression of 6.48 months.[27] Overall, the treatment was very well tolerated, with minimal toxicities, such as transient elevation of transaminases, noted.[27] A phase I/II trial investigating the combination of tremelimumab plus interventional procedures, such as radiofrequency ablation, TACE, and cryoablation, for non-resettable HCC patients is currently underway in the USA.[30] Initial results show no dose limiting toxicities and of the 19 patients that were suitable for evaluation, 5 (26.3%) achieved partial responses outside of the areas treated with TACE or ablation.[27] The median progression-free survival period was 7.4 months, with the median survival period of 12.3 months.[25]

Combined targeted therapy

Atezolizumab + bevacizumab

Novel combination treatment with atezolizumab, an anti-PDL-1 antibody, and bevacizumab, an anti-vascular endothelial growth factor (VEGF) therapy, is under investigation for treatment of advanced HCC and has shown to be effective in combination when treating other cancers.[50] HCC tumors over-express VEGF and PD-L1 and there is evidence of increased vascularity, which makes it a good target for this combination therapy that targets both sites.[51–53] Two global I/Ib studies showed promising results when looking at this combo in patients with unrespectable HCC who had not received prior systemic therapy but some of who received TACE and/or radiotherapy.[54] Data from the non-randomized arm of 119 patients showed an objective response rate of 36% (95% confidence interval, 26–46), with 12% of enrolled patients having complete response to treatment and a median overall survival of 17.1 months.[54]

The second arm of the study randomized the same patient population to atezolizumab and bevacizumab versus atezolizumab monotherapy.[54] Of those who received the

combination therapy, 20% had confirmed responses and 47% had stable disease, compared to 17% and 32% of patients, respectively, in the atezolizumab monotherapy group.[54] This accounted for the 0.55 hazard ratio (80% confidence interval, 0.40–0.74; p=0.0108) reported.[54] Additionally, median progression-free survival in the combination arm was 5.6 months compared to 3.4 months in the monotherapy group.[55]

There were increased grade 3/4 adverse events in the combination group (34% vs. 14%) with the most common adverse events being proteinuria, fatigue, and rash.[54] Based on data that showed an objective response rate of 36% (95% confidence interval, 26–46), the Food and Drug Administration granted Breakthrough Therapy Designation for atezolizumab/bevacizumab combination therapy to be a fist-line therapy for advanced or metastatic HCC.[55] In addition, enrollment for a new phase III study, IMbrave150 (NCT03434379), has completed enrollment and will be comparing atezolizumab/bevacizuma versus sorafenib in unresectable HCC patients.[55]

Ipilimumab + nivolumab

Ipilimumab is an anti-CTLA-4 antibody and multiple studies have recently investigated its use in combination with nivolumab. In the USA, the CheckMate040 trial results were recently published (NCT01658878).[56] The trial enrolled HCC patients who had failed treatment with sorafenib and randomized the patients to receive one of three different protocols of ipilimumab plus nivolumab. The combined treatment reportedly showed an acceptable safety profile, with an objective response rate that was twice that of nivolumab monotherapy (31% compared to 14%) and having median overall survival of 18 months.[56]

In the UK, the PRIME-HCC clinical trial is underway, assessing the efficacy of combination treatment pre-operatively with nivolumab and ipilimumab in HCC patients for whom liver resection is planned.[27] Participants will receive two doses of nivolumab and a single dose of ipilimumab in the weeks before their surgery. This is a single-arm, open-label study to be conducted in 32 patients at a small number of UK hospitals. The study has two parts. Part 1 will confirm, in a small number of patients, that the treatment regimen is safe and does not result in delay to liver resection, while Part 2 will expand the number of patients studied and assess survival over 2 years post-resection. The decision to proceed to Part 2 will be taken with advice from an independent, expert committee.[27]

SHR-1210 + Apatinib

The combination of SHR-1210, a novel anti-PD-1 antibody, and apatinib, a tyrosine kinase inhibitor selectively acting on VEGF receptor 2, is currently being investigated.[27] A phase 1 trial was completed in 2018 and showed acceptable tolerability of this combination and a response rate of 38.9%, with a median progression-free survival of 7.2 months for the 18 patients with HCC.[44] Overall, adverse events were relatively tolerable, with only one patient discontinuing the treatment due to treatment-related grade 3 hyperbilirubinemia.[44] Currently, a phase II trial is underway in the USA, comparing this combination to systemic chemotherapy in advanced HCC.[57] No results are available currently.

Other targets in immunotherapy

ACT is a new approach to look for treatments that allow a patients' own lymphocytes to attack cancer cells.[58,59] Adoptive immunotherapy for HCC includes cytokine-induced killer cells, tumor-infiltrating lymphocytes, natural killer cells, and chimeric antigen receptor (CAR) T cells. The safety of ACT in patients with HCC have been investigated in many preclinical studies, thus laying a foundation for its clinical applications.[58,59]

CAR-T cell therapy, in particular, has been a very successful novel method of treating CD19-positive hematological malignancies, and its application has recently been considered in the treatment of solid tumors, including HCC.[33] Currently, there are no clinical trials investigating CAR-T cell therapy for HCC, as there have been many concerns about the drug causing cytokine-release syndrome, which affects up to 90% of patients and can cause cardiovascular, pulmonary, and central nervous system complications.[59–61] Additionally, there is currently a lack of specific tumor antigens to target in HCC, limited trafficking and penetration of CAR-T cells to tumor sites, and an immunosuppressive tumor microenvironment.[33] To overcome these difficulties, numerous strategies have been developed, including enhancing the selectivity of CARs and controlling CAR-T activity.[33] In a recent study by Guo *et al.*,[62] the investigators concluded that gene-edited CAR T cells with PD-1 deficiency have stronger antitumor activity than wild-type CAR T cells and future development of CAR T cells with modified gene-editing may help improve CAR T cell efficacy as a treatment for HCC.[62] In another basic research study, Li *et al.*[63] demonstrated that GPC3-targeting CAR T cells, in particular CAR.hYP7, are a promising therapeutic intervention for liver cancer that can be translated for human use.[63] There is hope that with further investigation and clinical trials, CAR-T cell therapy will become a viable option for HCC treatment.

Interferon monotherapy has also been explored as adjuvant therapy, to both prevent tumor recurrence as well as to inhibit development of HCC in patients with chronic hepatitis B and C infections.[64–66] According to a published report by Lai *et al.*,[67] interferon-α increased survival rates and encouraged tumor regression in patients diagnosed with advanced HCC. According to Obi *et al.*,[68] 16% of HCC patients with portal vein invasion who received a combination of 5-flurouracil and interferon-α treatment had complete response, while 36% had a partial response, as interferon-α can induce apoptosis and inhibit cell growth in HCC tumors.

Different than ACT, cell-mediated immunotherapy is a novel approach that has been used to exploit the unique pattern of proteins that are expressed specifically on tumor cells as targets. Tumor-targeted antibodies are mutant or aberrantly expressed antigens on the surface on cancer cells and can be potential targets of the adaptive human immune system.[36] Tumor-targeted antibodies are being investigated for HCC immunotherapy, with a focus on alpha feto-protein-directed treatments.[69] Another approach is to use oncolytic viruses to attack HCC tumor cells.[70–72] Theoretically, these viruses can selectively replicate in tumor cells and cause lysis without harming normal tissues.[26] Oncolytic virotherapy-mediated oncolysis not only leads to tumor regression but also provides important immune responses. Most investigations into oncolytic virotherapy are currently in the preclinical or early clinical stages but are promising.[70]

Conclusions

Immunotherapy is a promising new frontier for HCC treatment, with many novel new strategies currently under development. The incidence of HCC is rapidly increasing, and it is a major cause of morbidity and mortality both nationally and internationally. Though HCC can be a devastating disease, the best hope for prolonged survival is early screening and diagnosis. Although there is some debate regarding the ideal methods of surveillance, ultrasound with and without alpha feto-protein every 6-12 months is generally accepted as standard practice in the proper patient population. Currently, multiple therapeutic modalities are available and research investigating innovative options is ongoing. Most patients are best served in liver transplant centers, where a multidisciplinary approach can take place under the guidance of experienced transplant hepatologists and gastroenterologists. Advances in HCC prevention, detection, and treatments have resulted in improved survival for a disease that was, until recently, considered terminal. Randomized phase I-III trials of nivolumab, atezolizumab, durvalumab, ipilimumab and tislelizumab as monotherapy or combination therapy are currently being conducted. However, there is still much to be revealed regarding checkpoint inhibitors as well as immunotherapy involving gut microbiota and monocytes in the peripheral blood, so clinical trials are necessary to determine their full benefit. Immunotherapy and targeted molecular therapies have personalized medical therapy, while improving patient care, and hopefully future research can continue this endeavor.

Funding

None to declare.

Conflict of interest

Nikolaos T. Pyrsopoulos declares separate research grants from Allergan, Bayer, Beigine, Bristol Myers Squibb, Confirm, Conatus, Intercept, Mallinckrodt, Novartis, Resusix, Saro, Valeant, Gilead, Exelixis, Hologic, Shire, Genfit and Prometheus. Also, Nikolaos T. Pyrsopoulos is an advisory consultant for Bayer, Exelixis, Gilead and Novartis. The other authors have no conflict of interests related to this publication.

Author contributions

All authors contributed equally to this paper in conception and design of the study, literature review and analysis, drafting, critical revision, editing, and giving approval of the final version.

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Review Article

Erythroid Lineage Cells in the Liver: Novel Immune Regulators and Beyond

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Abstract

The lineage of the erythroid cell has been revisited in recent years. Instead of being classified as simply inert oxygen carriers, emerging evidence has shown that they are a tightly regulated in immune potent population with potential developmental plasticity for lineage crossing. Erythroid cells have been reported to exert immune regulatory function through secreted cytokines, or cell-cell contact, depending on the conditions of the microenvironment and disease models. In this review, we explain the natural history of erythroid cells in the liver through a developmental lens, as it offers perspectives into newly recognized roles of this lineage in liver biology. Here, we review the known immune roles of erythroid cells and discuss the mechanisms in the context of disease models and stages. Then, we explore the capability of erythroid lineage as a cell source for regenerative medicine. We propose that the versatile lineage of erythroid cells provides an underappreciated and potentially promising area for basic and translational research in the field of liver disease.

Citation of this article: Yang L, Lewis K. Erythroid lineage cells in the liver: Novel immune regulators and beyond. J Clin Transl Hepatol 2020;8(2):177–183. doi: 10.14218/JCTH.2019.00031.

Introduction

The human body relies mainly on the bone marrow for steady state erythropoiesis. Under erythroid stress conditions, such as chronic inflammation, spleen and liver are used to expand the erythropoietic capacity because suppressed erythropoiesis is induced in bone marrow due to inflammatory pathways. But whether erythropoietic cells directly participated in inflammatory processes was rarely studied, until very recently. Besides being inert oxygen carriers, erythroid lineage cells emerge to be a modulator of innate and adaptive immune response.[1] This review summarizes the most recent advances in newly found features of erythroid lineage cells, largely focusing on their immune modulatory functions, especially in neonatal immunity, as evidenced by both *in vivo* and *in vitro* studies in mouse and human. In addition, we also shed some light on the emerging trends of erythroid cells in the fields of microbiome study and regenerative medicine.

Keywords: Erythroblast; Immune suppression; Liver inflammation; Hepatogenesis.
Abbreviations: EMP, erythroblasts-macrophage protein; EPO, Erythropoietin; HSC, hematopoietic stem cells; Tregs, regulatory T cells.
Received: 29 July 2019; Revised: 19 December 2019; Accepted: 25 February 2020
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Erythroid lineage cells: Natural history in the liver

Cellular markers for staging of erythroid cells

There are different stages during erythropoiesis. The cells of interest for this review, referred as "erythroid lineage cells" or "CD71+ erythroid cells", represent a mix of erythroblasts, including basophilic, polychromatic, and orthochromatic erythroblasts. A widely used assay relies on the cell-surface markers CD71 and Ter119, and on the flow-cytometric 'forward-scatter' parameter, which is a function of cell size.[2] However, because CD71 is expressed on all proliferating cells,[3] the adhesion molecule CD44 has been used in some studies to distinguish between erythroblasts at successive developmental stages.[4] It is well established that during murine erythropoiesis *in vivo*, one proerythroblast undergoes three mitoses to generate (sequentially) two basophilic, four polychromatic, and eight orthochromatic erythroblasts.[5] Using this set of cell surface markers, along with the features of cell size and presence or absence of nucleus, erythroblasts can be easily distinguished from pro-erythroblasts (Ter119-) and successive reticulocytes (without nucleus) (Fig. 1).

Emergence of -omics approaches and the optimization of *ex vivo* erythroid cultures in this field will greatly enable us to investigate the continuous yet hierarchical structure of hematopoietic network, and uncover novel growth factor receptor regulators of the erythroid trajectory.[6,7]

Erythroid cell origins and dynamics in developmental liver

Erythropoiesis occurs mainly in the bone marrow; but, that is true only for the adult stage. In fact, erythropoiesis involves many tissue origins and shifts locations during the early development stage. Therefore, to understand erythroid cell origins and dynamics in developing liver is key for us to understand their various biological roles. Differentiation and proliferation of erythroid lineage cells have been extensively studied over the years. Hematopoiesis, defined as the formation of cellular components in blood, occurs during embryonic development and throughout adulthood to replenish the blood system. Specifically, erythropoiesis, which refers to the

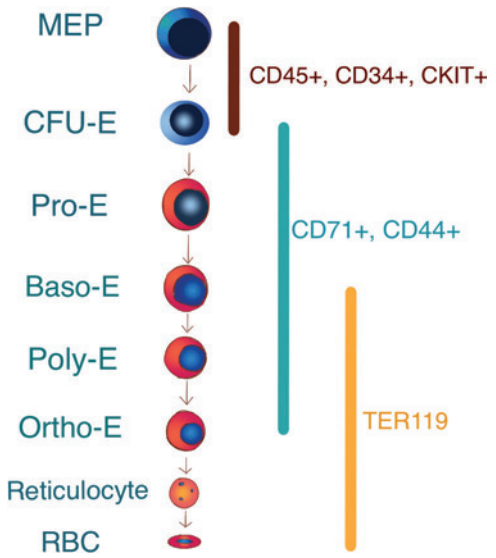

Fig. 1. Cellular markers for staging of erythroid cells. Representative cell surface markers for different stages of erythroid lineage cells. Most of the progenitors share CD45+, CD34+ and CKIT+. As they mature, they lose surface marker of CD71 and gain the marker of TER119. Eventually, reticulocyte and RBC lose their nucleus before being released into system circulation.

Abbreviations: MEP, megakaryocyte/erythroid progenitor; CFU-E, Colony Forming Unit-Erythroid cell; Pro-E, proerythroblast; Baso-E, basophilic erythroblast; Poly-E, polychromatic erythroblast; Ortho-E, orthochromatic erythroblast; RBC, red blood cell.

expansion and maturation of erythroid lineage cells, and is the earliest and largest population of cells in hematopoiesis.

We have learned from mouse models that there are two waves of hematopoiesis that occur during embryo development. The initial wave, called primitive hematopoiesis, starts at E7.5 in the extraembryonic yolk sac. The successive wave, called definitive hematopoiesis, starts at E9.5 in both the yolk sac and the intra-embryonic aorta-gonadmesonephros region.[8] Later, those hematopoietic progenitors migrate and seed the fetal liver, as the yolk sac microenvironment does not support terminal differentiation into definitive blood cell lineages; it is, thus, here that they can efficiently generate blood cells for the fast-growing embryo.[9] In detail, at E9.5-10.5, the liver rudiment is colonized by myeloerythroid progenitors. At E11.5, hematopoietic stem cells (HSCs) appear in the fetal liver, a time slightly later than that of the myeloerythroid progenitors.[8] Notably, the early fetal liver does not produce HSC *de novo* but is believed to be the main site of HSC expansion and differentiation.

The early fetal liver is rich in colony-forming unit-erythroid and proerythroblasts, reflecting an active erythropoiesis state early on, whereas myeloid and lymphoid progenitors accumulate later in life. In mouse models, HSCs plateau at E15.5-16.5 and start to decline in fetal liver, where the microenvironment can no longer meet the changing needs of lineage differentiation and HSC expansion.[9] The spleen starts to produce blood cells at E14 and continues to be a site of hematopoiesis after birth, at time of stress. At E18, the soft embryo starts to have solid bony structures, and bone marrow provides the suitable environment for the HSC and hematopoiesis throughout adulthood.[10]

The dynamics of erythropoiesis in developmental liver remains much less defined in humans. A recent study by Fanni *et al.*[11] shed some light on simplifying the time span into four stages, as follows: stage I lasts for the first 9 weeks (free of any clear sign of hematopoiesis); stage II from 10 weeks to 12 weeks (small and irregular erythrocytic foci); stage III from 13 weeks to 22 weeks (bigger foci in hepatic parenchyma); and, stage IV from 23 until 39 weeks (few round and isolated foci remains).

Immune regulating potency of erythroid cells

The immune regulatory capability of erythroid cells has been researched much less in previous years. However, this new emerging topic is the focus of this review. In the following sections, we will provide the updated evidence showing the interplay of erythroid cells with other immune cells and discuss which disease models have already been tested for their suppressive functions, with additional details given about the controversies of hypotheses in the biology of their immune potency (Fig. 2). Also, we will discuss the recent studies related to the microbiome and how it can be regulated by erythroid lineage cells. Finally, we will summarize the potential regulators of immunosuppressive erythroid cells that have been proposed in this field.

Interplay of erythroid cells with other immune cells

Erythropoiesis has been isolated and studied independently from other hematopoietic immune-related lineage cells, for decades. From the developmental perspective, the generation of lymphoid progenitors concurrently occurs with the development of myelo-erythroid progenitors, implying that the crosstalk of myeloid lineage and erythroid lineage is possibly quite common during development.[12]

Various cell types have been shown to interact with and be modulated by erythroid cells, including both lymphoid immune cells and myeloid immune cells. It is shown that nucleated erythroid cells exert a potent natural suppressor activity for both B and T cell-mediated immune processes.[13] Also, the erythroid cells from neonate spleen had the capacity to modulate the differentiation of CD4+ T cells into effector cells and provide a bias towards a Th2 type instead of Th1 type by producing IL-6.[14] CD71+ erythroid cells can also directly interact with immune regulatory T cells (known as Tregs), promoting the development and function of Treg cells through TGF-beta.[15]

The ability of erythroid cells to interact with myeloid immune cells has also been investigated in recent years. For example, it was found that the interaction between macrophage and erythroid cells happened throughout normal, stressed and pathological conditions, mediated by the adhesive molecule, erythroblast-macrophage protein, expressed on both cells.[16] Another report provided data to show that nucleated red blood cells could also induce IL-10/IL-19 production by monocytes, even without cell-to-cell contact, to suppress a vigorous harmful innate immune reaction in fetuses.[17]

Disease models involving suppressive erythroid cells

The immune suppressive function of erythroid lineage cells has been shown in different disease models in recent studies. Dunsmore *et al.*[18] reported that CD71+ cells compromise

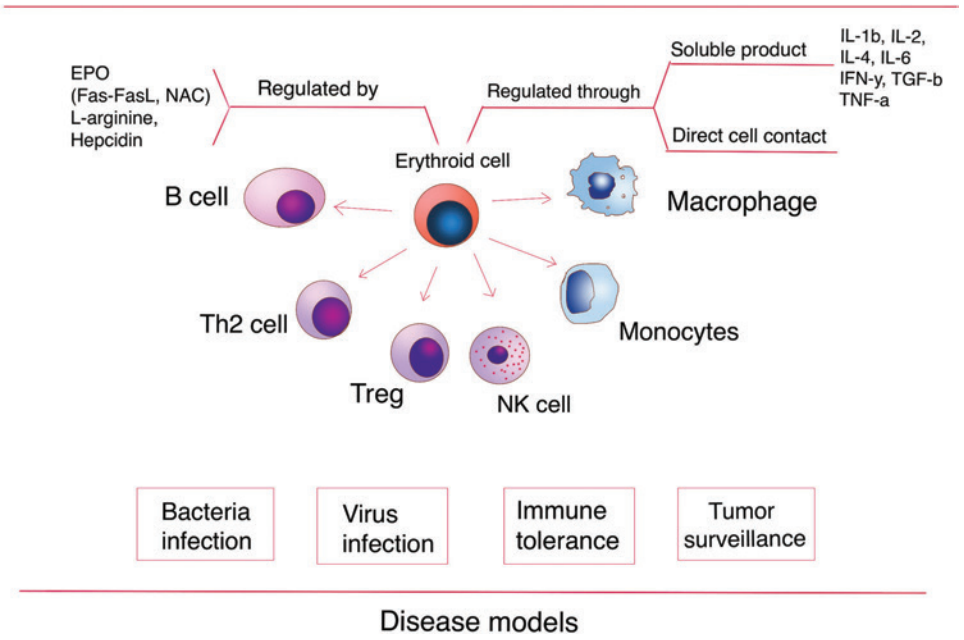

Fig. 2. Mechanism of immune regulation of erythroid cells. Erythroid cells can regulate on different cell types including both lymphoid cells (B cell, Th2 cell, Treg, NK cells and myeloid cells (macrophage, monocyte), through either soluble cytokines, or direct cell contacts. The upper stream regulators of erythroid cell include EPO (through Fas-FasL and NAC), L-arginine and Hepcidin. The immune regulatory roles of erythroid cells have been evidenced across disease models, including bacteria and virus infection, as well as immune tolerance and tumor surveillance.

Abbreviations: EPO, erythropoietin; Fas-FasL, Fas-Fas ligand; NAC, N-acetylcysteine; NK cell, natural killer cell; Treg, regulatory T cell; TNF-a, tumor necrosis factor alpha; TGF-b, transforming growth factor beta; IFN-y, Interferon gamma.

innate immune responses against *Bordetella pertussis* infection in the lung. Apart from bacterial infection, two studies have shown that CD235a + CD71+ erythroid cells also modulate immune response against virus infection, including the role of erythroid cells in peripheral blood in human immunodeficiency virus-infected people,[19] and in a biliary atresia model induced by rhesus rotavirus.[20]

Besides immunity against pathogens, erythroid lineage cells also participate actively in immune tolerance and surveillance. Umbilical cord CD71+ erythroid cells have been shown to play a role in spontaneous preterm labor and maternal-fetal tolerance.[21,22] In the enlarged spleen of hosts bearing advanced tumors, CD71+ erythroid cells were also found to be enriched and to facilitate tumor progression by secreting the neurotrophic factor artemin into the blood.[23] In both patients with advanced cancer and treatment-naive mice bearing large tumors, CD71+ erythroid cells contributed to the impaired T cell responses, especially that of the CD8+ T cells.[24]

Controversies: modulation or suppression, direct or indirect

In the field of research into the function of erythroid lineage cells, controversial results have been observed in a few studies, leading to debate over whether they are immune suppressive or modulatory and whether the interaction is direct or indirect. In a mouse sepsis model induced by endotoxin or polymicrobial challenge, neonatal CD71+ erythroid cells failed to modify sepsis mortality.[19] Another study in the pathogenesis of preterm labor showed neonatal CD71+ erythroid cells to be immunomodulatory, rather than immunosuppressive.[25]

The mechanism of erythroid cells' immune suppression activities, whether through direct cell contact or soluble products, is also an ongoing matter of debate. One human study showed direct contact, instead of soluble products, between neonatal CD71+ erythroid cells and maternal mononuclear immune cells and characterized it as the key step to release of pro-inflammatory cytokines and decrease of the anti-inflammatory cytokine TGF-beta.[25] Another study showed the opposite result; the investigators successfully used erythrocyte-derived conditioned media to induce a type-1 interferon response in macrophages, supporting an integrative role for soluble products in the immune response.[26] The spectrum of cytokines produced by erythroid cells is, surprisingly, quite widespread and includes IL-1beta, IL-2, IL-4, IL-6, IFN-gamma, TGF-beta1 and TNF-alpha.[27,28]

Microbiome and erythroid lineage cells

It was Elahi *et al.*[29] who first reported that CD71+ erythroid cells suppress the exaggerated inflammatory process and establish immune tolerance towards colonized commensal microorganisms after birth, which in turn compromised host defense against pathogens. They also showed that arginase II from CD71+ erythroblasts is essential for neonatal innate-immune suppression, which was further validated by another group, demonstrating a key role of arginine in mucosal immunity, especially of susceptibility to gut-derived pathogens.[30] In a more recent human study about inflammatory bowel disease, the role of erythroid cells in regulating the gut microbiome was further investigated. Data showed that

reduced frequency and/or impaired functionality of CD71+ erythroid cells during pregnancy may predispose inflammatory bowel disease patients to a more pro-inflammatory milieu in their gastrointestinal tract and induce dysbiosis.[31] Other recent mechanistic research has suggested heat-stable microbial products which circulate in the bloodstream might link to inflammatory signaling modulated by hematological compartments.[32]

Potential regulators of immunosuppressive erythroid cell

Erythropoiesis is a highly regulated process of erythrocyte production. However, limited studies were done to investigate potential regulators for the immune suppressive features of those erythroid cells. Here, we summarize some of those proposed potential regulators, which could be promising for future therapeutic applications.

L-arginine which overrides immunosuppression of neonatal CD71+ cells that express the enzyme arginase-2 could also be a potential regulator of the immune response of erythroid cells.[29] Hepcidin expression was shown to be mediated by the transferrin receptor 1 TfR1 (also known as CD71) expression on erythroid precursors, which might be a potential regulator of immunosuppressive erythroid cells.[33]

The erythropoietin (EPO) receptor is expressed abundantly on proerythroblasts and early-stage erythroblasts, indicating that EPO can be a potential regulator of the immune suppressive function of erythroid lineage cells. Several lines of evidence have demonstrated that liver is the predominant production site for EPO during development and is the major cellular sites of EPO gene expression.(33) Erythroid lineage progenitors in fetal liver are shown to be more sensitive to this effect of EPO than are those of adults,[34] implying an active regulatory role of EPO in the hepatic milieu. EPO has been proven to modulate the immune responses and dynamics of oxidative status in various studies both *in vivo* and *in vitro*,[35] through either the Fas and FasL pathway, modulation of N-acetyl-cysteine, a reactive oxygen species scavenger,[36] or by directly reducing production of neutrophils, accompanying accelerated erythropoiesis.[34] EPO can also signal through macrophages to promote apoptotic cell clearance and immune tolerance.[37] Targeting EPO and EPO-receptor have been shown to have great potential in regulating immune injury of various liver diseases, suggesting promising future clinical applications.[38–41]

Erythroid lineage cells and neonatal immunity

The accumulation of erythroid lineage cells in the liver perinatally suggests they are more related to neonatal immunity than any other developmental stage later in life. Unsurprisingly, high frequencies of erythroid cells were found (median: 31%) in cord blood samples from term and preterm neonates. These erythroid cells disappear rapidly by 1 week of age.[42] In neonates, the frequent onset of infection might not be attributed to an inherent immaturity of neonatal immune cells but rather to the immune suppression by CD71+ erythroid cells, which leave newborns vulnerable to infection.[43] Furthermore, the selective accumulation of erythroid cells in the spleen during development may explain differences of immune responses generated in infants and neonates.[14]

Immune potency of these CD71+ erythroid cells was also observed in neonatal salmonella infection but it seemed to have both positive and negative consequences for host immunity.[44] Immunosuppression mediated by CD71+ erythroid cells is also crucial for homeostasis in the perinatal period, as it has been shown to bring down TNF-alpha and IFN-gamma production through arginase-2 activity and PD-1/programmed death ligand-1 (PDL-1), contributing to feto-maternal tolerance.[22] The critical window of erythropoiesis in the neonatal period suggests erythroid lineage cells may play an important role in neonatal immunity.

Lineage crossing and regenerative medicine

Erythroid progenitors replenish other cell types

Neonate liver does not have a quiescent state, as adult livers do; they are continually undergoing massive transitional changes, even when not confronted by any external stimulus.[45] Thus, any single gene in the liver may serve distinct functions in different stages. One of the emerging themes is that many of the same signaling pathways, transcription factors and even cell types are used reiteratively.[46,47] Erythroid progenitors possess such versatility, making them capable of replenishing other cell types developmentally, as illustrated by the following examples. The non-hematopoietic cell fraction of the bone marrow, which contains heterogeneous stromal cell populations, has been shown to be generated from a hematopoietic rather than mesenchymal origin.[48] Adult connective tissue-resident mast cells, which are associated with various inflammatory processes, have been shown to originate from late erythro-myeloid progenitors.[49] Erythro-myeloid progenitors also constitute a source of endothelial cells.[50] The blood islands formed thereafter contain not only red blood cells but also endothelial cells.[51] The ability of erythroid progenitors to replenish other cell types suggest its potential to be used in regenerative medicine.

Erythroid cells crosstalk with hepatogenesis

In the past, erythrocyte-related genes and erythrocytes were frequently excluded from research analysis, as presumably the cells only carry oxygen and do not interact with other cells or the environment.[52] Specific retrieval and isolation protocols were constantly used to eliminate the majority of circulating erythroid cells to increase purity for "cells of interest".[53] For those reasons, how erythroid cells participate specifically in hepatogenesis is largely unknown.[54] As demonstrated in the previous paragraph, there are continuous lines of evidence showing that erythroid progenitors can replenish other cell types; therefore, their involvement in hepatogenesis needs to be further examined.

The biological process of erythropoiesis is weaved into hepatogenesis chronologically and spatially during liver development.[55] Hepatoblasts (hepatic endoderm cells) delaminate from epithelium and invade the adjacent septum transversum mesenchyme to form the liver bud at E9.5-E10.5.[56] After that, the liver bud undergoes a period of accelerated growth, as it is vascularized and colonized by hematopoietic cells.[57] The encounter of hepatoblasts and hematopoietic cells in the liver bud raises the possibility of cell-crosstalk during this developmental milestone of hepatogenesis. There are a handful of lines of evidence showing the

existence of such interactions between erythropoiesis and hepatogenesis in the early developmental stage.

An earlier study on the joining of the bile ducts during hepatogenesis of the mouse embryo has demonstrated that reciprocal cell interactions can possibly occur between the biliary epithelial cells from embryonic endoderm and erythroid cells from nearby mesoderm.[58] Using dynamic transcriptomic and proteomic profiling, relationships and extensive crosstalk between hematopoiesis and hepatogenesis in the mid-trimester fetal liver was characterized.[59] As we would expect, one possible means of crosstalk mediation can be cytokine secretion. Indeed, it has been shown that hematopoietic cells in the liver secrete cytokine oncostatin M, which in combination with the glucocorticoid hormones human growth factor and WNT, promotes hepatocyte differentiation and maturation.[60]

Compared to crosstalk between erythropoiesis and hepatogenesis during the early developmental stage, the interaction between erythroid cells and parenchymal cells during liver regeneration in the mature liver is more debatable. It is, however, well known that mature liver still maintains great self-regeneration capability. As discussed above, in the liver bud, hematopoietic cells are in direct contact with hepatoblasts, the common precursors of hepatocytes and cholangiocytes. However, in the mature liver, erythroblasts reside in the sinusoid and lose direct contact with cholangiocytes; blood from the portal vein enters the sinusoid space and comes into direct contact with the basal surface of the hepatocyte,[61,62] while bile is secreted from the apical surface of adjoining hepatocytes into the bile canaliculi (grooves in the cell surface).[63] The interaction between erythroid cells and regenerative parenchymal cells, if there is any, remains largely undefined.

Recent advances in single-cell RNA sequencing have provided new evidence that erythroblasts can cross lineage and participate in hepatogenesis. In fetal liver, two distinct ALB+ expressing populations and several nonhepatic populations, resembling erythroblast cell transcriptionally, types were found.[64] One of the biggest challenges and concerns of using induced pluripotent stem cells for regenerative medicine is the carcinogenesis potential of uncontrolled development of the seeding stem cells.[65,66] As such, discoveries of safer progenitor cells for regeneration purposes in many organ systems have shifted focus to *in situ* precursors.[67–70] Based on their versatility, hepatic erythroid cells can used as an integral component of regenerative modeling in future studies (Fig. 3).

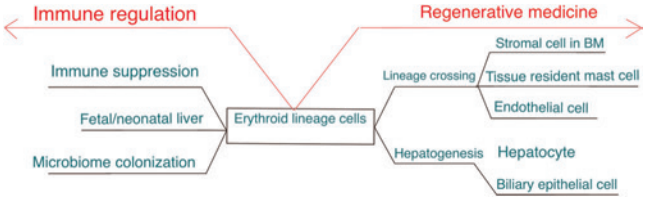

Fig. 3. Emerging roles of erythroid lineage cells in the liver. Erythroid lineage cells have shown potential of immune regulation and regenerative medicine. Future investigations may highlight their role in immune suppression, fetal/perinatal liver disease and microbiota-host interactions. On the arm of regenerative medicine, their role to participate in lineage crossing and hepatogenesis will be further investigated.

Conclusions

There are a few unique advantages of erythroid lineage cells. They are not permanently anchored and can be easily mobilized from sinusoid, having good potential for self-renewal, and being sensitive to external hazards and immune potent. They may function proactively through the developmental stage, the maturation of the immune and non-immune cells, and the different progressive stages of the disease. Those cells hold promise in research to highlight their utility in immune-related diseases and can be innovative targets for therapeutic options.

Acknowledgements

We would like to thank Dr. Diamantis Konstantinidis, from the Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, for his insightful discussion on physiology of erythropoiesis before this manuscript was drafted.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived and designed the review, and designed the figures (LY), co-wrote the manuscript (KL, LY).

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Review Article

Pathogenesis of Thrombocytopenia in Chronic HCV Infection: A Review

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Abstract

A large proportion of patients with chronic hepatitis C have associated thrombocytopenia (TCP). Due to bleeding risks, TCP, when severe, can limit diagnostic and therapeutic procedures, treatments, and increases risk of complications, especially excessive bleeding. It is important to understand the mechanisms that cause TCP in order to manage it. In general, TCP can be due to increased destruction or decreased production. Proposed mechanisms of increased destruction include autoantibodies to platelets and hypersplenism with sequestration. Proposed mechanisms of decreased production include virus-induced bone marrow suppression and decreased TPO production. Autoantibodies directed against platelet surface antigens have demonstrated an inverse correlation with platelet counts. Hypersplenism with sequestration involves the interaction of portal hypertension, splenomegaly, and platelet destruction. Decreased production mechanisms involve appropriate and inappropriate levels of TPO secretion. There is limited evidence to support viral-induced bone marrow suppression. In contrast, there is strong evidence to support low levels of TPO in liver failure as a major cause of TCP. TPO-agonists, specifically eltrombopag, have been shown in hepatitis C patients to increase platelet counts without reducing portal hypertension or splenomegaly. We conclude that TCP in hepatitis C virus-induced liver disease is often multifactorial, but an understanding of the mechanisms can lead to judicious use of new drugs for treatment.

Citation of this article: Rawi S, Wu GY. Pathogenesis of thrombocytopenia in chronic HCV infection: A review. J Clin Transl Hepatol 2020;8(2):184–191. doi: 10.14218/JCTH.2020.00007.

Introduction

Thrombocytopenia (TCP) is most often defined by platelet (Plt) counts less than 150×10^9/L.[1] The average Plt survival time has been reported to range from 7.5 to 9.5 days.[2] It has been estimated that at least 71 million individuals in the world and 2.7-3.9 million people in the USA have chronic hepatitis C infection.[3–4] From 0.16% up to 76% of these patients have associated TCP.[5] Populations with high percentages of advanced cirrhosis reportedly have increased prevalence of TCP. TCP is important to recognize because it can increase the risk of complications during invasive diagnostic or therapeutic procedures, need for treatment with interferon (IFN), and in the management of patients on orthotropic liver transplantation waiting lists. It can be a risk factor for bleeding esophageal varices, in chemotherapy for solid tumors or hematological malignancies, and surgery.[6,7] Bleeding risk increases as Plt levels decrease below 50×10^9/L, with major bleeding associated at levels below 10×10^9/L. However, Plt counts alone do not always reflect bleeding, as other factors such as Plt function, the presence of anti-Plt antibodies, and levels of coagulation factors may also be involved.[8]

In order to better understand how to treat or manage TCP, it is essential to understand the pathophysiological mechanisms that cause it. Such knowledge can lead to selection of appropriate therapy.

TCP can be caused by increased destruction (including increased storage) of Plts or decreased production of Plts (Fig. 1).[1] Conditions that cause increased destruction of Plts include autoimmune responses to antibodies against Plts, idiopathic TCP purpura (ITP), and hypersplenism. Hypersplenism is characterized as splenomegaly and TCP.[9] TCP in this case can be due to increased storage of Plts, with Plt sequestration and destruction of Plts by phagocytosis, as well as autoimmune responses. In this paper, the term 'destruction-sequestration' is used to encompass the mechanisms behind TCP caused by hypersplenism. This is an important distinction because Plt 'destruction', 'degradation', and 'sequestration' may have different mechanisms of action, some of which have not been proven, but are often used interchangeably in other articles/studies.

On the other hand, conditions that cause decreased production of Plts include virus-induced bone marrow suppression and decreased thrombopoietin (TPO) production. Not infrequently, several mechanisms may coexist. The purpose of this review is to present the current state of knowledge regarding the pathogenesis, diagnosis and treatment of TCP in patients with hepatitis C virus (HCV).

Increased destruction

Auto-antibodies

The relationship between autoimmune disease and TCP in HCV has been studied by analysis of anti-tissue antibodies (antinuclear and anti-smooth muscle antibodies),

Keywords: Thrombocytopenia; Chronic hepatitis; Autoantibodies; Hypersplenism; Thrombopoietin.

Abbreviations: HCV, hepatitis C virus; IFN, interferon; ITP, idiopathic thrombocytopenia purpura; PAIgG, platelet-associated immunoglobulin G complexes; Plt, platelet; TCP, thrombocytopenia; TIPS, transjugular intrahepatic portosystemic shunt; TPO, thrombopoietin.

Received: 30 January 2020; Revised: 25 March 2020; Accepted: 31 March 2020

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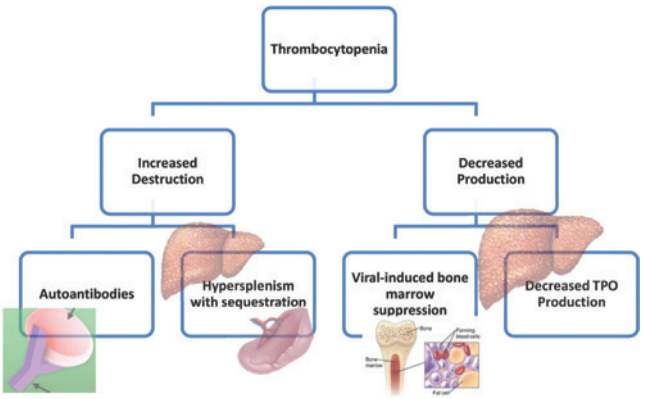

Fig. 1. Pathophysiologic mechanisms of thrombocytopenia.

cryoglobulinemia, rheumatoid factor, and kidney and microsomal antibodies.[10,11] Autoantibodies directed against Plt surface antigens have been identified. These antibodies are thought to become Plt-associated immunoglobulin G complexes (PAIgG) recognized by macrophages in the spleen or liver.[11] Once recognized by macrophages, premature destruction of the Plts occurs by phagocytosis within the reticuloendothelial system.[11–14] Up to 40% of chronic hepatitis C patients have autoantibodies[10] and up to 30% of patients with chronic ITP have been found to have chronic HCV.[15,16]

Nagamine *et al.*[17] compared chronic hepatitis C patients with chronic hepatitis B patients and controls, with the purpose of determining the relationship between PAIgG and Plt counts. Up to 88% of patients with chronic HCV have had high levels of PAIgG compared to their controls ($p<0.01$), with an inverse correlation of Plt levels ($p<0.05$).[11,17] The PAIgG levels increased with histological progression of hepatitis C, further supporting their endpoint. A weakness of the study is that at the time the study was performed, there was no assay for detection of Plt-specific autoantibodies; instead, total PAIgG levels were used. On the other hand, the PAIgG levels did not correlate with serum IgG levels. The study also suffered from a small patient population ($n<100$ per group) and a lack of measurement of specific autoantibodies.

In another study, two groups of patients, one with low Plt counts ($<150\times10^9$/L) and the other with normal Plt counts, were studied to determine differences in levels before and after IFN therapy.[18] In addition, that study measured levels of PAIgG in each subgroup to determine the relationship between TCP and an autoimmune response. Patients with cirrhosis, splenomegaly, chronic alcohol use and other autoimmune chronic liver diseases were excluded. At baseline, PAIgG levels were 181% higher in the TCP group (197.3 ±130.2 ng/10^7 Plt) compared to the non-TCP group (70.9 ±31.1 ng/10^7 Plt) ($p<0.01$). Although high levels of PAIgG were found in the TCP group, the study was not designed specifically to detect the relationship between immune dysfunction and TCP in HCV. PAIgG levels were lower in patients observed after IFN treatment. Unfortunately, exact numbers/percentage decreases were not studied and/or reported. These data are required to establish the existence of HCV-induced autoimmune responses.

HCV may contribute to or trigger the development of ITP, mediated in particular by circulating immune complexes. Pockros *et al.*[16] studied 3440 new HCV cases to determine the prevalence of new HCV-ITP cases. In that study, HCV-ITP was diagnosed by the following criteria: the diagnosis of HCV either before or concurrent with documentation of TCP, TCP out of proportion to the severity of the liver disease, a positive anti-Plt antibody test, and a response to agents known to be effective in the treatment of ITP (e.g., corticosteroids, cyclophosphamide) in patients who required therapy (six of the seven patients). Within a 54-month interval, there were seven new HCV-ITP cases associated with antibodies against GPIIb/IIIa. All of these patients were treated with steroids initially and only two responded by return to normal Plt counts. Three required steroids and intravenous immunoglobulin, and two required cyclophosphamide and splenectomy. Even with these treatments, the Plt counts were still low and the highest count was only around 50×10^9/L. Statistical analyses showed that those differences in HCV-ITP prevalence were highly significant. However, there were no data on treatment of the virus itself to determine its effect on Plt counts and antibody levels. It is important to note that all patients in the study were also positive for other autoimmune factors, such as antinuclear antibodies, anti-smooth muscle antibodies, or cryoglobulins, which are confounding factors. The study also had a very small patient population, which reduces its external validity. Overall, it is difficult to justify the conclusions of the study.

Honma *et al.*[19] prospectively studied 187 patients who were treated with direct-acting antivirals. PAIgG levels as well as Plt counts were measured prior to treatment and post-treatment. Among the patients, 91.4% had elevated PAIgG levels before the study. However, only 18.2% of the patients had TCP, which the study defined as $<100\times10^9$/L. Because of this low cutoff value, the number of individuals with TCP is likely to have been an underestimate. Among the 34 thrombocytopenic patients, 97.1% had elevated PAIgG levels. That study claimed that 12 and 24 weeks after end of treatment with direct-acting antivirals, there was a statistically significant increase in Plt counts and decrease in PAIgG levels, with the largest change in the individuals who had the lowest baseline Plt count and highest baseline PAIgG level ($p = 0.021$ and $p = 0.002$, respectively). Their data showed a statistically significant negative correlation between Plt count and PAIgG levels before and after direct-acting antivirals ($p<0.001$), which supports an autoimmune role in Plt destruction. However, larger studies are needed to confirm this conclusion.

Other studies or components of studies dispute the autoimmune theory of Plt destruction. By measuring PAIgG levels specifically, Pereira *et al.*[12] studied 35 patients with chronic liver disease to determine whether anti-Plt antibodies were involved in the development of TCP. Twenty-three of the thirty-six patients (64%) had anti-GP IIb/IIIA or GpIb antibodies found by using a specific glycoprotein immunoassay of IgG bound to those complexes. The specific antibody levels correlated with elevated levels of PAIgG. However, elevated levels of PAIgG were not inversely correlated to Plt counts. The data supported autoimmune destruction of Plts and not viral suppression. Autoimmune destruction is mediated by the reticuloendothelial system, whereby phagocytosis of Plt immune complexes is mediated by Fc receptors.

In another study, 78 patients with chronic HCV but without other autoimmune diseases were studied to determine the relationship to PAIgG formation and to compare PAIgG levels before and after IFN treatment.[20] Of these patients, 83.3% had elevated PAIgG titers (median titer of 400 ng/10^7 Plt)

compared to the upper normal value of 100 ng/10^7 plt. Of these patients, 23 underwent IFN treatment. Sixteen patients (69.5%) were responders (sustained virologic response) and were found to have significantly higher PAIgG titers (622 ng/10^7 plt) compared to non-responders (283 ng/10^7 plt; p = 0.002). The Plt counts decreased in the responder group from 195×10^9/L to 146×10^9/L (p = 0.0001).[20] That study also measured PAIgG levels before and after treatment. Strengths of the study included exclusion of other autoimmune disorders and assessment of the effect of eradication of HCV on PAIgG levels. These data argue against HCV-induced autoimmune antibodies leading to Plt destruction because the PAIgG titers increased while Plt counts decreased after eradication of HCV. However, the study could have been improved by evaluating specific effects of IFN on Plt counts after treatment.

Overall, autoimmune antibodies may be a contributory factor to TCP but there is not enough evidence to suggest it to be a sole cause of TCP in most cases of chronic HCV.

Hypersplenism with sequestration

As portal hypertension develops, the spleen enlarges and resistance to portal flow increases, causing redistribution/pooling of Plts in the spleen and therefore decreased Plts in circulation (sequestration).[21,22] The incidence of splenomegaly in cirrhosis has been reported to range from 36-92%, while the incidence of hypersplenism in cirrhosis ranged from 11-55%.[23] Fig. 2 shows the relationships between liver cirrhosis, TCP, and hypersplenism.

By application of radiolabeled Plts in patients with splenomegaly, it has been shown that the primary site of sequestration is the spleen rather than the liver. In one study, there was a 34% increase in Plt sequestration in patients with splenomegaly compared to those without. The mean Plt survival time was also studied and showed a 2.2±0.2 day decrease in survival time in patients with splenomegaly compared to their normal controls.[24,25] However, rate of sequestration was not studied, and although the term 'Plt destruction' was used, the evidence for this was not clear. There is evidence that Plt destruction-sequestration caused by hypersplenism is an autoimmune process. In this case, Plts are bound by anti-Plt antibodies and destroyed by macrophages.[23,26] In the study by Sekiguchi *et al.*,[26] 24 patients with HCV cirrhosis were compared to 17 HCV asplenic cirrhotic patients and 21 non-HCV cirrhotic patients to determine the relationship between spleen size, PAIgG, and Plt counts. Individuals with HCV and splenomegaly had a 61% increase in average PAIgG titers (247.9±197) compared to those splenectomized (125.6 ±87.8) and non-HCV cirrhosis patients (152.4±127.4). There was an inverse correlation between PAIgG and Plt counts. When titers were as high as 400 ng/ 10^7 cells, the Plt counts decreased to below 60 × 10^9/L. Furthermore, in patients who were splenectomized, there was no association with PAIgG and Plt counts, and there was a decreased T cell response reflected in decreased CD4/CD8 ratios. Thus, there appeared to be a relationship between splenomegaly and an autoimmune form of destruction-sequestration, resulting in decreased circulation of Plts. Overall, the data from that study was convincing for this relationship when comparing three separate groups. However, it was a retrospective study with the inherent weaknesses of such.

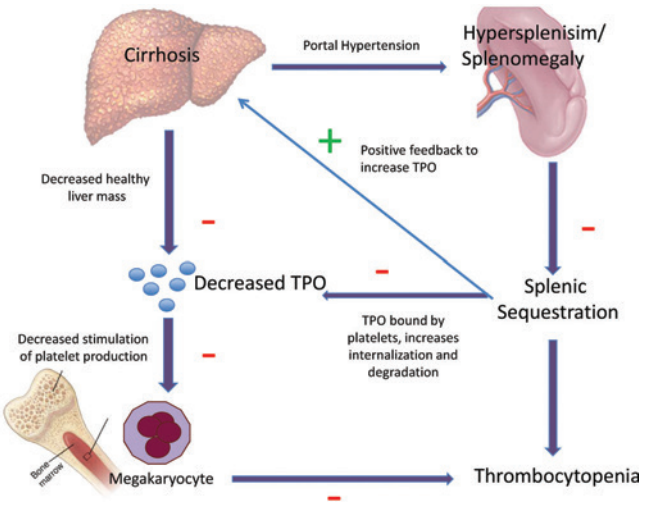

Fig. 2. Relationships between cirrhosis and thrombocytopenia.

In another study, 209 patients with chronic viral hepatitis were investigated, of which 85 patients had splenomegaly and 124 had a normal spleen size. HCV was the etiology in 93% of the cases. Of the patients with splenomegaly, 71% had TCP. As expected, there was an inverse relationship between spleen size and Plt count, along with a direct relationship between spleen size and portal vein diameter. On the other hand, there was no correlation between liver fibrosis and spleen size, which was unexpected as progression to cirrhosis and portal hypertension were expected to cause splenomegaly. Moreover, in that study, as fibrosis increased, the Plt count decreased (p<0.001). In the subgroup of patients with grades 3 and 4 fibrosis without splenomegaly, 76% had TCP and the median Plt count was 108-126×10^9/L.[27] The study had appropriate exclusion criteria and a sufficient number of patients studied, with overall equal demographics. However, the study only had 34% of patients with grades 3 or 4 fibrosis, which could have falsely lowered their results. Overall, this study demonstrated that splenomegaly leading to sequestration could not be the sole mechanism of TCP.

Furthermore, studies have shown that there was no significant difference in Plt counts between patients with and without splenomegaly. In patients who were already thrombocytopenic, the Plt counts were significantly different in those patients with enlarged spleens compared to without (mean Plt: 77±26×10^9 vs. 115±30×10^9 cells/L (p<0.0001)).[27] Splenomegaly may contribute to TCP but is often not sufficient to cause TCP.

To examine the relationship between portal hypertension and Plt counts, studies on patients with transjugular intrahepatic portosystemic shunt (TIPS) were undertaken. In one study, 55 TIPS patients were compared with 110 control patients to determine the effect of portal decompression on Plt counts pre- and post-procedure, over 12 months. The median Plt counts of patients post-TIPS increased by 19.7% (104×10^9/L to 124.5×10^9/L), whereas the control Plt counts decreased by 17.1%.[28] The greatest change in Plt counts occurred in the subgroup that had baseline TCP of <100×10^9/L. There were 20 TIPS patients and 43 control patients analyzed due to deaths or loss to follow-up. In that population, by month 12 post-TIPS, the median Plt counts increased by 36.8% and Plt counts increased by at least 25% above baseline in every patient in this group. The

median increase in Plt count was by 26.5×10^9/L, whereas in the moderate TCP group (Plt count $101\text{-}149\times10^9$/L) there was only a 1×10^9/L increase. In the control group, the Plt counts decreased by 14.3%. Also, there was an increase in Plt counts in patients that had a smaller decrease in portosystemic shunt gradient after TIPS (≥12 mmHg) compared to those that had a larger gradient difference (<12 mmHg).[28] Appropriate patient populations were excluded, limiting confounding factors for TCP. This was a prospective study with a control group, which many other studies have lacked.[29,30] However, only patients with severe TCP showed significant improvement in Plt counts and no group reached normal counts. The data from the portocaval shunt gradients lead to conclusions opposite to that expected based on spleen size alone. These data support the conclusion that hypersplenism with sequestration cannot be the sole mechanism of TCP.

Decreased production

Bone marrow suppression

Viral bone marrow suppression has been hypothesized to be a mechanism of development of TCP in chronic HCV patients. However, data on bone marrow suppression by HCV is extremely limited. One study compared Plt counts in HCV patients before and 6 months after IFN therapy. They found that the post-treatment Plt counts increased by 25% relative to the baseline value (p = 0.027). Twelve of twenty-two who responded to IFN therapy with clearance of HCV showed an increase in Plt count. Yet, in the 10 patients whose HCV did not respond to IFN, Plt counts decreased by 25%.[18] The improvement in TCP after completion and cessation of IFN therapy could have been due to elimination of IFN-suppression of bone marrow or improvement in liver function, as described in a subsequent section, and not necessarily HCV-induced bone marrow suppression. A strength of the study is that cirrhosis and splenomegaly were excluded. This is an important distinction, as the authors controlled for hypersplenism and decreased TPO production as causes of TCP. However, although they measured TPO antibodies before IFN treatment of HCV, they did not remeasure levels after treatment. TPO levels were higher prior to initiation of IFN, which was expected due to IFN-bone marrow suppression. It would have been helpful to have data on TPO levels after treatment of HCV with and without eradication of HCV. Although this was a relatively small study, it does support the role of HCV-bone marrow suppression but requires further confirmation.

Decreased TPO production

In response to an increased demand for Plts, the number and size of megakaryocytes increase under the stimulation of TPO, a hematopoietic factor that regulates this response at various levels. The primary site of TPO mRNA and protein synthesis is the liver. Lesser amounts are found in the kidney, brain, and testes. There is no significant storage of TPO. It is synthesized and immediately released. With persistent TCP, TPO levels increase exponentially and reach a steady state. TPO levels increase within 24 hours after the onset of TCP and levels are inversely and exponentially proportional to the Plt count. Also, in the absence of Plts, there is little clearance of TPO by Plts, levels rise, bone marrow megakaryocytes are stimulated, and Plt production increases. TPO is cleared by attachment to Plts.[31]

As shown in Fig. 3, TPO levels are appropriately high in aplastic anemia and ITP (due to positive feedback).[7,32] In the liver, as fibrosis advances to cirrhosis, liver mass decreases, resulting in levels of inappropriately low TPO. Adinolfi *et al.*[27] studied 124 patients and compared Plt counts and TPO concentrations at different stages of fibrosis. The TPO levels were measured and compared in 54 of those patients. As shown in Fig. 4, the median TPO levels (pg/mL) per stage of fibrosis were reported as follows: stage 0-1: 58, stage 2: 48, stage 3: 36, and stage 4: 27. The median TPO levels of stage 3-4 fibrosis were significantly lower compared to stage 0-2 (p<0.001).

One study sought to determine the effect of reducing hypersplenism by decreasing spleen size after splenic embolization, and improving synthetic liver function by liver transplantation on TCP. A total of 33 cirrhotic patients were studied, 24 of whom had cirrhosis due to HCV. Compared to the controls, the cirrhotic cohort had significantly lower TPO levels (median of 120.7 pg/mL compared to 756.4 pg/mL in non-cirrhotics) (p<0.001).[33] That population was then subdivided into two sub-cohorts of 22 patients who underwent splenic embolization and 11 who had liver transplants. In the splenic embolization sub-cohort whose spleen size decreased but had no change in liver mass, TPO levels increased by 94% and Plt counts increased by 84% at day 90, respectively (TPO on day 0 was 153.7 pg/mL and 282.1 on day 90, p<0.05). Plt counts on day 0 were 50.5×10^9/L and 98×10^9/L on day 90, p<0.05).[33] This is consistent with decreased Plt sequestration. However, TPO levels unexpectedly increased after splenic-embolization. After liver transplantation, which decreased spleen size but increased liver mass, TPO levels increased by 288% and Plt counts increased by 175% at day 90 (TPO on day 0 was 43 pg/mL and 166.9 on day 90). Plt counts on day 0 were 56×10^9/L and 154×10^9/L on day 90, p<0.05).[33] With liver failure, TPO levels are inappropriately low prior to transplantation. After transplantation, spleen size is decreased, and the synthetic function of the liver increased. With normalization of Plt counts at 90 days, TPO levels were found to be appropriately increased from

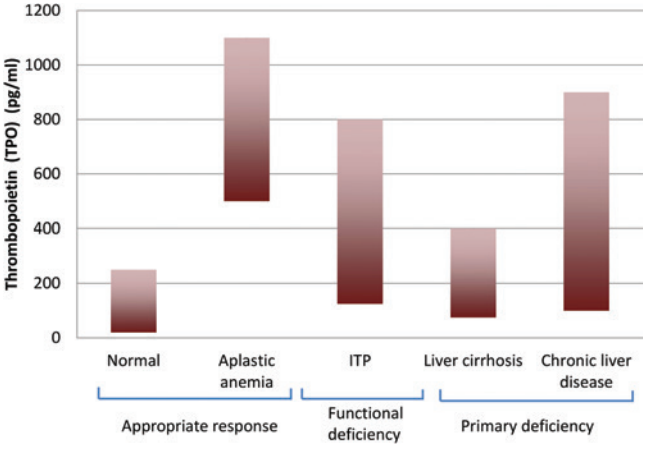

Fig. 3. Mean ranges of thrombocytopenia levels in various disease states: aplastic anemia, idiopathic thrombocytopenia purpura (ITP), liver cirrhosis, and chronic liver disease.

Modified from Giannini *et al.*[7,32]

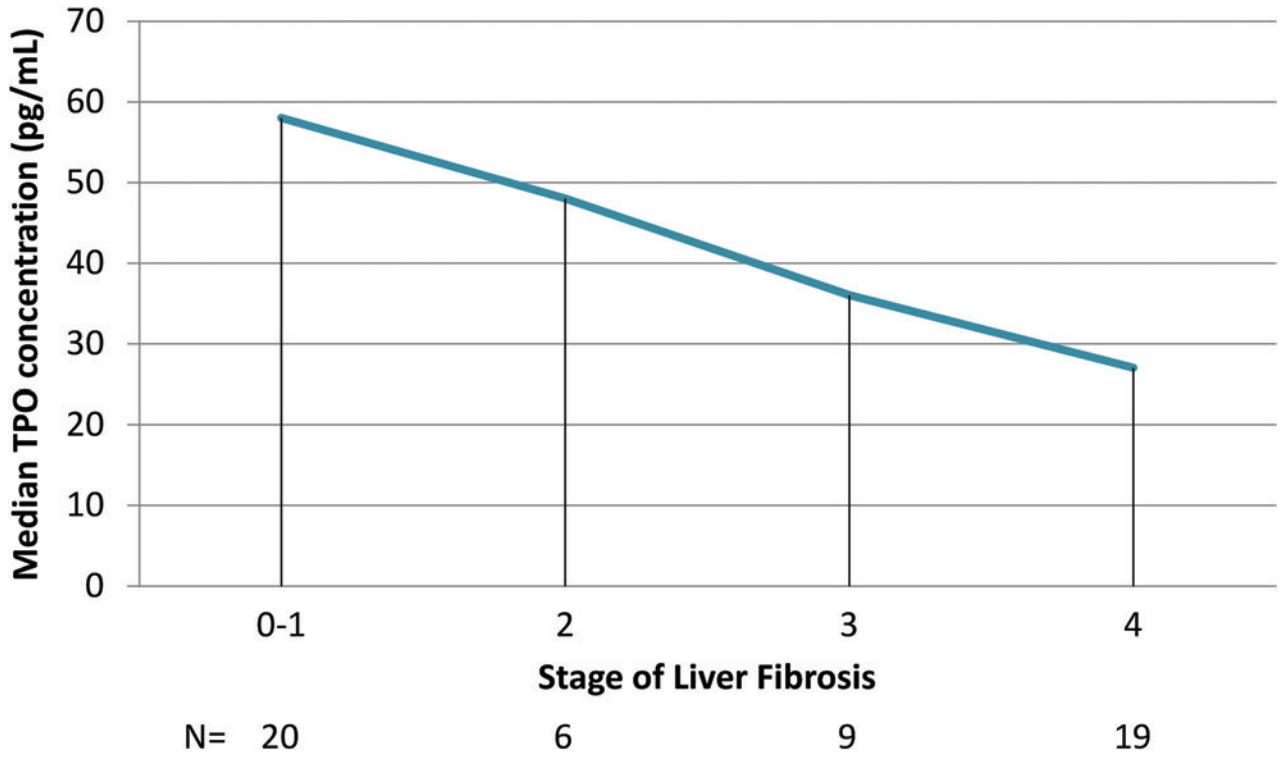

Fig. 4. Relationship between serum thrombocytopenia concentration and liver fibrosis.
Modified from Weksler *et al.*[11] and Adinolfi *et al.*[27]

baseline. It would be helpful to have had a longer study period to determine if TPO levels returned to normal at later points after transplantation. In this study, it is difficult to conclude that decreased TPO production is the leading cause of TCP. However, it seems likely that the combination of decreased TPO production and splenic sequestration due to splenomegaly from portal hypertension are involved in the pathogenesis of TCP, rather than hypersplenism alone.

Eltrombopag, an oral TPO-receptor agonist, has been shown to increase Plt counts in hepatitis C patients without reducing portal hypertension or splenomegaly.[34] Eltrombopag interacts with the trans-membrane domain of the TPO receptor, activating JAK2/STAT signaling pathways and increasing proliferation and differentiation of human bone marrow progenitor cells into megakaryocytes. McHutchison *et al.*[34] studied 45 chronic HCV cirrhotic patients and their median Plt counts over 112 days. The selection of patients was based on the following inclusion criteria: Plt count less than 70×10^9/L, diagnosis of chronic HCV, and evidence of compensated cirrhosis (through either liver biopsy, imaging, or endoscopic evidence of portal hypertension). Exclusion criteria included a history of thrombosis, pregnancy, or coinfection with hepatitis B or human immunodeficiency virus. Study patients received either varying doses of eltrombopag or placebo. If Plt counts increased above 70×10^9/L, those individuals were eligible to be treated with IFN per investigator's discretion. It was found that as the IFN dose increased, Plt counts decreased. However, with eltrombopag treatment, counts never declined below the minimum threshold necessary for treatment. This study showed that eltrombopag can increase Plt counts allowing patients to be treated for longer periods of time and at higher IFN doses. The study was a multicenter, double-blinded, centrally randomized, intention-to-treat analysis, and placebo-controlled with an effective power for the primary endpoint of increasing baseline Plt count to 100×10^9/L. During the study, eltrombopag was discontinued if Plts reached 200×10^9/L and was then reinitiated if they dropped again below 100×10^9/L. Another point that supports the conclusions was that the controls' Plt counts never increased more than the treated groups, even after pegylated-IFN treatment. However, there were adverse events (62 events and 7 serious events reported), which can complicate the use of eltrombopag for chronic HCV. Overall, the study not only showed an effective treatment for TCP in chronic HCV, but also substantiated the pathophysiological mechanism of decreased TPO production leading to TCP in this population.

Sanjo *et al.*[35] compared patients with chronic hepatitis, liver cirrhosis and controls. They concluded that their TPO levels were not significantly different. However, their PAIgG levels were elevated up to 144.6±113.6 ng/10^7 cells (compared to the controls which had levels of 18.9±2.5 ng/10^7 cells, $p<0.001$), thus supporting autoimmune mechanisms of TCP. However, they also reported that the liver volumes between the three cohorts were not significantly different, suggesting that the healthy liver mass producing TPO was not significantly different as well. This suggested that low TPO was not a major cause of TCP in this population, and that TPO deficiency cannot be the sole factor in the development of TCP. However, this conclusion is not completely justified because changes in liver volumes were not measured. If there were no differences in liver volume between the

controls and the chronic hepatitis/cirrhosis groups, that would suggest only patients with low stages of fibrosis were enrolled in this study.

Treatment

At present, four TPO-agonists (eltrombopag, romiplostim, avatrombopag and lusutrombopag) have been approved for HCV-related TCP.[34,36–39] Eltrombopag, as mentioned above, is an orally active TPO-agonist that increases differentiation of bone marrow progenitor cells leading to production of megakaryocytes.[34,36,40] Romiplostim is a polypeptide composed of four TPO mimetic peptides that dimerize TPO, leading to increase Plt production.[36,37] The two newest medications, avatrombopag and lusutrombopag, are both non-peptide oral TPO agonists.[39] By July 2019, the USA's Federal Drug Administration and European Medicines Agency had approved all medications, except romiplostim, for treatment of TCP in chronic HCV patients.[41–46] Eltrombopag and romiplostim were additionally approved for ITP.[41,42] Studies on the use of eltrombopag, including large phase 3 randomized, controlled, open-labeled studies called ENABLE-1 and ENABLE-2, have primarily focused on improving Plt counts in order to improve ribavirin and IFN HCV therapy.[34,47,48] The effect of eltrombopag on Plt count was studied in a phase 1 trial; however, neither chronic HCV nor thrombocytopenic populations were included.[40] Eltrombopag shows potential benefit for invasive procedures or surgery in patients with TCP due to liver disease.

Romiplostim, on the other hand, was studied over 90 days preoperatively in 35 patients with chronic liver disease and TCP secondary to HCV infection.[37] The goal of that study was to improve Plt counts greater than 70×10^9/L in order to allow the patient to undergo surgical interventions. The patients in that study were refractory to other standard treatment (e.g., Plt transfusion, folic acid, antioxidants), non-splenectomized, and scheduled for non-emergent surgical procedures. Mean Plt counts increased more than three-fold over baseline after 3 weeks of therapy, and remained at least one and a half times above the baseline even 2 months after discontinuation of the drug. Plt counts peaked at days 18 to 39, with a maximum level of 99×10^9/L. However, the study was a small, single-center study in Egypt, limiting extrapolation of data from this specific demographic to others around the world. Another disadvantage of the reported data is that it took weeks of treatment to achieve a positive change. The study did show that all the patients who failed other treatments responded to romiplastin, and only two patients failed to meet their target Plt count. There were also no reported adverse effects or serious events within 60 days of the surgeries.[37] There appears to be great potential in the use of TPO-agonists for increasing Plt counts for invasive procedures, and this led to two large randomized control studies that tested the use of avatrombopag and lusutrombopag prior to elective procedures.[39] Avatrombopag and lusutrombopag have been studied for the primary purpose of reducing preprocedural Plt transfusions. Up to 88% and 65% of patients in each trial reached this goal, respectively. Their secondary endpoint was to determine whether Plt counts increased to above 50×10^9/L, which was also achieved in up to 69% and 68% of patients on procedure day, respectively.[49,50] A strength of that study was the patient population and placebo group. A weakness of the study included restriction to severe TCP (Plt counts $<50\times10^9$/L), which was also the Plt transfusion criterion. Further studies on the use of these medications for chronic HCV-TCP population at moderate levels as well as severe levels for chronic elevation of Plt counts are needed. Furthermore, long-term benefit and risks need to be determined.

To evaluate possible virus-induced bone marrow suppression, Moussa and Mowafy[37] performed bone marrow biopsies on patients with HCV who were due for surgery. Samples were taken on days 0 and 90. On day 0, no patient had hypocellular bone marrow, thus indirectly showing that HCV did not cause decreased production of megakaryocytes. On day 90, there were five patients who began with a hypercellular state and changed to a normocellular state. Four patients changed in the opposite direction. Again, no patient had hypocellular marrow, which again showed that neither HCV nor romiplostim had significant effect on bone marrow production at this point of therapy.

Other TPO-agonists have been studied including interleukin 11, AMG-531 and PEG-TPOmp, non-peptide compounds like AKR-501, monoclonal antibodies, synthetic recombinant human TPO, pegylated-recombinant human megakaryocyte growth and development factor, recombinant human erythropoietin, danazol, and L-carnitine.[36,51–53] There are insufficient data to draw meaningful conclusions, and more clinical trials are needed.

Discussion

Currently, although several TPO-agonists are approved by the Federal Drug Administration and available, not all societal guidelines have recommended their routine use. For example, neither the American Association for the Study of Liver Diseases nor the Infectious Diseases Society of America have specific HCV TCP guidelines. However, the 2019 American Gastroenterological Association Clinical Update states, as part of their best practice guidelines, that TPO receptor agonists should be used for treatment of TCP in cirrhotic patients if the patient has sufficient time for the agent to be effective.[54] In addition, there is no recommendation for routine measurement of TPO before or during treatment.

It would seem to be beneficial to check TPO levels prior to use of a TPO-agonist to determine if the patient actually requires further megakaryocyte stimulation or not. It would be ineffective and wasteful to treat patients who already have high levels of TPO, and could expose patients unnecessarily to side effects.

TPO-agonists have reversible side effects as well as long-term effects. It is important to note that there have been studies that have associated a high incidence of thromboembolic events, including portal vein thrombosis, with the use of eltrombopag.[55,56] There were initial concerns for bone marrow fibrosis and increased risk of malignancy due to the constant stimulation of stem cells/megakaryocytes. However, after 10 years of observation of eltrombopag and romiplostim, this has not been found.[56] On the other hand, rebound TCP after abrupt discontinuation of romiplostim has been reported[31] as well as cataracts and aminotransferase elevations with eltrombopag use.[56] Due to these side effects and limitations, other TPO-agonists, avatrombopag and lusutrombopag, have been developed.

At this time, the use of these new medications must be limited to elective procedures, as they require at least 5 to 7 days to increase Plt counts. Plt counts also quickly decline after initial dosing,[49,50] so patients would require intermittent

or continuous use of the medication for long-term elevation of Plt counts. The risks of long-term elevation of Plt counts in this population are not known. On the other hand, the benefits of using these drugs to avoid surgery in high-risk surgical patients with low Plt counts can be substantial. In addition, such therapy could decrease the number of pre-procedural transfusions, thus reducing cost, risk of allergic reactions, and acquired infections. Data on successful treatment of TCP prior to surgery in patients with liver disease are convincing. Future studies will be needed to determine the long-term effects and the expansion of the use of avatrombopag and lusutrombopag.

Conclusions

The pathogenesis of TCP in chronic HCV patients is multifactorial and multifaceted. Four general pathogenic mechanisms have been proposed. Two mechanisms deal with increased destruction which include autoimmune antibody Plt destruction and hypersplenism with sequestration. Two mechanisms involve decreased production, including virus-induced bone marrow suppression and decreased TPO production. Of these, virus-induced bone marrow suppression has the least support. There are data supporting the coexistence of several mechanisms causing TCP due to chronic HCV and cirrhosis. More research is needed to better understand the factors involved in development of TCP in patients with liver disease and for the production of other agents with differing mechanisms of action in the treatment of TCP.

Acknowledgements

The support of the Herman Lopata Chair in Hepatitis is gratefully acknowledged.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Wrote the manuscript and prepared the figures (SR), proposed the idea for the review and critically revised the manuscript (GYW).

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Review Article

Management of Hepatorenal Syndrome: A Review

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Abstract

Acute kidney injury (AKI) occurs frequently in patients with cirrhosis, and hepatorenal syndrome (HRS) is second most common etiology of AKI after volume responsible pre-renal etiology. AKI in these patients negatively impacts pre- and post-transplant patient survival and healthcare burden. Reduced effective blood volume with consequent reduced renal blood flow, along with systemic inflammation in patients with decompensated cirrhosis, result in susceptibility to HRS. In this article, we will review updates over the last 5 years on the changing definition with diagnostic criteria and nomenclature of AKI and HRS, data on medical treatment with vasoconstrictors, and urinary biomarkers in diagnosis of etiology of AKI. We will also discuss the significance of liver transplantation evaluation once the diagnosis of HRS is established and the post-transplant immunosuppression management. We will also review one of the challenging issues that remains among transplant-eligible patients, that of allocation of simultaneous liver kidney transplant. Finally, we will review the new implemented policy from the Organ Procurement Transplant Network on simultaneous liver kidney allocation.

Citation of this article: Tariq R, Singal AK. Management of hepatorenal syndrome: A review. J Clin Transl Hepatol 2020;8(2):192–199. doi: 10.14218/JCTH.2020.00011.

Introduction

Hepatorenal syndrome (HRS) among patients with cirrhosis is one of the most devastating complications, with high mortality if not promptly recognized and properly treated.[1,2] Portal hypertension in cirrhosis leads to splanchnic arterial vasodilation, which results in reduced systemic vascular resistance and effective circulating blood volume.[3] Compensatory increase in cardiac output by activation of the renin-angiotensin-aldosterone and sympathetic nervous systems results in vasoconstriction of renal arteries with reduced renal blood flow. These physiological changes combined with hypoalbuminemia from reduced synthetic function of liver lead to sodium and water retention, manifesting as ascites and edema and setting the stage for development of acute kidney injury (AKI) and HRS. Inflammation with systemic inflammatory response syndrome in acute on chronic liver failure (ACLF) and decompensated cirrhosis is emerging as another major mechanism for the development of HRS.

In this article, we will review recent updates on the definition and terminology, criteria for diagnosis, emerging biomarkers [in differentiating HRS from intra-renal cause of AKI, especially acute tubular necrosis (ATN)], medical management, and role of liver transplantation (LT), especially for criteria for allocation of simultaneous liver kidney (SLK) transplantation

Prevalence and healthcare burden

HRS is common among patients with cirrhosis and its occurrence increases with its severity and duration. For example, in a prospective study, the incidence of HRS was 18% at 1 year and 39% at 5 years of follow-up.[4] Another study described the prevalence of HRS in about 48% of patients listed for LT.[5] Apart from negative impact on patient survival and outcomes, HRS is associated with huge healthcare cost and significant socio-economic burden.[6] For example, in a retrospective study on 2542 patients hospitalized with HRS, mean length of hospital stay per patient was 30.5 days, with $91,504 per admission.[7]

Definition of AKI and HRS

Serum creatinine estimation in patients with cirrhosis may not provide true renal function, due to a) malnutrition and muscle atrophy that occur with reduced synthesis of creatinine, b) increased renal tubular secretion of creatinine, c) dilution of serum creatinine due to increased volume of distribution in cirrhotic patients, and d) measurement error when there is cholestasis with elevation of serum bilirubin levels.[8,9] However, in routine practice, serum creatinine continues to be used for monitoring renal function and diagnosing AKI and HRS. This is because the test is simple, inexpensive, readily performed, widely available, and can be repeated frequently during the day. Over the last 10-15 years, the old definition of AKI using serum creatinine cut-off at 1.5 mg/dL has been changed, since even a minor change from baseline of as little as 0.3 mg/dL has been found to be associated with worse patient survival among hospitalized patients.[10] Currently, AKI is defined as increase in serum creatinine of ≥0.3 mg/dL within 48 h among hospitalized patients, or ≥50% increase over baseline level within the last 3 months among outpatients, or urine volume <0.5 mL/kg/h for about 6 h. Further, severity of AKI is stratified into three stages: stage 1 defined by increase in serum creatinine ≥0.3 mg/dL or 1.5- to 2-fold from baseline; stage 2 defined by increase by 2- to 3-fold; and, stage 3 defined by >3-fold

Keywords: HRS; Management; Liver transplant for HRS.

Abbreviations: ACLF, acute on chronic liver failure; AKI, acute kidney injury; ATN, acute tubular necrosis; HRS, hepatorenal syndrome; LT, liver transplantation; NO, nitric oxide; SLK, simultaneous liver kidney; TLR4, Toll-like receptor 4.

Received: 14 February 2020; Revised: 23 April 2020; Accepted: 8 May 2020

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increase or absolute serum creatinine of ≥4 mg/dL or initiation of renal replacement therapy.[11]

Beyond the well-known types of AKI, namely, pre-renal, intrarenal and post-renal, patients with cirrhosis may develop a specific type of renal dysfunction of HRS.[12] Traditionally, HRS is stratified into types 1 and 2, with 75% of cases being due to type 1 HRS (rapid rise of creatinine to >2.5 mg/dL over 1-2 weeks) with a median survival of 50% at 2 weeks.[5,13] In contrast, type 2 HRS, which presents as indolent decrease in renal function is often associated with refractory ascites, with median survival of about 6 months.[11,14,15] Recently, the nomenclature of HRS types has been modified with 'HRS-AKI' replacing HRS type 1 and 'HRS-CKD' replacing HRS type 2 (Table 1).[12] Being most common, the current review will focus on the HRS-AKI type.

Pathophysiology of HRS

Portal hypertension in cirrhosis results in splanchnic vasodilation, with pooling of blood and reduced effective circulating blood volume.[16] In early stages of cirrhosis, compensatory increase in cardiac output maintains the circulatory volume. However, the susceptibility of such afflicted patients to reduced renal blood flow and AKI is increased with a) hypovolemia (nausea, vomiting, diarrhea, poor oral intake, diuretics, gastrointestinal bleeding, use of non-steroidal anti-inflammatory drugs or radiocontrast agents), b) progressive disease with increasing severity and decompensation of cirrhosis, and c) cirrhotic cardiomyopathy in 40–50% of patients with cirrhosis and diastolic dysfunction.[17] The reduced circulating blood volume results in activation of the renin-angiotensin-aldosterone and sympathetic nervous systems, with sodium water retention and reduced renal blood flow occurring due to the vasoconstriction of renal arteries, with development of HRS-AKI (Fig. 1).

Recently, there is a growing line of evidence on the role of inflammation and systemic inflammatory response syndrome in the development of HRS.[18,19] Systemic inflammation induced either by pathogen-associated molecular patterns or by damage-associated molecular patterns plays a key role in the development of acute decompensation in patients with cirrhosis.[12]

Bacterial translocation from the gut due to increased intestinal permeability with activation of Toll-like receptor 4 (commonly known as TLR4) on hepatic macrophages results in inflammatory response.[20] Additionally, studies have suggested the up-regulation of renal tubular TLR4, which is associated with the development of renal dysfunction and tubular damage.[12]

The activated inflammatory cascade leads to release of proinflammatory cytokines (tumor necrosis factor-alpha or interleukin-6) and vasodilators [nitric oxide (commonly referred to as NO)]. Studies have also suggested that bacterial translocation plays a predominant role in causing the arterial vasodilation that is seen in advanced liver cirrhosis, occurring by stimulation of NO production and up-regulation mediated by tumor necrosis factor-alpha.[21] About 30% of patients with HRS have systemic inflammatory response syndrome, due to sterile inflammation in the absence of bacterial infection.[18]

Diagnosis of HRS

As soon as the diagnosis of AKI is established, steps are taken to expand the intravascular circulating blood volume, including withholding diuretics and administering intravenous fluid (1.5 L of normal saline or 1 gm/kg of albumin).[22] Simultaneously, efforts should be made to determine specific intrarenal or post-renal etiology with urine examination and renal ultrasound respectively (Fig. 2). Additionally, patients with ATN versus HRS could be distinguished based on fractional excretion of sodium. It appears that fractional excretion of sodium less than 0.2% may be clinically useful for distinguishing HRS from ATN.[23] If renal function does not normalize or improve with/within 48 h of this strategy and approach, a diagnosis of HRS is established if the work-up is negative for other etiologies of AKI (Fig. 2 and Table 1).[15]

There is emerging data on the utility of plasma and urine biomarkers of renal injury, such as neutrophil gelatinase-associated lipocalin, human endothelin-1, uromodulin, fatty acid binding protein, epidermal growth factor kidney injury molecule-1, and interlukin-18. In a prospective study, urinary concentration of neutrophil gelatinase-associated lipocalin measured at day 3 of development of AKI was found to be accurate for differentiating ATN from other causes of AKI, with c-statistic of 0.87 (95% confidence interval of 0.78-0.95). In this study, neutrophil gelatinase-associated lipocalin was also found to independently predict AKI progression and 28-day mortality.[24] Further studies are needed to validate the utility of neutrophil gelatinase-associated lipocalin before implementing this in routine management of patients with AKI.

Pre-transplant management of HRS

The medical management of HRS has been shown to improve short-term outcomes; however, long-term outcomes are poor without LT. The aim of the medical therapy is to stabilize the patient until LT and to optimize their pre-transplant condition. The medical therapy includes early treatment of AKI and use of vasoconstrictors.[1]

Early treatment of AKI

Early recognition and treatment is key to improving both pre- and post-transplant outcomes of patients with cirrhosis. The

Table 1. New definition and nomenclature of HRS

Old name	Old definition	New name	New definition
Type 1 HRS	➢ ≥50% increase in serum creatinine from baseline ➢ Cut-off serum creatinine value ≥1.5 mg/dL	**HRS-AKI**	➢ Increase in serum creatinine within <48 h ➢ ≥50% increase in serum creatinine from baseline within ≤3 months
Type 2 HRS	➢ Smoldering increase in serum creatinine to ≥1.5 mg/dL	**HRS-CKD***	➢ Estimated glomerular filtration rate <60 mL/min per 1.73 m^2 for ≥3 months in the absence of other (structural) causes

*Acute kidney disease if increase in serum creatinine is <50% from baseline and/or estimated glomerular filtration rate <60 mL/min for <3 months.

Abbreviations: AKI, acute kidney injury; CKD, chronic kidney disease; HRS, hepatorenal syndrome.

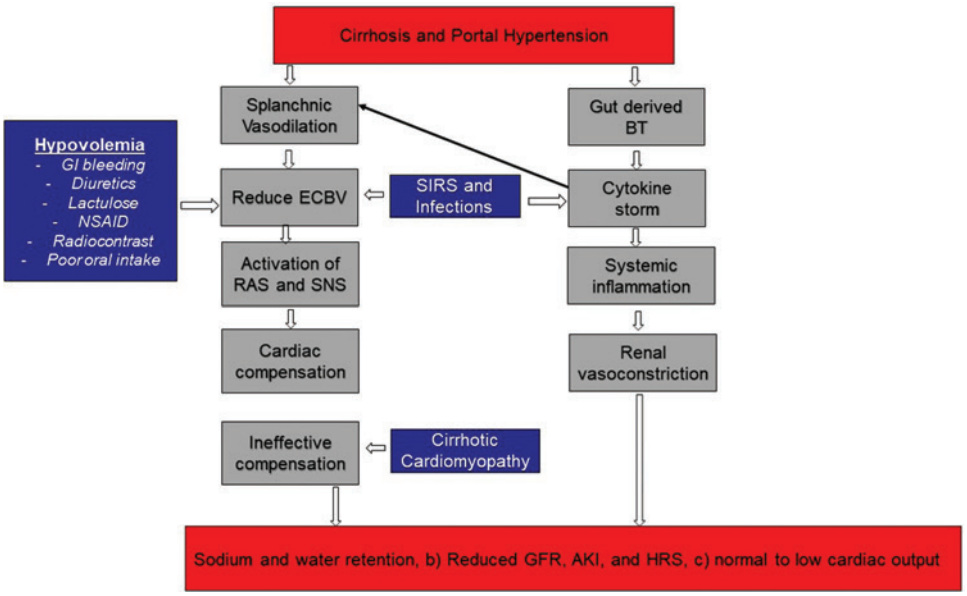

Fig. 1. Pathophysiology of renal dysfunction and HRS in cirrhosis.

Abbreviations: AKI, acute kidney injury; BT, bacterial translocation; ECBV, effective circulating blood volume; GFR, glomerular filtration rate; HRS, hepatorenal syndrome; NSAID, non-steroidal anti-inflammatory drug; RAS, renin-angiotensin system; SIRS, systemic inflammatory response syndrome; SNS, sympathetic nervous system.

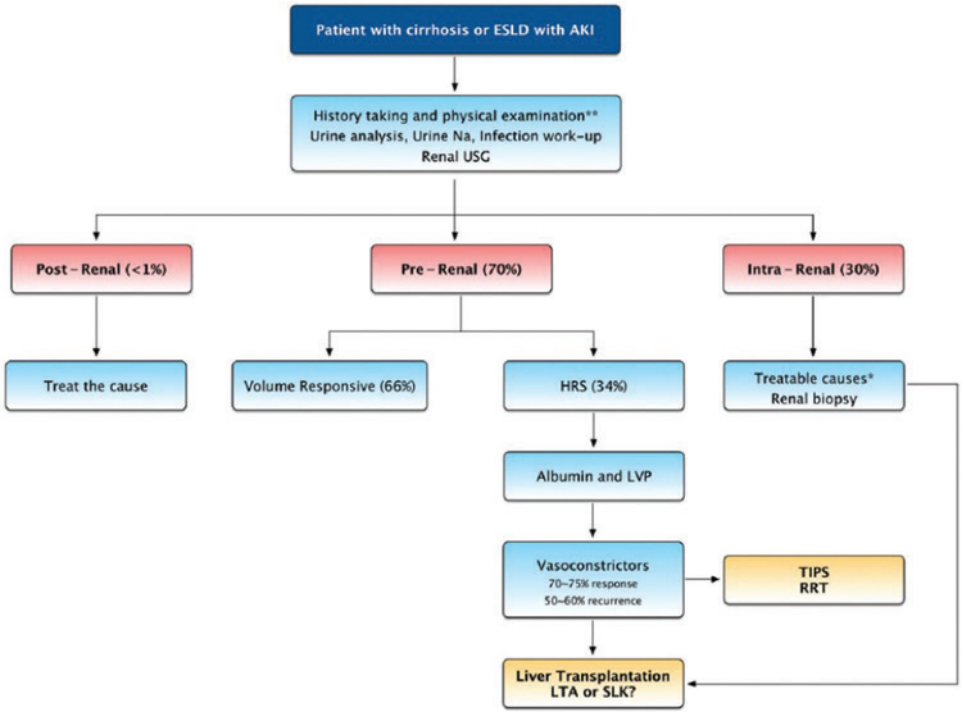

Fig. 2. Management approach and algorithm for AKI in patients with cirrhosis.

Abbreviations: AKI, acute kidney injury; ESLD, end-stage liver disease; LTA, liver transplant alone; LVP, large volume paracentesis; RRT, renal replacement therapy; SLK, simultaneous liver kidney; TIPS, transjugular intrahepatic portosystemic shunt. Reproduced with permission from Russ K *et al*.[2]

main aim is to identify and treat reversible factors, like dehydration, nephrotoxic medications (diuretics, non-steroidal anti-inflammatory drugs, aminoglycosides, and angiotensin-converting enzyme inhibitors), infection and sepsis, and gastrointestinal bleeding.[25] If large volume paracentesis is needed, especially over 3-5 L, intravenous

albumin replacement should be used with 6-8 g of albumin for every 1 L of ascitic fluid removed. Patients with spontaneous bacterial peritonitis should also receive intravenous albumin (1.5 g/kg on day 1, followed by 1 g/kg on day 3), along with antibiotic to improve outcome of these patients.[26]

HRS is a common complication that can occur during acute alcohol hepatitis, having a mortality of about 90% within 3 months, unless the patient receives liver transplant. Hence, early recognition and treatment for acute alcohol hepatitis is needed with alcohol abstinence supplemental nutrition, and, for select patients, pentoxifylline or corticosteroids.[27]

Prevention of HRS

Physicians managing patients with cirrhosis should be cognizant of reduced effective circulatory blood volume and renal blood flow, especially with the onset of portal hypertension. These patients should avoid nephrotoxic medications, especially non-steroidal anti-inflammatory drugs. Radiocontrast agents should be used judiciously. Optimization of diuretics should be performed with close follow up of basic metabolic panel and renal function. Further, early identification and treatment of AKI prevents progression and improves patient outcomes. The threshold should be low in using intravenous albumin for expanding fluid volume, especially in hospitalized patients with AKI and spontaneous bacterial peritonitis. For example, in a randomized controlled trial, use of intravenous albumin prevented type-1 HRS in patients with spontaneous bacterial peritonitis; the trial suggested decreased incidence of HRS (28% vs. 41%) and an improvement in 3-month survival (94% vs. 62%) in this population, when compared to placebo.[28]

Vasoconstrictor therapy

Vasoconstrictors cause constriction of splanchnic vessels, resulting in increasing the effective circulating blood volume, which in turn increases renal perfusion and glomerular filtration.[11] Vasoconstrictors work better when used with intravenous albumin.[29] Terlipressin is the most common vasopressor used and acts on the V1 receptors on vascular smooth muscle cells.[30] In a systematic review and meta-analysis of eight randomized trials, terlipressin was associated with 15% and 9% reduction of overall and HRS-related mortality respectively.[31] Another meta-analysis of 309 patients showed mortality benefit with terlipressin, with relative risk of 0.76 (95% confidence interval of 0.61-0.95).[32] Although, used extensively throughout the world, terlipressin is not yet approved by the FDA for use in the USA.[11] A recent randomized placebo controlled trial from North America (CONFIRM trial) involving 300 participants (199 receiving terlipressin), HRS reversal was documented in 29.1% of terlipressin-treated patients compared to 15.8% of patients receiving placebo ($p<0.012$).[33] Major side effects of terlipressin included abdominal cramps and diarrhea in about 20% patients and tachyarrhythmias or chest pain in 6% of patients. Rarely, ischemia of bowel or skin and extremities can occur.[32] These side effects are less frequent with use of terlipressin as continuous intravenous infusion, as compared to when the drug is applied in intravenous boluses, due to the less daily total dose needed when used as an infusion.[34]

As terlipressin is currently not available in the USA, other vasoconstrictors like norepinephrine, midodrine, and octreotide, are used for the treatment of HRS. Norepinephrine, a catecholamine with predominantly alpha-adrenergic activity, is an inexpensive alternative and widely used as an infusion for the treatment of HRS.[35] In a meta-analysis of seven trials of norepinephrine compared with terlipressin, the drugs were found to be equally effective in reversal of HRS (53 vs. 55%, *p* indicated non-significance).[36]

Midodrine, an alpha-adrenergic agent administered orally in combination with subcutaneous octreotide, is another alternative. In a case-control study, use of this combination on 75 HRS patients improved transplant-free survival, overall survival, with better renal function at 1 month compared to historical cohort of 87 HRS patients who did not receive this specific pharmacologic vasoconstrictor therapy.[37]

In the most recent meta-analysis of 13 randomized controlled trials on use of vasoconstrictors for HRS, terlipressin was the most effective agent for HRS reversal and norepinephrine was as effective as terlipressin. However, both these drugs were superior to midodrine and octreotide combination for HRS survival.[38] None of the drugs showed any benefit on HRS relapse or on patient survival. Based on these data, until terlipressin is available for use in the USA, norepinephrine remains the drug of choice, especially for patients treated in the intensive care unit, and the midodrine/octreotide combination is reserved for patients treated on the medical floor (Table 2).

Most patients are treated for 2 weeks at least before declaring non-response and discontinuation of the specific medication. As mentioned earlier, to achieve maximum efficacy, vasoconstrictors are used in combination with intravenous albumin infusion. Among responders, midodrine is usually continued indefinitely or until LT. In one study, outpatient terlipressin infusion as a bridge to LT has been reported in six patients after HRS reversal was documented, with three patients successfully bridged to LT.[39] Further prospective studies are needed to evaluate the role and regimen of this approach as basis for maintaining renal function and bridging patients to LT. The role of vasoconstrictors for type 2 HRS or HRS-CKD remains unclear and most studies have been performed on HRS-AKI patients. In a non-randomized study, terlipressin was associated with improved renal function in patients with type 2 HRS.[40] Further good quality randomized data is needed to evaluate the efficacy and long-term safety of these agents in patients with HRS-CKD.

Miscellaneous therapies

Few studies have evaluated the efficacy of transjugular intrahepatic portosystemic stent-shunt for HRS. Two small case series found improvement in renal function and survival in patients who underwent transjugular intrahepatic portosystemic stent-shunt for HRS.[41,42] However, transjugular intrahepatic portosystemic stent-shunt is a risky procedure and patients with HRS are usually too sick to undergo this procedure. Until benefit of transjugular intrahepatic portosystemic stent-shunt is documented in randomized controlled trials, the procedure is not recommended in the management of HRS. Renal replacement therapy can be used as a bridge to LT in patients who fail medical therapy.[14] The indications for renal replacement therapy in these patients are the same as for any other cause of AKI and include volume overload with 10% or more weight gain, hyperkalemia, symptomatic uremia, pericarditis, and acidosis. Risks of dialysis include hypotension, infection, and bleeding. Additionally, the exact mode of dialysis for these patients remains unknown. There is

Table 2. Studies describing various therapies for HRS

Study name	Type of study	Intervention	Outcome assessed
Hiremanth *et al.*[31]	Meta-analysis	Terlipressin	15% reduction in overall mortality.
Gludd *et al.*[32]	Meta-analysis	Terlipressin	Overall reduction in mortality 0.76 (95% CI: 0.61-0.95).
Israelesen *et al.*[36]	Meta-analysis	Norepinephrine vs. terlipressin	Equally effective in reversal of HRS (53 vs. 55%, *p*=NS).
CONFIRM trial[33]	RCT	Terlipressin vs. placebo	HRS reversal was documented in 29.1% of terlipressin-treated patients vs. 15.8% patients receiving placebo ($p<0.012$).
Skagen *et al.*[37]	Case control	Midodrine and octroetide	Transplant-free survival was higher compared with the control arm (median survival 101 days vs. 18 days, $p<0.0001$).
Nanda *et al.*[38]	Meta-analysis	All drugs available for HRS	Terlipressin plus albumin was more efficacious than placebo plus albumin (OR=4.72; 95% CI: 1.72-12.93; $p=0.003$) or midodrine plus albumin and octreotide (OR=5.94; 95% CI: 1.69-20.85; $p=0.005$), for HRS reversal. No significant difference was noted comparing terlipressin plus albumin versus noradrenaline plus albumin.

Abbreviations: CI, confidence interval; HRS, hepatorenal syndrome; NS, non-significant; OR, odds ratio.

no evidence on survival benefit with renal replacement therapy among patients not eligible for LT.[43] Molecular absorbent recirculating system by extra-corporeal albumin dialysis has been proposed as a treatment of refractory ACLF. In a randomized study of 166 patients, survival was similar in patients receiving standard of care ($n=81$) and patients treated with extra-corporeal albumin dialysis ($n=85$). However, extra-corporeal albumin dialysis was superior in improving encephalopathy, reducing bilirubin, and improving serum creatinine. Based on these data, extra-corporeal albumin dialysis may be an alternative option to bridge patients with HRS to LT.[44]

Liver transplantation for HRS

Liver transplantation is the definitive treatment for HRS and can be considered as soon as diagnosis of HRS is established. HRS patients, even after successful medical therapy and reversal of HRS, have poorer post-transplant outcomes than patients without HRS. In one study, of 104 patients, 33 with HRS had longer intensive care unit stay with higher use of hospital resources (including dialysis and blood transfusion), poorer renal function at 1 year, and worse patient survival. However, the patient survival rate at 5 years was satisfactory, at about 80%, justifying its use in these HRS.[45] It should be recognized that HRS patients with longer duration of renal dysfunction prior to LT may not recover renal function after LT. In another study, about 6% increased risk of non-recovery of renal function was shown with each additional day of pre-transplant dialysis.[46]

Simultaneous liver kidney allocation

Since the introduction of the model for end-stage liver disease scoring system, a proportion of all LT receiving simultaneous liver kidney (SLK) has increased from 4% in 2002 to 10% in 2016.[47,48] Selection of candidates for SLK is a challenge for the hepatology and nephrology transplant community, as there are no good predictors for recovery of renal function after LT alone.[49,50] In general, SLK transplantation provides survival benefit over LT alone to patients with serum creatinine >2 mg/dL and/or patients on hemodialysis. However, the data are scanty on the duration of renal dysfunction or of dialysis in predicting recovery of renal function after LT alone. Criteria for SLK allocation are therefore based on consensus recommendations and without good scientific data, which explains the increasing use of SLK and also the heterogeneity of their use across the regions and also between centers within the region (Table 3).[49]

The Organ Procurement Transplant Network introduced a new policy in 2016 for SLK allocation, with the following criteria: A) for chronic kidney disease: a) glomerular filtration rate of <60 mL/min for 90 days and subsequent glomerular filtration rate of <30 mL/min or initiation of dialysis, b) chronic kidney disease due to metabolic disease that can be corrected with a liver transplant (hyperoxaluria, atypical hemolytic uremic syndrome, familial non-neuropathic systemic amyloidosis, and methylmalonic aciduria); and B) for AKI: a) duration of AKI >6 weeks with persistent glomerular filtration rate of <20 mL/min, b) need of dialysis for >6 weeks, or combination of both the criteria meeting 5 weeks duration. Under this policy, the respective criteria need to be documented every 7 days to maintain listing for SLK.[51] A recent study examined the effects of the implementation of the Organ Procurement Transplant Network policy on 40,979 candidates, of which 1683 met the new criteria, 2452 met the old criteria, and 1878 met both the criteria. They found that patients meeting the new criteria were less likely to die post-transplant.[52] Further studies are needed for continuous

Table 3. Indications for considering SLK

A. Patients with ESRD listed for kidney and have liver disease (kidney pulling liver)
➢ ESRD patients with liver cirrhosis
➢ ESRD due to hyperoxaluria
➢ Polycystic kidney and liver disease with ESRD
B. Patients with ESLD listed for liver (liver pulling kidney)
➢ ESLD with chronic kidney disease
➢ ESLD with acute kidney injury

Abbreviations: ESRD, end-stage renal disease; ESLD, end-stage liver disease; SLK, simultaneous liver kidney.

monitoring of SLK outcomes with the implementation of the new policy.

Whether urinary or plasma biomarkers of tubular injury can improve optimal allocation of SLK was tested in a small open study. However, none of the biomarkers tested within 30 days prior to LT among patients with cirrhosis and AKI were useful in predicting recovery of renal function after LT alone.[53] There remains unmet need of accurate biomarkers for differentiation of HRS from ATN and predictors using clinical variables or biomarkers or combination of both for recovery of renal function after LT alone, as basis for optimal SLK allocation and use of already scarce donor kidney pool.

Post-transplant management

Common risk factors for the development of end-stage renal disease during the post-transplant period include calcineurin inhibitor nephrotoxicity, pre-transplant HRS, pre-existing renal insufficiency, and diabetes mellitus.[54–56] Additionally, episodes of acute renal failure, renal replacement therapy pre- and post-transplantation, hepatitis C infection, and increasing age have been shown to be associated with risk of chronic kidney disease in the post-transplant period.[57–59]

Given the significant nephrotoxic effects of calcineurin inhibitor, renal-sparing regimens have been used for preserving renal function in the post-transplant period among patients receiving LT for HRS. For example, use of renal-sparing approaches have been effective to preserve renal function during the post-transplant period, such as with a) interleukin-2 receptor antagonists (daclizumab, or basiliximab) or polyclonal antibodies (rabbit anti-thymocyte globulin) for induction of immunosuppression and delaying the introduction of calcineurin inhibitor, and b) mTOR inhibitors, such as everolimus or low-dose calcineurin inhibitor, with other agents, like mycophenolate, for maintaining the immunosuppression.[60–62]

Role of palliative care

Patients with progressive HRS and those ineligible for LT have high short-term mortality with huge healthcare burden. For example, in a study using the national in-patient sample on hospitalized cirrhosis patients who were denied LT, multiple somatic symptoms were experienced with poor quality of life, and this was associated with prolonged hospitalization and higher use of hospital resources. Only 11% of these patients received palliative care consultation.[63] Consideration should be given on a case-by-case basis, to discuss the goals of care with the patient and families.[63] Future research should evaluate timing and effects of palliative care on quality of end-of-life care in this population.

Conclusions

HRS is a serious complication among patients with liver cirrhosis and is associated with poor prognosis. With recent advances in therapeutic strategies due to better understanding of pathophysiology, there is a hope to reduce its prevalence and improve patient outcomes. Terlipressin and norepinephrine infusion are effective vasoconstrictors, and midodrine combined with octreotide is an alternative option. With the encouraging data from a recently completed multicenter trial in the USA, it is hoped that terlipressin will be approved by the Federal Drug Administration for clinical use in the USA. Vasoconstrictors provide better efficacy when combined with intravenous albumin. Neutrophil gelatinase-associated lipocalin at day 3 of onset of AKI is a promising tool for differentiating intrarenal etiology from HRS; however, larger prospective data are needed as basis for validation before implementing into routine clinical practice. Lack of accurate models for predicting renal function recovery after LT has resulted in increase in the use of SLK in these patients. It is hopeful that the recently introduced Organ Procurement Transplant Network policy for SLK allocation and listing would optimize the use of SLK and help the already scarce kidney donor pool. There remains a clinical unmet need for better and more accurate models predictive of renal function recovery after LT and non-invasive urine or plasma biomarkers for accurate diagnosis of HRS.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Drafted the first version of the manuscript (RT), edited and revised the manuscript, and contributed to conceptual development of the study (AKS).

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Review Article

Herb-induced Liver Injury in Asia and Current Role of RUCAM for Causality Assessment in 11,160 Published Cases

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Abstract

Herb-induced liver injuries (HILI) by traditional herbal medicines are particular challenges in Asian countries, with issues over the best approach to establish causality. The aim of the current analysis was to provide an overview on how causality was assessed in HILI cases from Asian countries and whether the Roussel Uclaf Causality Assessment Method (RUCAM) was the preferred diagnostic algorithm, as shown before in worldwide evaluated cases of drug-induced liver injury (DILI). Using the PubMed database, publications in English language were preferred to allow for reevaluation by peers. Overall 11,160 HILI cases have assessed causality using RUCAM and were published by first authors working in Asian countries. With 21 evaluable reports, most publications came from mainland China, with Hong Kong and Taiwan, followed by Korea (*n*=15), Singapore (*n*=2), and Japan (n=1), while other Asian countries were not contributory. Most publications provided case and RUCAM data of good quality. For better presentation of future cases, however, the following recommendations are given: (1) preference of prospective study design with use of the updated RUCAM version; (2) clear separation of HILI cohorts from those of other herbal products or DILI; (3) case series for epidemiology studies should contain many essential data, possibly also as supplementary material; (4) otherwise, preference of single case reports providing individual case data and RUCAM-based causality gradings, and applying liver test threshold values; and (5) publication in English language journals. In conclusion, China and Korea are top in presenting RUCAM-based HILI cases, other Asian countries are encouraged to follow.

Citation of this article: Teschke R, Zhu Y, Jing J. Herb-induced liver injury in asia and current role of RUCAM for causality assessment in 11,160 published cases. J Clin Transl Hepatol 2020;8(2):200–214. doi: 10.14218/JCTH.2020.00009.

Keywords: Liver injury; Drug induced liver injury; Herb induced liver injury; RUCAM.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; CAM, causality assessment method; DILI, drug-induced liver injury; DILIN, Drug-induced Liver Injury Network; EMA, European Medicines Agency; FDA, Food and Drug Administration; GTE, green tea extracts; HEV, hepatitis E virus; HILI, herb-induced liver injury; LTs, liver tests; PAs, pyrrolizidine alkaloids; PM, *Polygonum multiflorum*; RCTs, randomized controlled trials; RUCAM, Roussel Uclaf Causality Assessment Method; TCM, Traditional Chinese Medicines; ULN, upper limit of normal.

Received: 4 February 2020; Revised: 5 March 2020; Accepted: 31 March 2020

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Introduction

Herb-induced liver injury, with HILI as its acronym, was first introduced and proposed as a specific term in the scientific literature in 2011[1,2] and subsequently characterized.[3–6] Several review articles have addressed relevant issues of HILI also in relation with drug-induced liver injury (DILI).[7–9] Evaluating suspected HILI cases is complex, complicated, and can be a tricky undertaking because herbal medications exert an intrinsic liver injury type due to overdosed ingredients or improper herbal product quality, including adulteration or toxic contamination.[10] In addition, HILI emerges unpredictably in a limited number of susceptible individuals consuming herbs as medicines, based on an idiosyncratic reaction also known from drugs causing DILI.[11]

Contrasting to fragile HILI case evaluations in many publications, conditions are more stable for DILI by clearly defined conventional chemical drugs and the use of the Roussel Uclaf Causality Assessment Method (RUCAM) to assess causality, which has allowed an objective view on DILI characteristics based on 46,266 DILI cases published 2014-2019.[11] This success was the result of DILI evaluations, which incorporated the original RUCAM of 1993,[12,13] an early RUCAM version of 1990,[14] or more recently the updated RUCAM of 2016.[15] Additional information on RUCAM was provided in other publications,[16,17] associated with the encouragement to strictly adhere to published criteria directed to DILI and HILI cases.

The present review focuses on published HILI cases and case series provided by authors residing in Asian countries and regions such as China, Japan, Korea, and Singapore. The principal aim was to analyze to what extent specific causality assessment methods (CAMs) like RUCAM were used to back up HILI as robust diagnosis, ensuring further case characterization.

Literature search and source

The PubMed database (1964-December 30, 2019) was searched for articles on HILI in various Asian countries by using the following terms: herb-induced liver injury, HILI, RUCAM, Roussel Uclaf Causality Assessment Method, and China, Hong Kong, Japan, Korea, India, Taiwan, Thailand, and Vietnam; search terms were used alone or in combination. With a few exceptions, the search was confined to reports in the English language. Publications of Asian authors on HILI cases that had been assessed for causality using RUCAM were individually evaluated with respect to quality of reported RUCAM data. The final compilation consisted of original papers, consensus reports, and review articles, with the most relevant publications included in the reference list.

Definition

HILI is clinically defined as liver injury in association with the use of an herbal product, which may include herbal medicines such as traditional herbal medicines and herbal drugs that are under regulatory surveillance. Herbal products often represent a mixture of several herbs with abundant phytochemicals as ingredients and differ thereby from DILI caused by a single chemical that is on the market after regulatory approval. Differentiation of HILI from DILI is essential and incorporating HILI among a DILI cohort is misleading, not allowing for a separate characterization of HILI features.

Current state of RUCAM-based HILI reports published from the Asian region

China

Starting as early as 2006 with an analysis from Hong Kong,[18] an overall 21 reports of HILI cases were published which had been assessed for causality using RUCAM with results presented by groups with first authors having their working place in mainland China, Hong Kong, or Taiwan.[18–38] These publications merit further consideration. Assessed cases were commonly well presented with respect to case data completeness and evaluation (Table 1). Most reports provided data of cohorts consisting of HILI alone, but few combined results of both HILI and DILI cases, causing confusion due to mixed data.[24,26,30,36] In a few instances, publications erroneously mention in their title specifically only DILI, although HILI cases are also presented in the text,[24,26,36] ignoring thereby that HILI features are clearly different from those of DILI.[5–11] It seems that most reports were based on a retrospective rather than a prospective study design. Some studies included HILI cases not only with highly probable or probable causality gradings but also with a possible causality level based on RUCAM scores ≥3 (Table 1).[24,25,27,28,30,36,38] In other cases, RUCAM-based causality gradings were erroneously classified as definitive;[18] although this term was never proposed or approved in the RUCAM literature that determines highly probable as the highest grading,[12,15] the most appropriate term for results in biological systems like clinical liver injury. Occasionally, RUCAM-based causality gradings, classified initially as possible, had afterwards been upgraded to a probable level through a non-transparent maneuver[36]—an overall highly questionable and disputable approach as also discussed previously.[11] In rare instances, causality gradings were not reported[35] or RUCAM was used for causality grading but the respective publication remained unquoted,[26,34] even if the updated RUCAM was mentioned in the text.[34] Similar omissions of RUCAM quotation have been observed in some publications related to DILI.[11]

There are several excellent publications, which could serve as examples for future publications on RUCAM-based HILI cases (Table 1). The encouraging report of Zhang *et al.*[29] analyzed HILI cases in a perfect way, using the updated RUCAM of 2016, adopting a high threshold of liver tests (LTs) to avoid nonspecific liver injuries and providing for 26/28 cases a highly probable causality grading. As outlined in the report of Chau *et al.*,[20] the interrater agreement between experts and RUCAM was 81%, facilitating evaluations and exclusion of cases with alternative causes or unclear herbal product identification. In general, RUCAM-based HILI series are preferred that cover in more detail a single herb, such as *Gynura segetum* and other pyrrolizidine alkaloids (PAs)-containing herbs like in the reports of Lin *et al.*[21] and Gao *et al.*,[22,25] or *Psoralea corylifolia* like in the reports of Cheung *et al.*[19] and Li *et al.*,[34] or *Polygonum multiflorum* (PM), as shown in the reports of Dong *et al.*,[23] Wang *et al.*,[27] Zhu *et al.*,[28] Li *et al.*,[31] Jing *et al.*,[33] and Liu *et al.*[35] For instance, Li *et al.*[31] presented a perfect case report on HILI caused by PM, using the updated RUCAM of 2016.[15] Similarly, the case series of Dong *et al.*[23] focuses on PM on a single herb causing HILI in 18 patients, with each having received an individual causality grading of probable or highly probable. Since for 14/18 HILI patients, a highly probable causality grading was attributed, this is best explained by a careful case evaluation with complete data sets allowing for this extraordinary result. In addition and as shown in their report assessing causality by Gao *et al.*,[25] RUCAM was used for the first time in the hepatic sinusoidal obstruction syndrome caused by PAs in 23 patients, supporting the blood pyrrole-protein adducts as diagnostic biomarkers.[25] The reports of Hao *et al.*,[24] Chow *et al.*[32] and Tan *et al.*[37] are worth mentioning because these authors clarify, already in their title, that cases had been assessed for causality using RUCAM. Tan *et al.*[37] also carefully assessed the comedicated drug using a separate RUCAM sheet, as recommended earlier.[12,15] As potential confounding alternative diagnosis, hepatitis E virus (HEV) infection was excluded in all three patients, and RUCAM-based data had been presented in a transparent list.[37] In this study, most interesting was the finding of a high causality grading of probable, achieved with a score of 7; although liver injury by *Swietenia macrophylla* was unknown at the time of publication, providing a score of 0, not allowing additional scores. Therefore, lack of previous knowledge of liver injury does not prevent high causality gradings. Similarly, lacking unintentional readministration, which provides a score of 0, nevertheless allowed for a high causality grading.[37] This again underscores the value of RUCAM by taking care of liver injury cases lacking some elements.

Japan

In Japan, the report of Tsuda *et al.*[39] used the RUCAM of 1993 but there are no other RUCAM-based cases of HILI to be used for comparison with worldwide RUCAM-based HILI cases.

Korea

First authors of reports from Korea contributed as experts were numbered overall 15, and thereby represented a substantial number of publications on 526 HILI cases that had been assessed for causality using RUCAM (Table 1).[40–54] These included single case reports, case series and review articles. Respective articles were mostly of good quality, with minor shortcomings. These included, for instance, the use of a RUCAM version modified by the authors for unknown reason(s) without own method re-validation,[40,44,51,53] the inclusion of cases with a possible causality grading that impairs the focus on cases with a probable or highly probable causality grading[40,41,42,50,52,54] using the RUCAM algorithm but leaving individual causality grading unreported for unknown reason(s),[51,54] forgetting quotation of the used RUCAM publication,[51,54] and classifying the original highly probable causality grading erroneously as definite.[47] It seems that most reports followed a retrospective study approach (Table 1),[40–54] whereas RUCAM instructions clearly recommend the use of RUCAM for prospective studies.[15]

Table 1. Asian countries with a selection of published HILI cases assessed for causality using RUCAM, occasionally reported together with DILI cases

Country	Author	Year	HILI (*n*) DILI (*n*)	Products	Comments
China	Yuen[18]	2006	HILI (7)	Several herbs	Using RUCAM for causality assessment, gradings were highly probable (not definitive!) in 3 patients, probable in 2 cases, and possible in 2 patients
	Cheung[19]	2009	HILI (3)	*Psoralea corylifolia*	RUCAM-based causality assessment provided with scores of 6-8 in all cases a probable causality grading
	Chau[20]	2011	HILI (27)	Multiple herbs	With RUCAM, causality gradings were highly probable in 5 cases, probable in 16, possible in 5, and unlikely in 1 case
	Lin[21]	2011	HILI (1)	*Gynura segetum*	Using the original RUCAM, a score of 6 corresponding to a probable causality grading was reported
	Gao[22]	2012	HILI (5)	*Gynura segetum*	Based on evaluations by the original RUCAM, scores were reported as ≥5 corresponding to a probable or possible causality grading
	Dong[23]	2014	HILI (18)	*Polygonum multiflorum*	RUCAM provided 4 x a probable and 14 x a highly probable causality grading
	Hao[24]	2014	HILI (87) DILI (13)	Multiple herbs and drugs	RUCAM was used to assess causality, whereby HILI or DILI cases with a score of ≥3 (possible or higher) were included
	Gao[25]	2015	HILI (23)	PA-containing herbs	RUCAM was used, cases with a score of >5 were included (causality grading of possible or probable, score: 5.52±0.67)
	Ou[26]	2015	HILI (130) DILI (361)	Multiple herbs and drugs	Unspecified, not quoted RUCAM version used for HILI, providing a probable or highly probable causality grading
	Wang[27]	2015	HILI (40)	*Polygonum multiflorum*	Within a subgroup: 9 highly probable, 15 probable, 16 possible RUCAM gradings
	Zhu[28]	2015	HILI (158)	*Polygonum multiflorum*	The original RUCAM with a score of ≥3 was used for the included cases
	Zhang[29]	2016	HILI (54)	Multiple herbs	Use of the updated RUCAM, providing a highly probable causality grading in 26 cases and a probable one in 28 cases
	Zhu[30]	2016	HILI (563) DILI (870) both (552)	Multiple herbs and drugs	Cohorts consisted of HILI, DILI, or both, RUCAM gradings were highly probable or probable, rarely possible
	Li[31]	2017	HILI (1)	*Polygonum multiflorum*	By using the updated RUCAM, reported causality grading was probable
	Chow[32]	2019	HILI (1,552)	Many single herbs (1,428) or mixtures with multiple herbs (124)	RUCAM-based causality gradings were: probable or higher for only 138 cases of HILI by single herbs and for 56 cases by herbal mixtures, possible for 226 cases of HILI by single herbs and for 27 cases by herbal mixtures, but lacking causality for 1,064 cases of HILI by single herbs and for 41 cases by herbal mixtures
	Jing[33]	2019	HILI (145)	*Polygonum multiflorum*	With the updated RUCAM, causality gradings were highly probable (11%), probable (82.8%), and possible (6.2%)
	Li[34]	2019	HILI (1)	*Psoralea corylifolia*	The updated RUCAM was used but not specifically referenced, with score of 10 as a highly probable causality grading
	Liu[35]	2019	HILI (331)	*Polygonum multiflorum*	Cases had been assessed by RUCAM without providing causality gradings
	Shen[36]	2019	HILI (6,971) DILI (18,956)	Multiple herbs and drugs	RUCAM-based assessment with scores ≥3 in all HILI cases but causality grading was not differentiated from additional, RUCAM-based 18,956 DILI cases
	Tan[37]	2019	HILI (3)	*Swietenia macrophylla*	RUCAM score was 7 for all 3 patients, in line with a probable causality grading.
	Zhu[38]	2019	HILI (488)	Multiple herbs	Using the updated RUCAM, causality grading was highly probable in 52 cases (10.5%), probable in 370 cases (74.8%), and possible in 66 cases (13.3%)

(*continued*)

Table 1. (*continued*)

Country	Author	Year	HILI (*n*) DILI (*n*)	Products	Comments
Japan	Tsuda[39]	2010	HILI (1)	Saireito	Use of the original RUCAM of 1993 provided a RUCAM score of 8 and thereby a probable causality grading
Korea	Ahn[40]	2004	HILI (64)	Various herbs	Use of RUCAM with modifications by the authors, providing mostly probable and highly probable causality gradings
	Seo[41]	2006	HILI (17)	Various herbs	RUCAM was used, and cases with a score of at least 3 were included
	Kang[42]	2008	HILI (66) DILI (38)	Various herbs and drugs	RUCAM provided scores ≥4 for all HILI cases corresponding to a possible grading or higher
	Sohn[43]	2008	HILI (24)	Various herbs	RUCAM was applied in all HILI patients undergoing a liver transplantation
	Kang[44]	2009	HILI (1)	*Corydalis speciosa*	Reported was the use of a RUCAM version, modified by the authors, providing a score of 9 and thereby a highly probable causality grading
	Kim[45]	2009	HILI (2)	Arrowroot	The use of RUCAM provided a score of 10 and thereby a highly probable causality grading
	Bae[46]	2010	HILI (1)	*Polygonum multiflorum*	With RUCAM, a score of 10 was achieved corresponding to a highly probable causality grading
	Yang[47]	2010	HILI (3)	*Aloe vera* or *arborescens*	RUCAM-based scores of 7 in 2 cases provided a probable causality grading, and a score of 9 was achieved in the third patient corresponding to a highly probable causality grading (a definitive one as erroneously stated in the text does not exist in the RUCAM system)
	Jung[48]	2011	HLI (25)	*Polygonum multiflorum*	With RUCAM, the scores were 6-8, corresponding to a probable causality grading in 15 patients and were >9 corresponding to a highly probable causality grading in 10 patients
	Kim[49]	2012	HILI (1)	*Hovenia dulcis*	Using RUCAM, a score of 6 was obtained for this case corresponding to a probable causality grading
	Suk[50]	2012	HILI (149) DILI (101)	Various herbs and drugs	RUCAM-based evaluation for HILI cases provided an average score of 7, with a range of 3-12 and thereby a possible or probable causality grading
	Lee[51]	2015	HILI (27)	Various herbs	Use of a modified RUCAM, lack of any quotation and of causality grading
	Lee[52]	2015	HILI (97)	Various herbs	Using RUCAM, scores were 8.2±1.4; individual cases received mostly a highly probable or probable causality grading
	Woo[53]	2015	HILI (5)	Various herbs	A simplified RUCAM was used that provided a probable causality grading
	Cho[54]	2017	HILI (6)	Various herbs	RUCAM was used without specification of its version and referencing, providing a probable causality grading in four HILI cases and a possible grading in two cases
Singapore	Wai[55]	2006	HILI (15) DILI (14)	Various herbs and drugs	RUCAM was used in HILI patients for causality assessment, but individual causality gradings were not reported; it was mentioned that all cases fulfilled all RUCAM criteria collected in the course of a prospective study, which suggests a causality grading of at least probable due to the expected data completeness
	Teo[56]	2016	HILI (10)	Various herbs	RUCAM was used in 10 assessable cases, with scores from 0 to 2 for 9 patients and a score of 5 for 1 patient

Prefect studies were provided among others by Suk *et al.*,[50] who followed a prospective design for their nationwide HILI study in Korea, and by Kim *et al.*,[45] Bae *et al.*,[46] Yang *et al.*,[47] Jung *et al.*,[48] Kim *et al.*,[49] and Woo *et al.*,[53] who all provided cases limited to a probable or highly probable causality grading, suggesting complete case data sets or prospective data collection in single case reports. Valuable is, also, the report of Kang *et al.*,[44] who described a patient with a positive re-exposure result, as evidenced by a striking increase of serum alanine aminotransferase (ALT) activity shown in a separate figure and likely following the test criteria published earlier.[15]

Singapore

Groups from Singapore presented two reports, with altogether 25 HILI cases that had been assessed for causality using RUCAM.[55,56] In the first report published 2006 by Wai *et al.*,[55] a prospective study design was used that allowed for complete case data, conditions commonly facilitating high causality gradings. The second study published 10 years later by Teo *et al.*[56] presented data from a retrospective analysis of spontaneous reports submitted to the national registry; respective causality gradings were extremely low due to incomplete case data, not unexpected under these study conditions.

Other Asian countries

There are virtually no valid reports on RUCAM-based HILI cases from authors residing in other Asian countries like Vietnam, Indonesia, Thailand, or India. Some reports could have been published in local language but not in English; it is also possible that RUCAM had not yet achieved a larger acceptance. With respect to RUCAM-based liver injury by Indian Ayurvedic medicines, two reports were published by authors outside of India, namely from Germany[57] and the USA.[58] In the report from Germany, the original RUCAM of 1993 was used and referenced for causality assessment, having provided scores of 6-8 as a probable causality grading for four concomitantly used herbal medicines, preferring one single herb with the highest score of 8.[57] The USA report discussed RUCAM without providing a correct reference and attributed a score of 5 corresponding to a possible causality grading,[58] while some questions including posology and product quality have been raised.[59] It is well recognized that reports of Indian Ayurvedic medicine-related liver injury are sparse in the literature,[60,61] which we found to include not only herbs but also other complementary and alternative medicines.[61] An exemption refers to 8 cases of HILI by Indian products as reported in a RUCAM-based prospective study by the Indian group of Rathi *et al.*[62] that was classified as a report of excellence.[63] With respect to Ayurvedic and herbal medicine-induced liver injury, there is a refreshing statement by Devarbhavi:[64] *Is it time to wake up and take notice*. Indeed, the quality of HILI case evaluation is insufficient in many countries, including Asian ones, a topic that merits further discussion as outlined below.

Actual issues

Increasing use of RUCAM in Asia

There is now increasing awareness of the benefits provided by RUCAM among various countries, including China,[18–38] Korea,[40–54] and Singapore,[55,56] as evidenced by reports initially published in 2004 from Korea[40] and in 2006 from China[18] and Singapore,[55] with subsequent articles (Table 1). On top among the Asian countries is currently China, best explained by the large population and heavy use of herbal traditional Chinese medicines (TCMs), with increasing numbers of publications and cases until 2019.[18–38] Korea ranks at the second position, followed by Singapore in third place (Table 1). Scientists from other Asian countries are more cautious using RUCAM, either to avoid disturbances with the politics of the national TCM-based health system, hospital-related issues, scientific society-based requirements, or that they just prefer their own CAMs (but this should not be the preferred solution and must be declined).

RUCAM essentials

RUCAM has a remarkable scientific run among experts of HILI and RUCAM as an appreciated diagnostic algorithm for assessing causality in liver injury cases, shown alone by the large list of RUCAM-based DILI and HILI cases published until 2015.[15] Additional support for RUCAM came from a recent study of 46,266 DILI cases, which had been assessed for causality using RUCAM and were published from 2014 to 2019.[11] For assessing causality in DILI or HILI cases, no other method exists with such a background of worldwide use and acceptance.[11,15]

Appreciation of RUCAM is also substantiated by the reports evaluated for the current analysis of 11,160 HILI cases (Table 1)[15–56] that are validated by RUCAM for robust causality assessment. RUCAM is continuously used without problems,[11,15] except for some minor questions, addressed and clarified in previous RUCAM publications.[15–17] The updated RUCAM is as good as physicians and assessors are handling this method and strictly apply published recommendations.[15] RUCAM has not been designed for chronic DILI and HILI or when a suspected injury occurs on pre-existing liver disease —both complex conditions where a more accurate approach especially for the timing of the events and the exclusion of alternative causes is needed. Problems were not found at the level of RUCAM itself but rather were related to poor quality case data or the users if they publish incorrect RUCAM-based causality gradings that had been lifted intentionally from possible to probable gradings. Otherwise, a recent analysis showed that RUCAM performs well provided the RUCAM users do a good job.[11]

The philosophy behind creating the original RUCAM of 1993 was to facilitate a valid diagnosis for patients with suspected liver injury. This led to the development of a liver-specific, quantitative, objective, transparent, and structured diagnostic algorithm[12] which was well validated using cases with positive re-exposure tests as gold standard.[13] An update was published later,[15] with two different scales, one for cases of hepatocellular injury (Table 2) and one for the cholestatic or mixed liver injury (Table 3).[15] This updated RUCAM is now in common use and should be applied for future cases replacing earlier versions.[12,14] Occasionally, groups reported the use of RUCAM versions with their own unclear modifications (Table 1), but this attempt must be rejected because such modifications would require a new method validation that has never been provided. A clear unmodified diagnostic algorithm, such as the updated RUCAM of 2016, is essential for complex diseases, as are DILI and HILI, to avoid subjective evaluations and arbitrary conclusions; the RUCAM-based method uniformity will allow

Table 2. RUCAM worksheet for hepatocellular injury

Suspected product: Items for hepatocellular injury	Date: Score	Result
1. Time to onset from the beginning of the drug/herb		
• 5-90 days (rechallenge: 1-15 days)	+2	□
• <5 or >90 days (rechallenge: >15 days)	+1	□
Alternative: Time to onset from cessation of the drug/herb		
• ≤15 days (except for slowly metabolized chemicals: >15 days)	+1	□
2. Course of ALT after cessation of the drug/herb		
Percentage difference between ALT peak and ULN		
• Decrease ≥50 % within 8 days	+3	□
• Decrease ≥50 % within 30 days	+2	□
• No information or continued drug use	0	□
• Decrease ≥50 % after the 30th day	0	□
• Decrease <50 % after the 30th day or recurrent increase	-2	□
3. Risk factors		
• Alcohol use (current drinks/day: >2 for women, >3 for men)	+1	□
• Alcohol use (current drinks/day: ≤2 for women, ≤3 for men)	0	□
• Age ≥55 years	+1	□
• Age <55 years	0	□
4. Concomitant drug(s)/herb(s)		
• None or no information	0	□
• Concomitant drug/herb with incompatible time to onset	0	□
• Concomitant drug/herb with time to onset 5-90 days	-1	□
• Concomitant drug/herb known as hepatotoxin and with time to onset 5-90 days	-2	□
• Concomitant drug/herb with evidence for its role in this case (positive rechallenge or validated test)	-3	□
5. Search for alternative causes Group I (7 causes)	Tick if negative	Tick if not done
• HAV: Anti-HAV-IgM	□	□
• HBV: HBsAg, anti-HBc-IgM, HBV-DNA	□	□
• HCV: Anti-HCV, HCV-RNA	□	□
• HEV: Anti-HEV-IgM. anti-HEV-IgG, HEV-RNA	□	□
• Hepatobiliary sonography / Doppler / CT /MRC	□	□
• Alcoholism (AST/ALT ≥2)	□	□
• Acute recent hypotension history (particularly if underlying heart disease)	□	□
Group II (5 causes)		
• Complications of underlying disease(s), such as sepsis, metastatic malignancy, autoimmune hepatitis, chronic hepatitis B or C, primary biliary cholangitis or sclerosing cholangitis, genetic liver diseases	□	□
• Infection suggested by PCR and titer change for		
• CMV: anti-CMV-IgM, anti-CMV-IgG	□	□
• EBV: anti-EBV-IgM, anti-EBV-IgG	□	□
• HSV: anti-HSV-IgM, anti-HSV-IgG	□	□
• VZV: anti-VZV-IgM, anti-VZV-IgG	□	□
Evaluation of groups I and II		
• All causes-groups I and II – reasonably ruled out	+2	□

(*continued*)

Table 2. (*continued*)

Suspected product: Items for hepatocellular injury	Date: Score	Result
• The 7 causes of group I ruled out	+1	□
• 6 or 5 causes of group I ruled out	0	□
• Less than 5 causes of group I ruled out	-2	□
• Alternative cause highly probable	-3	□
6. Previous hepatotoxicity of the drug/herb		
• Reaction labelled in the product characteristics	+2	□
• Reaction published but unlabeled	+1	□
• Reaction unknown	0	□
7. Response to unintentional reexposure		
• Doubling of ALT with the drug/herb alone, provided ALT below 5×ULN before reexposure	+3	□
• Doubling of ALT with the drug(s)/herb(s) already given at the time of first reaction	+1	□
• Increase of ALT but less than ULN in the same conditions as for the first administration	-2	□
• Other situations	0	□
Total score		

Adapted from a previous report of Danan and Teschke, 2016.[15]

The above items specifically refer to the hepatocellular injury rather than to the cholestatic or mixed liver injury (shown in Table 3).

Total score and resulting causality grading: ≤0, excluded; 1-2, unlikely; 3-5, possible; 6-8, probable; ≥9, highly probable.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CMV, cytomegalovirus; CT, computed tomography; EBV, Epstein-Barr virus; HAV, hepatitis A virus; HBc, hepatitis B core; HBsAg, hepatitis B antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HSV, Herpes simplex virus; MRC, magnetic resonance cholangiography; ULN, upper limit of the normal range; RUCAM, Roussel Uclaf Causality Assessment Method; VZV, Varicella zoster virus.

for valid comparison of case results between countries and continents.

RUCAM evaluates seven key elements characteristic for liver injury, which are individually scored, and their summed score provides a final score and a final causality grading;[15] for instance, final score of ≤0 excludes causality, of 1-2 is unlikely, of 3-5 is possible, of 6-8 is probable, and ≥9 is highly probable. The highest RUCAM-based causality level is not definite as erroneously described in some publications (Table 1) but clearly termed as highly probable,[15] respecting the biological nature-based variability of liver injury and the associated lack of any definite or certain condition. In general, the highest final scores and associated high causality gradings are obtained with complete case data sets and are best achieved by a prospective study design as the primary aim of any causality assessment of liver injury cases.[15] However, and if worse comes to worst, RUCAM is also applicable and prepared for liver injury cases assessed retrospectively, but this commonly leads to low final RUCAM scores and low causality gradings because RUCAM partially disqualifies missing data by low or negative scores to be subtracted from the final score. Low final scores often provide a possible causality grading, and respective cases should not be included in study cohorts of cases with a probable or highly probable causality grading, just to avoid a mix of cases with different causality gradings. Describing clinical features of liver injury cases should be based exclusively on cases with a probable or highly probable causality grading of RUCAM. This certainly applies for evaluations and descriptions of any new diagnostic biomarker, as well.[65] Some diagnostic biomarkers are well established for HILI and DILI, but others came under scientific fire due to recent actions of the European Medicines Agency (known as the EMA) through the correct and official retraction of its earlier Letter of Support to promote biomarker research and use.[65] The retraction by EMA was the consequence of faulty results based on studies misconducted by not-further identified liver injury experts.[11,65] This official retraction represents currently, and in near future, a tricky dilemma for the scientific liver injury community.

Additional notes on HILI in Asia or elsewhere relating to RUCAM are warranted for reasons of clarity and transparency.[66–71] A report of excellence is the careful systematic review on Chinese HILI and the use of RUCAM in 54 cases with high causality gradings, published by Zhang *et al*.[29] A robust diagnostic algorithm, such as RUCAM, is commonly used in cases of DILI[11] and HILI by TCMs,[18–62,66] with more details provided in a recent systematic review on clinical characteristics and outcomes.[66] This analysis compares the quality of three RUCAM-based study cohorts, preferring studies of single case reports which provide clinical data and RUCAM details of each patient with HILI by TCMs. The second choice are studies, which summarize the data of a series of patients with HILI by TCM. The third choice refers to studies of extremely low quality, which report the proportion of HILI by TCM in a mix with all DILI cases. This analysis also showed, for study cohorts with a fairly good case data quality, that RUCAM was used as a diagnostic tool in 97/203 studies (47.8%), whereby 154/203 studies (75.9%) were published in Chinese-language journals, which lacked individual references not open for re-evaluation by peers and without causality gradings; only 2/203 studies were prospective.[66] Consequently, over half of the studies published in China did not benefit from a good CAM, calling for substantial improvement in future cases. Shortcomings are also evident in a USA

Table 3. RUCAM worksheet for cholestatic or mixed liver injury

Suspected product: Items for cholestatic or mixed liver injury	Date: Score	Result
1. Time to onset from the beginning of the drug/herb		
• 5-90 days (rechallenge: 1-90 days)	+2	□
• <5 or >90 days (rechallenge: >90 days) Alternative: Time to onset from cessation of the drug/herb	+1	□
• ≤30 days (except for slowly metabolized chemicals: >30 days)	+1	□
2. Course of ALP after cessation of the drug/herb Percentage difference between ALP peak and ULN		
• Decrease ≥50 % within 180 days	+2	□
• Decrease <50 % within 180 days	+1	□
• No information, persistence, increase, or continued drug/herb use	0	□
3. Risk factors		
• Alcohol use (current drinks/day: >2 for women, >3 for men)	+1	□
• Alcohol use (current drinks/day: ≤2 for women, ≤3 for men)	0	□
• Pregnancy	+1	□
• Age ≥55 years	+1	□
• Age <55 years	0	□
4. Concomitant use of drug(s)/herb(s)		
• None or no information	0	□
• Concomitant drug/herb with incompatible time to onset	0	□
• Concomitant drug/herb with time to onset 5-90 days	-1	□
• Concomitant drug/herb known as hepatotoxin and with time to onset 5-90 days	-2	□
• Concomitant drug/herb with evidence for its role in this case (positive rechallenge or validated test)	-3	□
5. Search for alternative causes	Tick if negative	Tick if not done
Group I (7 causes)		
• HAV: Anti-HAV-IgM	□	□
• HBV: HBsAg, anti-HBc-IgM, HBV-DNA	□	□
• HCV: Anti-HCV, HCV-RNA	□	□
• HEV: Anti-HEV-IgM, anti-HEV-IgG, HEV-RNA	□	□
• Hepatobiliary sonography / Doppler / CT / MRC	□	□
• Alcoholism (AST/ALT ≥2)	□	□
• Acute recent hypotension history (particularly if underlying heart disease)	□	□
Group II (5 causes)		
• Complications of underlying disease(s), such as sepsis, metastatic malignancy, autoimmune hepatitis, chronic hepatitis B or C, primary biliary cholangitis or sclerosing cholangitis, genetic liver diseases	□	□
• Infection suggested by PCR and titer change for		
• CMV: anti-CMV-IgM, anti-CMV-IgG	□	□
• EBV: anti-EBV-IgM, anti-EBV-IgG	□	□
• HSV: anti-HSV-IgM, anti-HSV-IgG	□	□
• VZV: anti-VZV-IgM, anti-VZV-IgG	□	□
Evaluation of group I and II		
• All causes - groups I and II – reasonably ruled out	+2	□
• The 7 causes of group I ruled out	+1	□

(*continued*)

Table 3. (*continued*)

Suspected product: Items for cholestatic or mixed liver injury	Date: Score	Result
• 6 or 5 causes of group I ruled out	0	□
• Less than 5 causes of group I ruled out	-2	□
• Alternative cause highly probable	-3	□
6. Previous hepatotoxicity of the drug/herb		
• Reaction labelled in the product characteristics	+2	□
• Reaction published but unlabeled	+1	□
• Reaction unknown	0	□
7. Response to unintentional reexposure		
• Doubling of ALP with the drug/herb alone, provided ALP below 2×ULN before reexposure	+3	□
• Doubling of ALP with the drugs(s)/herbs(s) already given at the time of first reaction	+1	□
• Increase of ALP but less than ULN in the same conditions as for the first administration	-2	□
• Other situations	0	□
Total score		

Adapted from a previous report of Danan and Teschke, 2016.[15]

The above items specifically refer to the cholestatic or mixed liver injury rather than to the hepatocellular injury (shown in Table 2).

Total score and resulting causality grading: ≤0, excluded; 1-2, unlikely; 3-5, possible; 6-8, probable; ≥9, highly probable.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CMV, cytomegalovirus; CT, computed tomography; DILI, EBV, Epstein-Barr virus; HAV, hepatitis A virus; HBc, hepatitis B core; HBsAg, hepatitis B antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HSV, Herpes simplex virus; MRC, magnetic resonance cholangiography; ULN, upper limit of the normal range; RUCAM, Roussel Uclaf Causality Assessment Method; VZV, Varicella zoster virus.

Food and Drug Administration (FDA) study, which discusses issues of HILI and used the method of the Drug-Induced Liver Injury Network (DILIN)[67] for causality assessment, which comes along without any specific element scoring and provides only arbitrary causality gradings as percentage ranges;[68] additionally, other CAMs were used,[67] known for being not specific for liver injury cases and not based on typical, individually scored liver-related key elements, as amply discussed previously[15] and reiterated recently.[68] No question, the strength of this FDA report would have been increased if the updated RUCAM of 2016[15] would have been used rather than just referencing publications on RUCAM.[67] Critical is also the data source of used cases, which were partially retrieved from the USA's National Institutes of Health LiverTox database,[67] known for inclusion of liver injury cases lacking robust CAMs and being therefore disputed.[69–71]

Liver test thresholds

Liver injury is defined by increased serum activities of LTs: ALT of at least 5 times the upper limit of normal (ULN) and/or of alkaline phosphatase (ALP) of at least 2×ULN, best assessed simultaneously on the day of first presentation, as outlined in 2016.[15] In the original RUCAM of 1993, ALT thresholds were lower, with at least 2×ULN,[13] but should not be applied anymore to ensure exclusion of unspecific liver injury cases.[15] The currently favored ALT and ALP threshold values of 2016[15] have also been considered as perfect in China by Yang *et al.*[72] Therefore, and for reasons of comparability, in future publications on HILI, the use of the current thresholds and their mentioning in the text is urgently recommended, namely ALT ≥5×ULN and ALP ≥2×ULN. In fact, actual threshold information is often lacking in HILI publications (Table 1).[18–56] Disregarding thresholds impedes clear differentiation between liver injury and LT abnormality.[51] As expected, increasing ALT thresholds from ≥3×ULN to ≥5×ULN substantially reduces the case number of true HILI.[73]

Causality grading

RUCAM-based causality gradings are defined with highly probable being the top level.[15] Attempts to modify the commonly used RUCAM gradings must be resisted. For instance, efforts to use the RUCAM gradings concomitantly with the arbitrary percentage ranges of causality gradings have been published, so far being favored by the disputable vague DILIN system, and to incorporate it in the RUCAM algorithm[74]—an approach that will not work. Just the opposite direction should be taken by incorporating the RUCAM-based scoring system in the DILIN method, rendering it then an excellent quantitative CAM, unrelated to the intransparent, subjective global introspection method used currently in the USA. Problematic are also post hoc uptonings of RUCAM-based causality gradings from possible up to probable.[36] In addition and as confirmed in court, intentional uptonings of RUCAM scores from possible to probable gradings invalidate published conclusions,[75,76] disregarding ethics among the scientific community.[11]

Epidemiology

Epidemiology aspects of liver injury remain an issue.[51,54,73,77,78] A low HILI prevalence was found in a large retrospective single center study from Korea, in which 27/4769 patients (0.6%) with musculoskeletal disorders

received TCMs, as reported by Lee *et al.*,[51] with confirmed results through secondary evaluation by the same group.[73] For Korea again, Cho *et al.*[54] reported HILI prevalence results from a nationwide multicenter and prospective study with 6/1001 patients (0.6%). These results, from one single country and presented by two different groups, are surprising and require comments. With 0.6%, identical data of HILI prevalence were achieved;[51,54,73] although, one group used a retrospective design, commonly known for its low case quality,[51,73] whereas the other group followed a prospective protocol.[54] The low prevalence data were achieved by both groups using HILI cases with ALT thresholds of at least 3×ULN, which included many cases with unspecific LT increases.[51,54,73] With higher ALT thresholds of ≥5×ULN, HILI case numbers approached the zero range,[73] signifying that all is now perfectly done, with reasonable results and without the need of further studies. Indeed, since 2017, no other HILI-related reports were published from Korea (Table 1). HILI is seemingly not a problem in Korea,[51,54,73] similar to Germany, considering the low TCM-related HILI incidence data.[77] In that report, liver injury data were derived from a prospective, hospital-based and large-scale study of 21,470 patients who had no liver disease prior to treatment with herbal TCM. Among these, 26 patients (0.12%) experienced HILI on formal grounds, as evidenced by ALT values of ≥5×ULN, but a probable causality was attributable to only 8/26 cases, a possible one to 16/26 patients, and an excluded one to 2/26 cases, using the updated RUCAM.[77]

In China, with around 1.4 billion inhabitants,[36] conditions of HILI are more complex.[36,78] In particular, valid epidemiology data of HILI are not available for the population; although, herbal TCMs are integral constituents of the Chinese health system. An earlier vain epidemiology analysis was not RUCAM-based and used mixed cohorts of injury cases by drugs, herbs, or CAMs.[78] Instead, some improvements were evident in a more recent report, with the title focusing on incidence and etiology of DILI in mainland China, published in a 2019 issue of *Gastroenterology*.[36] At least, it was now recognized that the use of RUCAM, as a valuable diagnostic algorithm, can help assess causality in liver injury cases.[36] However, the respective cohorts were grouped under the term of DILI, and represented still not only DILI but also liver injury cases caused by herbal TCM and herbal dietary supplements, representing two different product categories and again providing conditions similar to the shortcomings of the earlier study[78] and not allowing for characterization of HILI epidemiology features.[36] Nevertheless, some progress is recognizable because other critical shortcomings have been well identified in the text under the limitation section.[36] What's more important, a new version of this study was already promised and will hopefully be published with inclusion of the updated RUCAM of 2016, now being without major flaws and after more careful peer reviews, preventing letters to the Editor. Under the current conditions, no valid statement is warranted on HILI epidemiology in China.[36] Nevertheless, China is well prepared to present valid data on HILI cases, all assessed by RUCAM, as listed in Table 1 and referenced.[18–38]

For future studies on epidemiology, a reminder may be useful: epidemiology includes incidence and prevalence; hence, these two parameters are to be considered separately.[79] The incidence of HILI and, of course, DILI is expressed as the total number of new injury cases during a certain period of time, divided by the number of individuals in the population initially at risk. The prevalence of liver injury by herbs or drugs is calculated as the total number of liver injury cases in the population at a given time, and it represents an estimate of how common liver injury can affect the general population at a fixed time. Consequently, incidence commonly provides information about the risk of acquiring new liver injury; whereas, prevalence signifies how widespread liver injury from herbs or drugs is. Prospective studies will provide best results on the incidence.

Case data quality

With a good study design, high-quality HILI cases are to be expected.[15–17] Only a prospective study design that includes the use of the updated RUCAM[15] will provide valid and complete data of HILI cases, with a high causality grading of probable or better highly probable. Case presentation should follow few principles.[80] No question, the updated RUCAM can be used even for HILI cases obtained from retrospective studies; although, this is not the preferred approach. Based on the present experience, editors of journals should prefer publication of only articles dealing with HILI cases presenting good case data quality obtained prospectively using the updated RUCAM.

Herbal product quality

Basic requirements: Whenever a patient with assumed HILI is further evaluated clinically, one of the key questions relates to product quality, including herb authentication (Table 4). RUCAM is not destined to check for product quality. Of concern are impurities and adulteration by synthetic drugs that might have been added erroneously or intentionally to increase the efficacy of the herbal product.[10,79] The quality of herbal medicines must be evaluated by toxicology methods, such approach is a routine measure in a TCM hospital in Germany, as described previously.[77] In this clinical setting, only herbal TCMs of verified quality are used by the patients under care, raising the question of whether this quality concept contributes to the low number of HILI cases observed under these specific hospital conditions. In addition, the quality of herbs is influenced by other factors[79] that are rarely considered in HILI case analyses published in Asian countries[18–56] or elsewhere, including those in the recent Special Issue on "Drug, Herb, and Dietary Supplement Hepatotoxicity", which presented much information in various articles on liver injury by herbal products.[81] Therefore, these important and so far largely neglected aspects are discussed in much more detail below.

Plant circadian clock system: The Nobel Prize in Physiology or Medicine 2017 was awarded to the three US scientists Michael W. Young, Michael Rosbash, and Jeffrey C. Hall for their discoveries on the molecular mechanisms controlling circadian rhythms (the physiological 24-hour body clock).[82] Their discoveries explain how plants, animals, and humans adapt their biological rhythm so that it is synchronized with the Earth's revolutions. They identified a gene which encodes a protein within the cell during the night that then degrades during the day. Sufficient evidence exists to mandate understanding plant physiology and consideration of plant circadian rhythm in manufacture of good quality herbal products.[82,83] In experimental studies using plant leaves and mimicking the daylight, exposure of ultraviolet-C (short wavelength) to the

Table 4. Proposal for good quality of herbal medicines, safe use, and requirements for regulatory approved herbal drugs

Specific international qualification required for regulatory approved herbal drugs
• Good Agricultural Practices
• Good Manufacturing Practices
• Definition of plant family, subfamily, species, subspecies, and variety
• Definition of plant part
• Definition of solvents and solubilizers
• Lack of impurities, adulterants, and misidentifications
• Minimum of batch and product variability
• Lack of variety to variety variability
• Brand name with details of ingredients, plant parts, batch number, and expiration date
• Manufacturer with address
• Regulatory specification of indication of herbal drug use
• Daily dose with details of the application form
• Maximum duration of herbal drug use
• Efficacy of the herbal drug proven by valid randomized controlled trials
• Description of adverse reactions and their frequency
• Information of risk/benefit profile
• Internationally approved regulatory surveillance
• Regulatory harmonization to use the updated RUCAM in order to assess causality in suspected HILI cases

Abbreviations: HILI, herb-induced liver injury; RUCAM, Roussel Uclaf Causality Assessment method.

Shell ginger (*Alpinia zerumbet*) of the ginger family (*Zingiberaceae*) modulates the relative chemical composition, changes the amounts of essential oils and total phenols, and alters the antioxidant activity.[83] The circadian clock system in plants controls many important metabolic pathways and functions, including photosynthesis, stomatal opening, and molecular processes leading to gene expression.[82] Transcriptional, translational, and post-translational processes are interlocked by feedback loops among morning- and evening-phased genes.[83] Changing circadian rhythms may be an approach to gain improved plant quality, to prevent poor quality, or both.[82,83] Better identifying their pathways and processes that are clock controlled and of benefit for the plants,[84] however, is still a major multidisciplinary challenge of plant chronobiology.

Plant stress: Herbal product quality is also modified by biotic or abiotic plant stress, affecting higher plants.[79] Biotic plant stress by pathogen attacks of other living organisms is caused by insects, larger grazing animals, parasites, bacteria, viruses, and fungi. Instead, abiotic stress is caused by environmental attacks, heavy ultraviolet radiation, draft, wounding, or soil contamination by salts or heavy metals.[83,85,86] At the molecular level, plant stress leads to oxidative stress through generation of reactive oxygen species, damaging the plant's integrity and impairing herbal product quality. This is triggered if radical scavenging chemicals, such as polyphenols, are absent in the plant under injurious stress.

Seasonal variation: Herbal quality is strongly dependent on the harvest time, shown recently as example for the roots of *Cyathula officinalis,* a popular TCM.[87] Using a metabolomic approach based on gas chromatography-mass spectroscopy, 166 metabolites had been identified in these roots, 63 of which showed significant quantitative changes in different growth years of up to 4 years. It was suggested to harvest in the fourth grow year in order to boost herbal quality, and extending these studies to other plants.[87] Such studies about variation of phytochemicals in different harvest times is in line with Good Agricultural Practice standards of Chinese traditional herbs in China. Fixing the harvest year will provide consistency of batches and herbal products with the desired phytochemicals as target ingredients.

Area of harvest: Unexpected were results obtained with PM, harvested from various regions of China and assessed for its hepatotoxic potential.[88] This is an important study, since PM is much used in China and elsewhere, and known for its liver toxicity. These results showed that liver toxicity was obviously different among the various areas of harvest, and the most toxic PM was from the Sichuan Province. It is noteworthy that emodin was not considered the main hepatotoxin anymore, as opposed to previous studies.[88–90] Preference is now given to both tetrahydroxystilbene-O-(galloyl)-hex and emodin-O-hex-sulfate as the primary offending agents.[88]

Case and herb listing: An optimum listing of several individual Asian herbs causing HILI should include cases with RUCAM-based causality assessment and high causality grading. Respective lists presented by authors of Asian countries in English language are scarce, partly due to the focus on DILI cases with neglect of HILI data (Table 1).[18–56] Similarly, in one of the largest studies of DILI with HILI published within the last year, little attention was paid to a separate robust listing of herbs causing liver injury in China.[37] Instead, a comprehensive list was provided by the exceptional study of Zhang *et al.*[29]

A few publications from authors outside of Asia have presented some case and herb listings of Asian HILI but with limited information. For instance, our group published initial lists of HILI by various herbal TCMs, with partially incomplete data regarding causality grading, RUCAM use, or quotation of respective reports.[15,89–95] In one publication of 2014, HILI lists contained herbal TCMs, references, and data of causality assessments using criteria of re-exposure tests but RUCAM-based causality gradings were not provided.[91] In the other report, these gradings were provided for a few HILI cases.[92] Reports of 2015 presented HILI lists of TCM herbs with established causality[93] or a large list of herbal TCMs causing HILI with exact case numbers but without RUCAM-based causality grading.[94] A large list with individual RUCAM-based causality grading for various herbal TCMs was

also published.[95] Reports of 2016 presented case lists of HILI with TCM herbs and causality assessment by RUCAM or positive re-exposure tests[89] or a country-wise case listing of HILI by herbal TCMs with exact numbers and references,[90] and a large list of RUCAM-based injury cases by herbal TCMs, other herbs and drugs, all listed within the publication of the updated RUCAM.[15] From outside of Asia, reports were published by authors of the USA on a few cases of HILI by TCMs.[3,67] The large group of cases included in the first USA report would have benefitted if better stratified regarding RUCAM assessment.[3] In contrast, cases presented by the second group of the USA FDA[67] were partially assessed using the updated RUCAM[15] or a unique, not validated evidence-based method. Cases were also derived from the LiverTox database,[67] with its published problems in assessing a correct causality in liver injury cases.[69–71] Data were also disappointing in another FDA report with attempted focus on the development of a database for herbal and dietary supplement-induced liver toxicity, but herbal TCMs and causality assessment by the updated RUCAM were explicitly not considered.[96]

Not included in this analysis were cases of HILI in association with the use of products derived from *Camellia sinensis*, consumed either as green tea beverage or green tea extracts (GTE) because respective publications by Asian authors are scarce; indeed, it is mainly a problem in Western countries, where many RUCAM-based reports were published on liver injury in connection with the use of GTE.[97,98] Key issues around liver injury by GTE are obviously settled now, as the United States Pharmacopeia and DILIN members finally made it and confirmed that GTE are potentially hepatotoxic by using the updated RUCAM and thereby breaking boundaries to good medicine based on evidence and a diagnostic algorithm in line with artificial intelligence proposals.[99]

Networks and regulatory databases: Generally problematic are reports presented as network data when case presentations and causality assessments are poor.[67,96,100] For instance, a network-based pharmacology study of the HILI potential of traditional hepatoprotective Chinese herbal medicines discusses aspects of liver injury without considering issues of causality assessment like the use of the updated RUCAM.[100] Clearly, shortcomings of methodological requirements invalidate studies like this one. Unexpectedly, not a single case of HILI was found in a retrospective study of adverse events due to complementary health products in Singapore from 2010 to 2016; adverse events were reported to the Health Sciences Authority, and analyzed were overall 147,215 adverse event reports suspected to be associated with pharmaceutical products and complementary health products, which included Chinese traditional medicines.[101] These data are at variance with another Singapore study of liver injury associated with CAM—a review of adverse event reports in an Asian community from 2009 to 2014, in which 10 assessable HILI cases provided weak RUCAM scores from 0 to 2 for 9 patients and a score of 5 for 1 patient.[56] In another report from Singapore, RUCAM was used in 15 HILI patients for causality assessment, whereby all cases reportedly fulfilled all RUCAM criteria but individual RUCAM-based causality gradings were not reported.[55] Data were collected in the course of a prospective study which suggest a causality grading of at least probable due to the expected data completeness. These data again underscore the complexity of accessing valid HILI data within a single country, but the overall conclusion can be reached that HILI is rare in Singapore. The reasons of these promising data are possibly related to the herbal product quality.[101]

Current and resolved controversies

In Korea, a HILI report published in 2015[51] contained shortcomings regarding the use of RUCAM (Table 1). There was intermittently a heavy dispute on the low HILI case frequency—forced by scientific societies, TV, and print press, and overall poor conditions for scientific discussions—but re-evaluation confirmed the initial conclusions and likely settled the disturbances, for now.[73] Focusing on another report[36] and the related Letters to the Editor[102–104] by various DILI experts from China,[102,104] India,[103] and Iceland,[103] discussions have emerged around the reported RUCAM-based DILI and HILI cases[36] but it seems that the problems can well be solved in a new, promised prospective study, whereby the use of RUCAM may again be helpful, now applying its updated version.[36,102–105] The cited problems focused, among others, on the retrospective design of the study[36] and it was argued that results gathered retrospectively do not allow valid conclusions.[102–104] This is why the updated RUCAM calls for prospective use.[15]

Guidelines

For China, guidelines exist with focus on the diagnosis of HILI (Fig. 1),[106] HILI by herbal TCMs,[107] and DILI.[108] Several

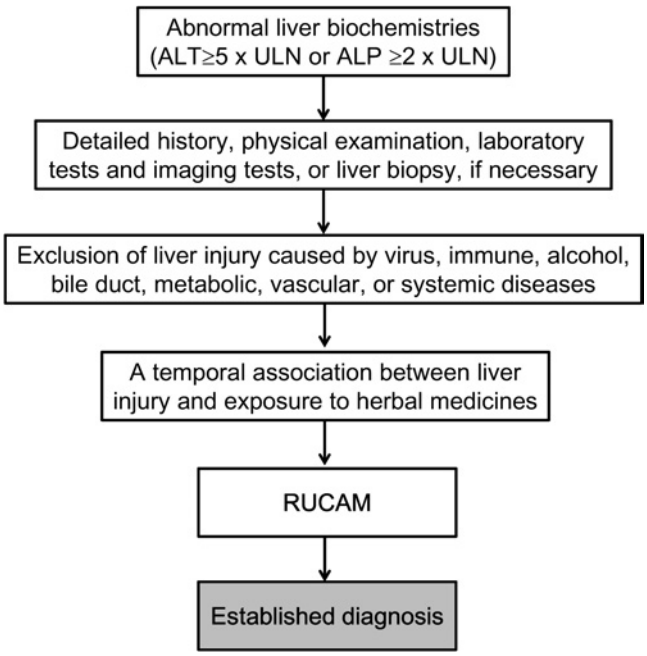

Fig. 1. Flowchart depicting the diagnosis strategy of herb induced liver injury, adapted from the Chinese guidelines for the diagnosis and management of herb-induced liver injury.[106] Thresholds of ALT and ALP are in line with the updated RUCAM.[15] Establishing the RUCAM-based diagnosis of HILI requires RUCAM scores of ≥6 that provide causality gradings of probable or highly probable. Additional search for herbal authentications, adulterations, toxin contaminations, and biomarkers may be needed.[106]
Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; RUCAM, Roussel Uclaf Causality Assessment Method; ULN, upper limit of normal.

criteria are identical, others are variable.[106–108] Therefore, and in future guidelines, some uniformity is desired to facilitate their use. This should include separate listing of RUCAM-based HILI and DILI cases without using concomitantly a non-RUCAM method to avoid confusion, providing RUCAM-based causality gradings for each case (Table 1), identical LT thresholds and liver pattern criteria, evaluating liver injury cases for typical features only if high causality grades such as highly probable or probable have been achieved, and the prospective use of the updated RUCAM with quotation of the corresponding publication.[15] New guidelines should specifically address only diagnostic recommendations using the updated RUCAM and not include clinical data like general liver injury features unless derived from cases assessed for causality by RUCAM with high causality gradings.

Guidelines with the updated RUCAM should also be used for evaluation of liver injury in patients with COVID-19 infections to analyze whether the injury is caused by the virus itself (found in the liver),[109,110] by other factors such as pre-existing liver disease,[18,33,36] or the use of potentially hepatotoxic conventional drugs or herbal TCMs,[33] conditions well described in publications from China.[18,11,36] Finally, since acute respiratory syndrome is a severe complication in these patients, the liver injury could be caused by respiratory insufficiency leading to respiratory hepatopathy due to hepatic hypoxia, in analogy to cardiac hepatopathy, as detailed earlier[111,112] and listed as important differential diagnosis of HILI and DILI.[15]

Conclusions

In Asian countries, herbal medicines are part of the national health system and in use for many centuries, obviously without major problems. More recently, however, much attention has been paid to their adverse effects on the liver. Proposals include: (1) diagnosis of HILI should be improved alongside guidelines that incorporate current ALT thresholds and the use of the updated RUCAM to validly assess causality; (2) for study purposes, a prospective design is urgently needed to prevent fruitless discussions on poor quality HILI publications; and (3) randomized-controlled trials are needed to establish a good benefit over risk balance for safe use by consumers.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (RT, YZ), acquisition of data (YZ, JJ), analysis and interpretation of data (RT, YZ, JJ), drafting of the manuscript (RT), critical revision of the manuscript for important intellectual content (YZ, JJ).

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Review Article

NAFLD Epidemiology, Emerging Pharmacotherapy, Liver Transplantation Implications and the Trends in the United States

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome. The spread of obesity worldwide in pandemic proportions has led to a rapid rise of NAFLD in developed and developing countries alike. There are no approved pharmacological agents to treat steatohepatitis or advanced fibrosis but obeticholic acid recently has shown some promise in phase III trial. Currently, NAFLD is the number one etiology for simultaneous liver and kidney transplantation in the USA, second most common indication for liver transplantation (LT) and projected to become number one very soon. LT for NAFLD poses unique challenges, as these patients are generally older, obese and more likely to have a number of metabolic risk factors. Bariatric surgery is an option and can be considered if a structured weight loss program does not achieve the sustained weight loss goal. Comprehensive cardiovascular risk assessment and aggressive management of comorbid conditions are crucial in the LT evaluation process to improve post-transplant survival. Recurrent nonalcoholic steatohepatitis after LT is not uncommon, and thus warrants primary and secondary prevention strategies through a multidisciplinary approach. Prevalence of NAFLD in a donor population is a unique and growing concern that limits the access to quality liver grafts.

Citation of this article: Gadiparthi C, Spatz M, Greenberg S, Iqbal U, Kanna S, Satapathy SK, *et al.* NAFLD epidemiology, emerging pharmacotherapy, liver transplantation implications and the trends in the United States. J Clin Transl Hepatol 2020;8(2):215–221. doi: 10.14218/JCTH.2020.00014.

Keywords: NAFLD; NAFL; NASH; Liver transplantation.

Abbreviations: ALD, alcoholic liver disease; CLD, chronic liver disease; CVD, cardiovascular disease; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; LT, liver transplantation; NAFLD, nonalcoholic fatty liver disease; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; UNOS, United Network for Organ Sharing; OPTN, Organ Procurement and Transportation Network; T2MD, type 2 diabetes mellitus.

Received: 1 March 2020; Revised: 15 April 2020; Accepted: 5 May 2020

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Introduction

Recent advances in chronic hepatitis B and C therapies, combined with increasing prevalence of the obesity epidemic and of other metabolic disorders, such as type 2 diabetes mellitus (T2DM) and hyperlipidemia, have led to a dramatic rise in nonalcoholic fatty liver disease (NAFLD). Today, NAFLD is a major global health problem and has emerged as the 2nd most common indication for liver transplantation (LT) in the USA, and is projected to become number one soon.[1] NAFLD is a spectrum of liver disease that includes two major types: nonalcoholic fatty liver (NAFL), when there is steatosis, and nonalcoholic steatohepatitis (NASH), when there is significant inflammation. Because of sedentary lifestyle and poor dietary habits, combined with advancing age, prevalence of NAFL and its progression to NASH cirrhosis, liver failure, and HCC, and ultimately the need for LT, are continuing to rise.

While LT is curative and has been shown to improve survival of patients with advanced liver disease of any etiology, there are unique challenges in NASH patients. First, there is no effective pharmacotherapy currently available to halt progression of NASH to advanced fibrosis stages, unlike viral hepatitis. Second, NASH patients are often older, obese and have numerous comorbidities compared to those with other chronic liver disease (CLD) etiologies, thus increasing the risk of mortality during and after LT. Third, increased prevalence of NAFLD in the donor population may adversely affect the availability and quality of liver grafts in future. Finally, recurrent NASH after LT in recipients can negatively affect graft and patient survival. In this evolving landscape, the purpose of this review is to discuss the burden of NAFLD, its risk factors, and its implications on LT.

Epidemiology of NAFLD

As noted previously, due to the growing obesity epidemic now affecting more than 1.9 billion adults globally, NAFLD has become one of the leading causes of CLD.[2] According to recent estimates, NAFLD affects as many as one billion individuals throughout the world. Similarly, in the USA, NAFLD affects nearly 80-100 million individuals, making it the number one etiology of CLD.[3] Nearly 25% of patients with

NAFL progress to NASH; however, the true prevalence of biopsy-proven NASH is difficult to determine, as the majority of NAFL patients do not undergo biopsy. Although the prevalence of NAFLD is increasing throughout the world, there appears to be a significant geographical variation. Overall global prevalence of NAFLD is reported to be 25.2%, according to a recent meta-analysis, with the highest rates being in the Middle East (32%) and South America (31%) and the lowest in Africa (14%).[4] The prevalence is 27% in Asia, followed by 24% in North America, and 23% in Europe.

The prevalence of NASH in the general population is estimated to be in the range of 1.5% and 6.45%.[2] However, the true prevalence of NASH is difficult to ascertain, primarily because of inaccuracies of diagnostic modalities used. Ultrasound fails to identify the mild form of liver steatosis; up to 50-80% of patients with NAFLD may have normal liver enzymes, and the gold standard liver biopsy suffers considerable sampling error.[5] Nevertheless, if the current trend continues, the future burden of NASH and its related complications are only going to rise astronomically. Novel methodologies used in modeling studies have given a glimpse into future projections. For example, in a modeling study utilizing obesity (by body mass index) and incidence of T2DM in eight countries, involving approximately one-quarter of the world's population, Estes and colleagues[6] estimated a significant rise in NASH and related complications by 2030. Specifically, they reported a 63% increase in NASH prevalence, 168% increase in incidence of decompensated NASH cirrhosis, 137% increase in hepatocellular carcinoma incidence, and 178% increase in liver-related death, with overall number of deaths as high as 800,000. Figure 1 summarizes the natural history and progression of NAFL/NASH to related CLD, LT and recurrent NAFL/NASH.

Risk factors of NAFLD

It is very well established that T2DM, obesity and related metabolic syndrome (hyperlipidemia, increased waist circumference, hypertension) play a major role in the pathogenesis of NAFLD. According to a large systematic review, involving 222,816 diabetic patients from 25 countries, the NAFLD prevalence in T2DM patients is as high as 61.1%.[7] Similarly, the prevalence of NASH and advanced fibrosis (≥F3) in biopsied diabetic patients was reported as 64% and 10.4% respectively.[7,8] NAFLD prevalence increases with increasing body mass index[8] and it is estimated that 95% of morbidly obese patients undergoing weight-loss surgery have NAFLD.[9] As the rates of obesity amongst children have risen from 5.0% in 1960 to 16.9% in 2010, NAFLD is increasingly diagnosed in children and adolescents.[10]

Among the non-modifiable risk factors, age, sex and ethnicity are implicated in the pathogenesis of NAFLD. In the USA, NAFLD is most prevalent in Hispanic Americans, followed by non-Hispanic Whites, and is least common in African Americans.[11] According to population studies, NAFLD is more common in males and prevalence increases with age. NAFLD also causes substantial economic impact due to health care dollar spending. The 10-year burden of NAFLD is estimated to reach more than 1 trillion dollars in the USA alone. In Europe, the 10-year burden is expected to reach 334 billion euros.[10]

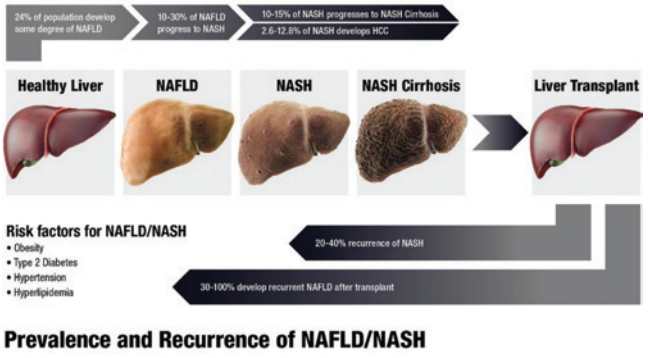

Fig. 1. Natural history of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis and recurrence after liver transplantation.

NASH as an indication for LT

As already noted, NAFLD is now the most common cause of CLD in the USA and Europe and is continuing to rise worldwide.[10] Among the top indications for LT in the USA, based on the United Network for Organ sharing (UNOS) data from 2003-2014, NASH experienced the highest rate of increase (162%) compared to alcohol (55%) and hepatitis C virus (HCV) (33%).[12] Subsequently in 2013, NASH became the 2nd leading indication for LT in the USA.[1] In the same year, the advent of direct acting antiviral agents, a highly effective and safer type of medications, has led to dramatic reduction in chronic HCV disease burden and rates of LT. The decline of HCV prevalence, combined with recent resurgence of alcoholism, has resulted in alcoholic liver disease (ALD) to become the number one cause for LT in the USA, surpassing HCV.[13] However, this trend is not expected to last very long, as NASH, with its current trajectory, is expected to replace ALD and become the leading indication very soon.

Although NASH patients undergoing LT are older and obese compared to those with other etiologies, studies have demonstrated that the short-term and long-term post-transplant survival rates are very similar. For example, 1- and 3-year post-transplant survival rates for NASH LT recipients were 84% and 78% compared to 87% and 78% (p=0.67) for other indications (HCV, ALD, and cholestatic and autoimmune hepatitis). In addition, the 3-year graft survival rate was 76% for NASH LT recipients.[14] In another study, the 5-year survival of LT recipients for NASH was superior to those with HCV (77.81% vs. 72.15%).[12] More recently, in a retrospective study of 26,121 LT recipients with HCC from 2002-2016, NASH patients were older (mean age of 62.9 years) compared to those with HCV (59.2 years), HBV (57.2 years) and ALD (60.6 years), obese (body mass index of ≥30, NASH 60.5% vs. HCV 32.9%, HBV 14.4% and ALD 40%) and more likely to be diabetic (NASH 60.3% vs. HCV 22.5%, HBV 19.3% and ALD 32.7%). The 1-year post-transplant survival rate was similar across all cohorts (p>0.5) but long-term mortality and graft loss were highest in HCV and lowest in HBV.[15]

Although HCV has remained as the most common cause of HCC in LT candidates, NASH was the most rapidly growing cause, with 11.8-fold increase from 2002 to 2016. Figures 2 and 3 demonstrate the temporal trends of annual waitlist additions and LT rates in the USA from 2008-2018 for the top 5 etiologies of CLD based on the most recent UNOS data. These graphs demonstrate a steady and upward trend for NASH-related LT in the USA.

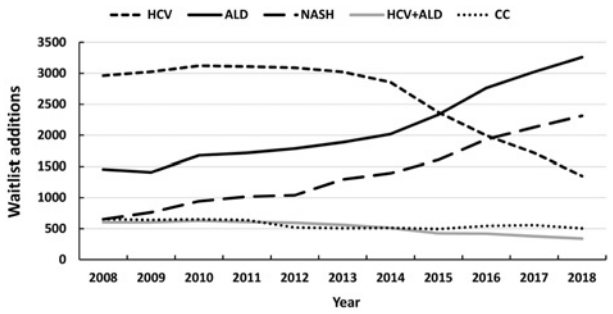

Fig. 2. Temporal trends in annual waitlist additions for top 5 etiologies in the USA UNOS 2008-2018.

Abbreviations: ALD, alcoholic liver disease; CC, cryptogenic cirrhosis; HCV, hepatitis C virus; NASH, nonalcoholic steatohepatitis; UNOS, United Network for Organ Sharing.

Strategies to prevent progression of NAFL/NASH

T2DM, insulin resistance, obesity and other metabolic risk factors are the main factors driving the prevalence of NAFLD and remain the primary targets in its prevention and progression. Lifestyle modifications, such as exercise and a healthy diet, resulting in sustained weight loss are the only proven and effective strategies available currently to curtail the NAFL/NASH burden.[2] NAFLD is considered to be the hepatic manifestation metabolic syndrome. Approximately 70% of patients with T2DM have NAFLD and these diseases share common pathophysiological pathways.

Antidiabetic drugs, as well as statins, can improve biochemical and histological features of NAFL/NASH.[16] Management of comorbidities is not only critical in decreasing progression of NASH but is also pivotal in decreasing cardiovascular mortality, which is the major cause of death in these patients. While weight loss is the single most effective intervention, it can be extremely challenging for patients to achieve the sustained weight loss goal. Structured weight management programs with a multidisciplinary team have had variable success. In morbidly obese patients, who are less likely to be considered for LT surgery, bariatric surgery may be necessary and has not shown to have any negative impact on the LT outcomes.[17] Additionally, bariatric surgery improves and sometimes eliminates other comorbid conditions in many patients and has shown to improve long-term survival from the two most common causes of death in NAFLD, malignancy and cardiovascular disease (CVD).[18]

Recent advances in pharmacotherapy

The two primary endpoints of numerous ongoing clinical trials are resolution of steatohepatitis and improvement in liver fibrosis or both, which are considered surrogate markers for slowing the progression of NASH. Despite the vast knowledge of risk factors and clear elucidation of pathophysiologic pathways in NASH, there has been no significant breakthrough in disease-specific pharmacotherapy yet. However, recent studies have shown some promise. Obeticholic acid, which is already approved for primary biliary cholangitis, is currently under review by the national Federal Drug Administration and could potentially be the first approved medication for NASH. In recent interim analysis of the REGENERATE trial, a phase III multicenter, randomized placebo-controlled study, biopsy-proven NASH patients with F1-F3 fibrosis, treatment with obeticholic acid at 25 mg a day has reached the primary end point of improvement in liver fibrosis score by $\geq$ 1 stage without worsening of NASH at 18 months follow-up compared to placebo (23% vs. 12%, p=0.0002).[19]

A number of other agents, such as cenicriviroc, elafibranor, aramchol and resmetirom, are in the pipeline with phase III trials. Primary end points are fibrosis improvement and prevention of worsening of NASH for cenicriviroc and NASH resolution and prevention of progression fibrosis for elafibrinor.[20] Elafibranor with its favorable safety profile and tolerability makes an attractive choice, but the phase III results of the RESOLVE-IT trial are delayed. Cenicriviroc's phase IIb results were promising, but poor preliminary efficacy results cast doubt over the success of the ongoing AURORA phase III trial.[21] The strong safety and efficacy found in a phase II trial as well as in preliminary results of the phase III MAESTRO-NASH trial has made resmetirom a hopeful alternative.[22] Finally, early results of aramchol are inconsistent but phase III trial is in progress.

Special considerations of NASH in LT

NASH is a multisystem disease associated, with clinical manifestations beyond the liver. NASH patients are at higher risk of mortality and morbidity due to increased prevalence of metabolic comorbidities. Importantly, CVD and malignancies contribute to higher mortality in NASH patients than liver-related morality (cirrhosis and HCC).[23] These factors should be considered in the LT evaluation process.

Cardiovascular risk assessment and management in LT

NASH independently contributes to increased cardiovascular mortality and morbidity, regardless of other cardiovascular risk factors. In addition to coronary artery disease, several other cardiovascular complications are reported in NAFLD patients, such as premature atherosclerosis to left ventricular dysfunction and hypertrophy, aortic sclerosis, congestive heart failure, and cardiac arrythmias (atrial fibrillation and prolonged QTc).[24] Based on the recent meta-analysis by Targher *et al.*,[25] including 34000 patients, presence of NAFLD is associated with 65% increase in fatal and non-fatal cardiovascular events at medial 7-year follow-up period. LT surgery is inherently stressful to the heart because of sudden changes in hemodynamic parameters and furthermore, post-operative complications can unmask underlying clinically silent CVD leading to poor outcomes and increased mortality.[26] Compared to other etiologies, post-transplant cardiovascular events are higher in LT recipients with NASH cirrhosis, especially in the immediate post-operative period.[27] Therefore, several societies recommend comprehensive cardiovascular risk assessment and testing during the LT evaluation process.

While it is important to thoroughly evaluate these patients, it is unclear what constitutes comprehensive cardiovascular evaluation and that itself varies significantly across the LT centers. However, the general approach should focus on identifying underlying CVD, congestive heart failure and portopulmonary hypertension, and optimize these conditions prior to LT surgery and exclude high-risk patients. Patients with clinically significant congestive heart failure should be excluded from LT due to the risk of decompensation and death. High-risk patients with coronary artery disease should

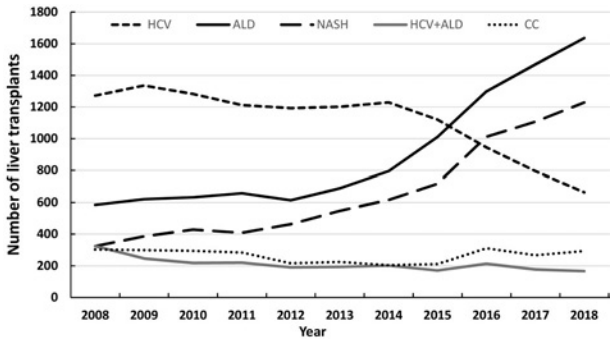

Fig. 3. Temporal trends of annual liver transplantations for top 5 etiologies in the USA UNOS 2008-2018.

Abbreviations: ALD, alcoholic liver disease; CC, cryptogenic cirrhosis; HCV, hepatitis C virus; NASH, nonalcoholic steatohepatitis; UNOS, United Network for Organ Sharing.

either undergo revascularization before transplant surgery or be excluded from the waitlist.[28] Additionally, patients with moderate to severe portopulmonary hypertension who fail to respond to vasodilator therapy are considered high-risk and should be excluded from LT.[29]

Both structural and functional cardiac evaluation is required in LT candidates. Cardiopulmonary exercise testing is challenging because cirrhotic patients may be deconditioned with poor performance status, ascites, malnutrition, and frailty. Doppler echocardiography is routinely performed to assess left and right ventricular and valvular function and to screen for pulmonary hypertension. Noninvasive stress testing is performed using dobutamine stress echocardiogram or myocardial perfusion imaging, and if abnormal, further evaluation with cardiac catheterization may be required. Although, dobutamine stress echocardiogram has shown to be a good noninvasive test to evaluate coronary ischemia in the general population, it suffers from poor sensitivity in cirrhotic patients due to difficulty in achieving target heart rate and double product, perhaps due to use of betablockers for variceal prophylaxis in many patients.[30] Because of these limitations, several centers routinely perform right and left heart catheterization as a part of transplant evaluation process of NASH patients. Although this approach is debated, it may be reasonable in these patients due to their inherently higher cardiovascular risk. Furthermore, this approach allows identifying patients with clinically silent coronary artery disease and provides an opportunity to revascularize them prior to LT. However, it is interesting to note that in a small study of 13 patients, 50% with severe coronary artery disease died due to cardiovascular causes after LT surgery despite undergoing revascularization (3 percutaneous coronary intervention and 6 coronary artery bypass graft surgery) prior to surgery.[31] This suggests that there may be other factors to consider before embarking on LT surgery in NASH patients.

Other traditional risk factors, such as hypertension, hyperlipidemia, T2DM and CKD should be screened and managed appropriately. Despite the small potential risk of hepatotoxicity, statins have far reaching benefits against progression of cirrhosis, portal hypertension and HCC beyond treating hyperlipidemia and thus should be strongly considered.[32] Finally, the LT evaluation process should include a multidisciplinary team approach, including cardiology, cardiac anesthesiology, nephrology, endocrinology and nutrition in addition to hepatology and transplant surgery, to appropriately risk stratify and optimize NASH patients to improve post-transplant outcomes.

Obesity and role of bariatric surgery

It is clear that obesity is highly prevalent in NASH patients. Obesity by itself and high body mass index are not absolute contraindications for LT surgery. Moreover, body mass index is not a reliable indicator and tends to overestimate obesity in the presence of ascites and volume overload. Studies on effects of obesity on LT outcomes have shown conflicting results. In a large meta-analysis published in 2015 that included 13 studies comparing 2275 obese patients with 72212 non-obese LT recipients, body mass index did not negatively impact the post-transplant survival.[33] Because body mass index is a less reliable surrogate for obesity, other parameters such as visceral adipose tissue and muscle mass should be included in the evaluation process to optimally predict post-transplant survival.[34]

Based on the Scientific Registry of Organ Transplants data from 1994 to 2013 that included over 85,000 adult LT recipients, body mass index values did not impact post-transplant survival, whereas T2DM in LT recipients in both pre-transplant (hazard ratio of 1.21, 95% confidence interval of 1.12-1.30) and post-transplant settings (hazard ratio of 1.06, 95% confidence interval of 1.02-1.11) and T2DM in donors (hazard ratio of 1.10; 95% confidence interval of 1.02-1.19) were associated with poor outcomes.[35] Therefore, it is evident that, not obesity alone, but the presence of other metabolic comorbidities in addition to obesity lead to poorer outcomes. Nevertheless, a body mass index of ≥40 is generally considered a relative contraindication for LT in most centers.

Bariatric surgery is a feasible option for morbidly obese patients. Due to lack of data regarding long-term follow-up, the optimal timing of bariatric surgery whether before, during or after LT surgery remains unclear. Although metabolic comorbidities seem to improve with bariatric surgery in the pre-transplant setting, studies have reported significantly higher post-operative complications.[36,37] Concomitant LT and bariatric surgery is an option in highly select patients. In a Mayo study, LT candidates with a body mass index of ≥35 who were unable to achieve weight loss goal in the pre-transplant setting underwent combined LT surgery and sleeve gastrectomy.[38] Compared to the LT alone group, the combined LT-sleeve gastrectomy group achieved more sustained weight loss at 3-year post-transplant follow-up and were less likely to develop insulin resistance, hepatic steatosis and hypertension. However, this approach is limited by prolonged surgery time, immediate need for immunosuppression post-transplant, and risk of poor nutrition status.[39] Overall, based on studies, sleeve gastrectomy appears to be a preferred option compared to Roux-en-Y gastric bypass surgery and has several advantages. Compared to Roux-en-Y gastric bypass, sleeve gastrectomy requires less operative time, does not cause malabsorption or altered anatomy, maintains adequate immunosuppression, and provides endoscopic access to the biliary system in the event of post-operative biliary complications.[26] While weight-loss is a desired goal in NASH patients, it is important to screen and manage malnutrition and sarcopenia, which are independent predictors of poor waitlist and post-transplant mortality. Studies

have shown that up to 25% of obese patients suffer from malnutrition.[40] Therefore, it is very critical to maintain proper nutrition and adequate protein supplementation in obese NASH patients who undergo bariatric surgery or are enrolled in weight-loss programs, to avoid malnutrition and sarcopenia.

Post-transplant outcomes of NASH compared other CLDs

Despite the higher prevalence of cardiovascular risk factors in NASH patients, several studies have shown that post-transplant outcomes of NASH patients are similar to those of other indications of LT. In a large systematic review comparing LT recipients with NASH (n=717) and non-NASH (n=3520), 1-year, 3-year, and 5-year post-transplant survival rates were similar between the two groups, although the NASH patients were older, with higher prevalence of women, had higher body mass index, and were more likely to have T2DM, hypertension and hyperlipidemia compared to non-NASH counterparts.[41] While NASH patients experienced higher mortality due to CVD (odds ratio of 1.65, 95% confidence interval of 1.01-2.70; p = 0.05) and sepsis (odds ratio of 1.71; 95% confidence interval of 1.17-2.50; p = 0.006), graft failure was lower (odds ratio of 0.21; 95% confidence interval of 0.05-0.89; p=0.03) compared to non-NASH LT recipients. Studies based on UNOS data, showed similar results. From 2001 to 2009, comparing 1959 NASH LT recipients and 33,822 non-NASH patients, 1-year and 3-year post-LT survival was similar: 84% and 78% for NASH respectively, 86% and 79% for cryptogenic cirrhosis, and 87% and 78% for other indications (p=0.67).[14] A more recent UNOS study from 2003 to 2014 showed outcomes of 63,061 adult LT recipients, including 20,782 HCV patients (32.96%), 9470 ALD patients (15.02%), and 8262 NASH patients (13.11%). Results of this study demonstrated that 5-year post-transplant survival was better in NASH patients compared to HCV (77.81% vs. 72.15, p<0.001) despite the NASH cohort being more likely to have obesity and higher rates of T2DM and CVD.[12]

In another retrospective study, Sadler *et al.*[42] showed that HCC patients with NASH (60/929, 6.5%) and non-NASH (869/929, 93.5%) had similar 1-year, 3-year, 5-year survival rates (98%, 96%, and 80% respectively in NASH vs. 95%, 84%, and 78% in non-NASH, p=0.1). Overall, based on multiple studies, both single-center as well as those involving large databases, NASH patients, despite the older age and higher prevalence of comorbidities compared to other etiologies, showed similar post-transplant survival. This could, in-part, be explained by better graft survival rates and rigorous LT selection process, where patients with higher cardiovascular risk are excluded from the LT waitlist.[26]

Recurrent NASH after LT

While recurrent NASH is an important complication in LT recipients with NASH, *de novo* NASH is a growing concern in non-NASH LT recipients. There are several reasons for this: patients in the post-transplant setting generally feel well, not in a catabolic state of cirrhosis, and are more likely increase the daily calorie intake, resulting in accelerated weight gain. In fact, use of corticosteroids and other immunosuppressive agents, including calcineurin inhibitors and mammalian target rapamycin inhibitors can result in metabolic derangement and development of obesity, insulin resistance, T2DM, hypertension, and hyperlipidemia.[43–46] Additionally, some studies have suggested that non-NASH indications, such as HCV and ALD, are also associated with development of *de novo* NASH after LT.[47,48] There exists a considerable heterogeneity among the studies that estimated the prevalence of recurrent and *de novo* NASH. In a study, 30% of the LT recipients with NASH developed recurrent steatohepatitis at 1-year; however, none of them developed cirrhosis in long-term follow-up.[49] The rates of recurrent NAFL in another study, comprising 257 NASH/cryptogenic cirrhosis LT recipients, at 1-year, 2-year, 5-year, and 10-year follow-up were higher (8.2%, 13.6%, 24.9%, and 32.9% respectively) compared to non-NASH/cryptogenic cirrhosis LT recipients (3.1%, 5.9%, 9.6%, and 10%).[50] However, the rate of recurrent NASH was much lower, at 5% (13 out of 257), and advanced fibrosis was rare, and in fact, post-LT survival was similar to that in the non-NASH/cryptogenic cirrhosis group. Nevertheless, CVD and infection-related complications were higher in patients with recurrent NAFL. Therefore, these patients should be closely monitored to prevent rapid weight gain, and screened for development of metabolic conditions and managed accordingly.

Presence of NAFLD in donor livers is another challenge, due to overall increasing prevalence of NAFLD in the general population. This ominous trend not only affects the quality and numbers of donor livers but it also may cause delayed or primary graft dysfunction, as well as graft loss and poor recipient outcomes ultimately.[51–54] Steatosis-induced microcirculatory and cellular dysfunction following reperfusion is thought to be a major cause for hepatocyte necrosis and graft loss. Thus, it is important to identify the extent of steatosis in donor livers. Mild steatosis (<30%) in the donor grafts is generally accepted and not associated with poor outcomes compared to more than >30%, which showed poor outcomes at 1-year after transplant.[51,52,54] Some transplant centers routinely perform donor rush liver biopsy prior to LT and discard high-risk grafts; however, this approach may prolong cold-ischemia time and is not widely available in all centers. Nonetheless, donor steatosis does not appear to affect rates of recurrent NASH in NASH-recipients more than non-NASH recipients and therefore, there is insufficient data to recommend different approach in each group at this time.

Conclusions

NAFLD is steadily raising throughout the world and is on a trajectory to become the number 1 indication of LT in the USA. Despite the prevalence of metabolic comorbidities, post-transplant survival for NASH is comparable to other etiologies. Obesity alone is not a contraindication for LT in the absence of other comorbid conditions and body mass index is not a reliable indicator of obesity in the presence of ascites and volume overload. Weight loss surgery before or during LT surgery can be considered in select patients but it is limited only to specialized centers, due to higher complication rates. Prevention of sarcopenia and malnutrition while achieving weight loss is a challenging task. LT cures end-stage liver disease but not the underlying metabolic risk factors associated with NAFLD; therefore, strategies to address these comorbidities are crucial to improve outcomes and prevent recurrence of NAFLD after transplantation. Prevalence of

NAFLD in donor livers is increasing and needs attention to expand the donor pool to meet the growing demand for LT.

Funding

None to declare.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design, acquisition and review of literature, and drafting and approval of the final manuscript (CG, MS and SG), drafting, supervision, critical revision and approval of the final manuscript (UI, SK, SS, AB and AA). All authors were involved in the final approval of the version of the manuscript submitted and have agreed to be accountable for all aspects of the work.

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Review Article

Hepatic Antifibrotic Pharmacotherapy: Are We Approaching Success?

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Abstract

The incidence rate and mortality of liver fibrosis caused by various etiologies are high throughout the world. Liver fibrosis, the subsequent cirrhosis and other serious related complications threaten the health of patients and represent a serious medical burden; yet, there is still a lack of approved methods to prevent or reverse liver fibrosis. Therefore, effective hepatic antifibrotic drugs are urgently needed. The activation and proliferation of hepatic stellate cells are still the mechanisms of fibrosis that remain the focus of therapeutic research. In recent years, significant progress has been made in the development and applicability of antifibrosis drugs. In this review, we summarize the effectiveness and safety of available antifibrosis drugs utilizing different targets. In addition, some characteristics of antifibrosis drugs in phase II and III trials are introduced in detail.

Citation of this article: Chang Y, Li H. Hepatic antifibrotic pharmacotherapy: Are we approaching success? J Clin Transl Hepatol 2020;8(2):222–229. doi: 10.14218/JCTH.2020.00026.

Introduction

Liver cells usually regenerate after injury, but when injury and inflammation persist, the liver cannot regenerate normally and fibrosis will occur. Liver fibrosis is a pathological outcome of the repair response to chronic liver injury caused by any etiology, such as hepatitis B or C virus infection (HBV/HCV), nonalcoholic fatty liver disease (NAFLD), alcoholic steatohepatitis, autoimmune hepatitis, or cholestatic liver disease. Tissue remodeling and repair can lead to the production and deposition of a large number of collagens, fibronectin, undulin, laminin, and other extracellular matrixes (ECMs) and eventually to the formation of scar tissue.[1] Long-term liver fibrosis will promote the accumulation of a fibrous matrix and destroy the normal function and structure of the liver. If left untreated, it will eventually progress to liver cirrhosis or carcinoma, which are the major causes of death due to chronic liver disease. Therefore, there is a dire need for an antifibrotic drug that can not only inhibit the progression of hepatic fibrosis but also reverse its progression.

However, to date, there is no effective chemical drug in the clinic for the treatment of liver fibrosis. Therefore, research on hepatic antifibrotic drugs is a 'hot topic'. At present, the main drug treatment strategies for fibrosis include the treatment of primary diseases, control of the inflammation, regulation of ECM synthesis and degradation, improvement in liver parenchyma cell injury, and apoptosis. Although there are no approved pharmacotherapies for fibrosis, sustained effort and remarkable progress have been made in the research on antifibrosis drugs in recent years, particularly for drugs for NAFLD-related fibrosis. The present review will emphasize the progress that has been made in efficacy and safety of potential drugs for the treatment of fibrosis and highlight underlying challenges in the future.

Activated hepatic stellate cells (HSCs) are still the primary effector cell of fibrosis

Myofibroblasts (MFs) are the main cells that produce ECM (e.g., collagens) in the process of chronic liver cell damage. MFs do not exist in normal liver tissue. The major source of MFs is HSCs, although a small part of MFs comes from portal vein fibroblasts,[2] hematopoietic stem cell fibroblasts, and bone marrow-derived fibrocytes.[3] Interestingly, in the model of cholestatic liver injury, portal vein fibroblasts are the major source of MFs at the onset of injury, but HSCs are still the main source of MFs in the later stages.[4] Nevertheless, it is controversial whether MFs originate from hepatocytes or cholangiocytes by the epithelial-to-mesenchymal transition or endothelial mesenchymal transition.[5]

In the healthy liver, HSCs show a quiescent phenotype. HSCs are located in the space of Disse, accounting for 5-8% of the total cells of the liver.[6] There is much evidence that the activation of HSCs plays a critical role in fibrosis. Transforming growth factor (TGF)-β, osteopontin, and platelet-derived growth factor (PDGF) are the most important cytokines that promote the activation of HSCs and the proliferation of ECM. Many other cytokines and intracellular signal transduction pathways are also involved in the activation of HSCs. Therefore, drugs targeting the activation of HSCs will become a therapeutic strategy for hepatic antifibrosis.

Reducing the number of activated HSCs is essential for reversing and treating liver fibrosis. The three main pathways

Keywords: Antifibrotic agents; Fibrosis; Liver; Reversal; Pharmacotherapy.

Abbreviations: AE, adverse event; ASK1, apoptosis signal-regulating kinase 1; CB1, cannabinoid receptor 1; CCR, CC chemokine receptor; ECM, extracellular matrix; FGF, fibroblast growth factor; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide-1; HBV/HCV, hepatitis B or C virus infection; HSC, hepatic stellate cell; HSP-47, heat shock protein-47; LOXL2, lysyl oxidase-like protein 2; MF, myofibroblast; MMP, matrix metalloproteinase; NA, nucleos(t)ide analog; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor-κB; NK, natural killer; OCA, obeticholic acid; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptor; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase.

Received: 6 April 2020; Revised: 10 May 2020; Accepted: 1 June 2020

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that can help eliminate activated HSCs are the return to quiescent phenotype, apoptosis, and senescence (Fig. 1).[7–9] At present, there is solid evidence that the reversal of HSC activation to the quiescent cell state plays a dominant role.[10] Thus, promoting the apoptosis of HSCs may be a potential antifibrotic target. In addition, multiple other cell types and factors play important roles in the process of liver fibrosis, such as immune cells, particularly macrophages,[11] liver progenitor cells, autophagy,[12] and epigenetics.[13,14] Pathways and signals derived from intrahepatic or extrahepatic events also provide some potential targets for the drug treatment of liver fibrosis.

Pharmacological therapy strategies for liver fibrosis

Currently, with a better understanding of the pathogenesis of fibrosis, an increasing number of potential drugs that reverse fibrosis are in phase II or III trials. Here, we briefly review the current status of promising antifibrotic drugs in clinical trials (Table 1). The following represent the latest advances in pharmacological therapy strategies for antifibrosis and are outlined in Fig. 2.

Curing or controlling the primary disease

There is no doubt that the control or cure of primary liver disease is an efficient and effective way to reverse the progression of fibrosis. Many studies have proven that if the underlying etiology is effectively controlled or eliminated, liver fibrosis can be reversed, the structure and function of the liver can be restored to normal, and the risk of developing cirrhosis and tumors can be decreased.[15]

The most complete clinical evidence comes from chronic viral hepatitis. Clearance of HCV or long-term effective inhibition of HBV with potent nucleos(t)ide analogs (NAs) can effectively reduce and even reverse the progression of fibrosis and cirrhosis.[16,17] It is worth noting that if there has been liver cirrhosis with significant portal hypertension, even after virologic cure, there may still be signs of clinical disease progression in a short period of time, including recurrent complications. In nonalcoholic steatohepatitis (NASH), a loss of up to 10% of total body weight can improve the fibrosis stage.[18] It was observed that, despite sustained virologic response, 8-12% of patients with HCV still showed progress

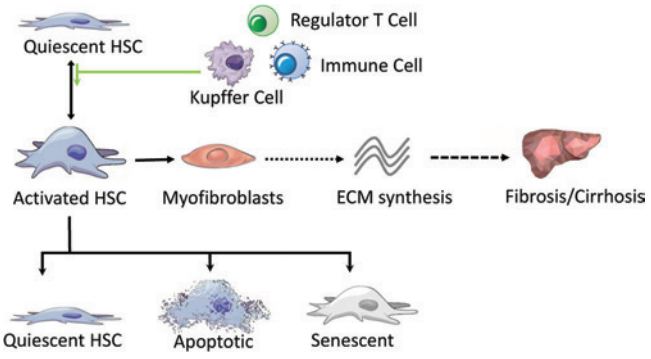

Fig. 1. Pathogenesis of liver fibrosis.

The schematic summarizes the fate of hepatic stellate cells and their role in liver fibrosis.

Abbreviations: HSC, hepatic stellate cell; ECM, extracellular matrix.

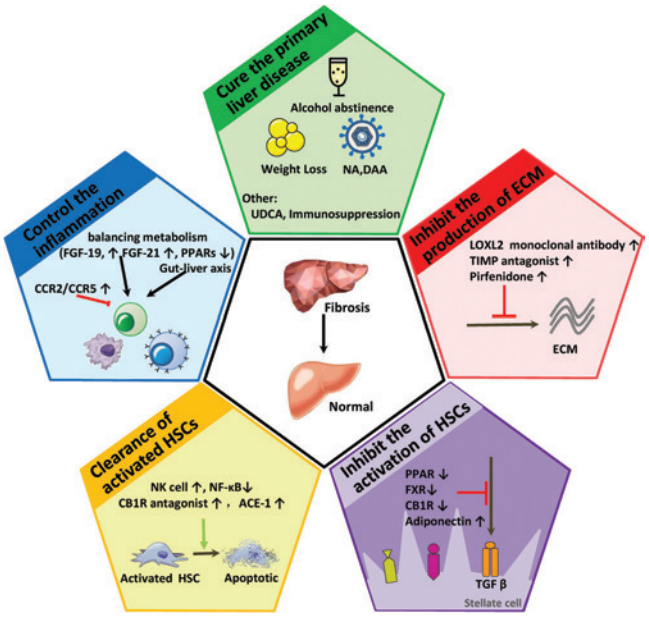

Fig. 2. Pharmacological therapy strategies for hepatic antifibrosis.

Abbreviations: ACE, angiotensin converting enzyme inhibitors; CB1, cannabinoid receptor 1; CCR, CC chemokine receptor; DAA, direct-acting antiviral agents; ECM, extracellular matrix; FGF, fibroblast growth factor; FXR, farnesoid X receptor; HSC, hepatic stellate cell; NA, nucleos(t)ide analog; NK, natural killer; PPARs, peroxisome proliferator-activated receptor; TGF, transforming growth factor; LOXL2, lysyl oxidase-like protein 2; TIMP, tissue inhibitor of metalloproteinase.

in the degree of fibrosis or cirrhosis[17,19] and still retained a 5% risk of liver cancer.[20] The possible cause of fibrosis progression or liver primary cancer is that antiviral therapy starts too late and is more likely to be associated with other underlying liver diseases, most of which are NAFLDs.

In recent years, liver fibrosis and cirrhosis caused by NAFLD have attracted increasing attention, as these will become the major etiologies of liver transplantation or hepatocellular carcinoma in the near future.[21] Insulin resistance, oxidative stress, and metabolic disorders are the main pathological bases for the occurrence of NAFLD and the progression of fibrosis. Many therapeutic strategies and new research drugs for NAFLD fibrosis mainly target reducing insulin resistance or abnormal metabolism to reduce the production of free fatty acids, lipotoxicity, excessive accumulation of triglycerides in hepatocytes, mitochondrial dysfunction, and endoplasmic reticulum stress.[22] Many agonists of receptors for the NAFLD metabolic pathway have been found to be effective in inhibiting fibrosis, such as farnesoid X receptor (FXR) antagonist, peroxisome proliferator-activated receptors (PPARs), and glucagon-like peptide-1 (GLP-1). On the one hand, FXR plays a central role in glucose and lipid metabolism. On the other hand, FXR can also down-regulate the adipogenesis inducer SREPB-1c to induce fibroblast growth factor (FGF) 19 and reduce the production of endogenous bile acids.[23] Obeticholic acid (OCA), a strong FXR agonist, has been demonstrated to improve biomarkers of inflammation and reduce the degree of fibrosis stage in patients with type 2 diabetes and NAFLD.[24,25] Of course, other nonsteroidal FXR ligands, including AKN-083 (Allergan,

Table 1. Promising pharmacological agents for hepatic antifibrosis in clinical trials

Mechanism of antihepatic fibrosis	Drug	Pharmaceutical company	Indication disease	Current phase	Trial name	Participants	Patient	Major AEs
FXR agonist	OCA	Intercept	NASH	III	REGENERATE	Fibrosis	2370	Pruritus; Drug toxicity
					REVERSE	Compensated cirrhosis	540	
CCR2/ CCR5 inhibitor	Cenriviroc	Allergan	NASH	III	AURORA	Fibrosis	2000	Fatigue; Diarrhea
					CENTAUR	Fibrosis (S 1-3)	289	
ASK-1 inhibitor	Selonsertib	Gilead	NASH	III	STELLAR- 3	Advanced fibrosis	800	Nausea, Headache, Nasopharyngitis, Upper abdominal pain, Sinusitis, Back pain, Fatigue
					STELLAR- 4	Compensated cirrhosis	883	
Pyridinone derivative	Pirfenidone	Shionogi	HCV	II	-	Chronic hepatitis C	34	Gastrointestinal, Skin-related AEs
FGF-21 analog	Pegbelfermin	Bristol Myers Squibb	NASH	IIa	-	Fibrosis stage 1-3	75	Diarrhea, Nausea
FGF19 analog	Aldafermin (NGM282)	NGM Biopharmaceuticals	NASH	IIb	-	Fibrosis (S 1-3)	82	Injection site reactions, Gastrointestinal symptoms
			PSC	II	-	Fibrosis	62	
PPAR α/δ agonist	Elafibranor	Genfit	NASH	III	RESOLVE-IT	F2-3	2000	Renal impairment/failure
			PBC	II	-		45	
siRNA against HSP47	BMS-986263	Bristol Myers Squibb	HCV	II	-	METAVIR Stage 3- 4	62	Mild to moderate infusion related reactions

Abbreviations: AEs, adverse events; ASK1, apoptosis signal-regulating kinase 1; CCR2/5, C-C chemokine receptor type 2; FGF, fibroblast growth factor; FXR, farnesoid X receptor; HSP, heat shock protein; NASH, non-alcoholic fatty liver disease; OCA, obeticholic acid; PBC, primary biliary cholangitis; PPAR, peroxisome proliferator-activated receptor; PSC, primary sclerosing cholangitis; siRNA, small interfering RNA.

Dublin, Ireland), troifexor (Novartis, Basel, Switzerland), LMB763 (Novartis), and GS-9674 (Gilead Sciences, Foster City, CA, USA), are also in vigorous experiments and studies, and are expected to become prospects for antifibrosis drugs. In addition to the FXR agonist, PPARs (PPARα, PPARβ/δ, and PPARγ) have been widely tested in NAFLD. Although pioglitazone (PPARγ agonist) has been found to reduce liver fibrosis in patients with NASH without type 2 diabetes,[26] the limitations of patients with heart failure and drug-related weight gain may limit its clinical application for liver fibrosis.

As reviewed above, PPARs can not only improve lipid metabolism and insulin sensitivity but can also reduce liver inflammation. In the phase II trial of 276 patients with NASH without fibrosis treated with elafibranor (PPAR α/δ agonist) for 1 year, the degree of fibrosis did not progress after receiving elafibranor (120 mg/d) versus the placebo group (19% vs. 12%; p=0.045).[27] Although the reported elafibranor was well tolerated, renal impairment (increase in serum creatinine, p<0.001) or renal failure needs to be vigilantly monitored and further observed. The phase III trial of elafibranor for patients with NASH with fibrosis (F2- F3) is ongoing. We look forward to the further results of effectiveness and safety. In addition, a phase II trial (NCT03124108) of the efficacy of elafibranor in patients with primary biliary cholangitis has recently begun. These patients with primary biliary cholangitis are under-responsive to ursodeoxycholic acid. Whether this can reflect the antifibrotic effect is also awaited.

The other most promising categories of antifibrotics for NASH are FGF19 analog or FGF21 analog. FGF19 is a hormone that potently regulates CYP7A1-mediated bile acid homeostasis, inhibits fatty acid synthesis and increases fatty acid oxidation to maintain glucose homeostasis. Aldafermin (formerly NGM282), a FGF19 analog, treatment induced histological improvement in patients with NASH who received subcutaneous 3 mg of aldafermin once daily for 12 weeks (-0.5 fibrosis score, p=0.035). Significant reductions in fibrosis scores and neoepitope-specific N-terminal propeptide of type III collagen are evident in 12 weeks (-22% and -33% in the 1 mg and 3 mg groups, respectively).[28] At least one adverse event (AE) occurred in 93% of patients in the phase II trial for the treatment of NASH. Injection site reactions (34%), diarrhea (33%), abdominal pain (18%), and nausea (17%) were the most common AEs.[29] Some results were also found in patients with primary sclerosing cholangitis after treatment with NGM282. Enhanced liver fibrosis scores were improved in 12 weeks (−0.29, p=0.028, in the 1 mg group; −0.37, p=0.009, in the 3 mg group); however, gastrointestinal symptoms were more frequent in the phase II trial.[30] Also, pegbelfermin, an FGF21 analog, can regulate energy metabolism. In a phase IIa trial, 75 patients with NASH with fibrosis stages 1-3 were treated with pegbelfermin 10 mg or 20 mg once per day. Data from 16 weeks of treatment showed that pegbelfermin led to a decrease in liver stiffness (-6.8%, p=0.0004, in the 10 mg group; -5.2%, p=0.0008, in the 20 mg group). However, some common AEs occurred (16% diarrhea, 14% nausea).[31] Although these side effects are mild, further observation is needed in future trials.

Although liver injury caused by different etiologies determines the initial mode of the liver fibrosis response, the pathological mechanism of fibrosis caused by different injury factors in the late stage of fibrosis is relatively consistent, such as bridging fibers between portal vein regions and cirrhosis.[32] In any case, removal of the causative factor, such as weight loss in NAFLD or suppression of viral replication in hepatitis B/C, is the basic treatment strategy to stimulate regression or reverse fibrosis. It should be noted that fibrosis may continue to progress in some patients in whom we are able to control or cure the primary disease,[17,19] although the control of the primary disease is very effective in the treatment of fibrosis. Therefore, the mechanism of liver fibrosis still needs to be studied, while other strategies for the treatment of liver fibrosis still need to be carried out.

Control of the inflammation

The inflammatory response activates a variety of inflammatory cells and releases inflammatory cytokines, which makes HSCs change from a static state to an activated and proliferative state. Furthermore, it can lead to the deposition of ECM, and at the same time, it can also cause the disorder of liver immune function and further aggravate the injury of hepatocytes. Therefore, the inhibition of inflammation and the immune response are also important links in the treatment of fibrosis. Targeting inflammatory mediators or inhibiting the infiltration of inflammatory monocytes can reduce the formation of fibrosis.

Chemokines released by stress hepatocytes, Kupffer cells, endothelial cells and HSCs can regulate the recruitment of inflammatory cells (monocytes, neutrophils, lymphocytes) in the liver.[33] The chemokine receptor CC chemokine receptor 2 (CCR2) is one of the core drivers of hepatic inflammation and fibrosis.[34,35] It has been observed that the degree of hepatic fibrosis can be inhibited by targeting CCR2 in patients with NASH.[36] The chemokine receptor CCR5 also contributes to fibrosis.[35]

Cenicriviroc, a dual inhibitor of CCR2/CCR5, has been tested in patients with fibrotic NASH, producing exciting results. In phase IIb trials (CENTAUR), a total of 126 patients with NASH with bridging fibrosis and/or NAS ≥5 treated with cenicriviroc 150 mg were observed to yield a reduction in fibrosis. Even though antifibrotic effects have been reported, the safety of cenicriviroc should be considered carefully, as 2.8% of patients experienced fatigue and 2.1% of patients experienced diarrhea in 289 patients at year 1.[37] The antifibrotic effect of cenicriviroc was also shown in the final data at year 2.[38] In addition, a phase III trial (AURORA, NCT03028740) is ongoing, and reports of the side effects of cenicriviroc are worthy of continuous follow-up. In the same way, cenicriviroc can also inhibit inflammation and reduce fibrosis by inhibiting hepatocyte death,[39] balancing metabolism,[40] or regulating the "gut-liver axis",[41] which are also promising treatment strategies.

Other drugs aimed at blocking the recruitment of inflammatory cells such as macrophages, antioxidants, and hepatoprotectants are also in full-swing preclinical trials and may enter clinical development in the near future.

Inhibition of cellular signaling pathways and cytokines to interfere with or block the activation of HSCs

Many experiments have demonstrated the biological efficacy of fibrogenic cytokines that act in an autocrine or paracrine manner. In particular, TGF-β is a master profibrogenic cytokine. The TGF-β proteins comprise 3 isoforms: TGF-β1, 2, and 3. Mechanistically, TGF-β1 is the predominant isoform in the pathogenesis of liver fibrosis.[42] With activated canonical TGF-β signaling, targeted HSCs are transdifferentiated to MFs, inducing ECM production. In fact, inhibiting the

overexpression and activity of TGF-β has become a promising target of antifibrosis therapy.[43,44] However, inhibition of TGF-β almost acts ubiquitously in all organisms, which may induce inflammation or tumors. Thus, limiting TGF-β to directed fibrotic organs has become a challenge.[45] These emerging cellular and signaling pathway mechanisms of liver fibrosis or cirrhosis provide the basis for research on antifibrotic strategies.

In addition, TGF-β's ligand-receptor binding and its signal transduction pathway may become potential targets for antifibrosis therapy. Research on such is in full-swing, and many experiments and clinical trials have already demonstrated that fibrosis can be slowed or reversed by inhibiting the activation of HSCs and regulating the signal-related pathway. Among them, cannabinoid receptor 1 (CB1) antagonist, angiotensin-converting enzyme inhibitor or angiotensin II receptor 1 blocker, endothelin 1 receptor antagonist, tyrosine kinase inhibitor, FXR antagonist, PPAR agonists, vitamin D receptor,[46] and adiponectin have shown potential for antifibrotic therapies. In particular, OCA (an FXR agonist) has demonstrated clinical benefit among patients with NASH in phase III clinical trial,[47] pioglitazone (a PPARγ antagonist),[48] rimonabant (CB1 antagonist) and other drugs are also undergoing trials.[49]

OCA, as described above, might be the most promising drug candidate that reduces fibrosis in patients with NAFLD. Although treatment of patients with NASH with OCA given orally at 25 mg daily for 72 weeks was found to be safe in a phase II (FLINT) trial; of note, 33 (23%) of 141 patients in phase II developed pruritus. Moreover, pruritus in the REGENERATE trial (phase III, NCT02548351) in 1968 patients with NASH with stage F1-F3 fibrosis accumulated (p=0.0002). Moderate to severe pruritus occurred in 336 (51%) patients in the OCA 25 mg group.[47] Other AEs caused by OCA are elevated total cholesterol or low-density lipoprotein. The study of the efficacy and safety of OCA in patients with NASH with compensated cirrhosis in the phase III trial (REVERSE, NCT03439254) is ongoing. Thus, long-term efficacy and safety treatment with OCA need to be further considered.

Although studies of fibrosis pay close attention to intrahepatic cells and signaling pathways, it is important to realize that hepatic fibrosis is also greatly affected by extrahepatic events, including signals from the gut, fat, and muscles. All targeted therapies are effective in preclinical studies. The reason may be that the target is clear, but the actual clinical requirements for drug side effects are also very high and there will be a compensatory mechanism when a single target is blocked. Therefore, a very effective target drug has not been found and commercialized as antifibrotic therapy. In the future, in addition to further intervention with effective targets, combination therapy may also be a possible direction.

Clearance of activated HSCs

Promoting the apoptosis of activated HSCs, deactivation or direct reduction in the number of MFs may prevent the progression of liver fibrosis. The increased expression of nuclear factor-κB (NF-κB) and the antiapoptotic protein Bcl-2 can lead to the continuous activation of HSCs.[50] Drug-induced apoptosis of HSCs by inhibiting NF-κB, including fraxetin (7,8-dihydroxy-6-methoxy coumarin)[51] and 4-hydroxy-2 (3H)-benzoxazolone,[52] has been identified in many animal experiments. Although these drugs may be potential antifibrotic agents, clinical trials have not yet begun. Therefore, it may be a long time before it can be used in the clinic. The clearance of HSCs or MFs by apoptosis can be controlled therapeutically. Recently, a novel molecular therapy that modulates Bcl-x alternative splicing by an antisense oligonucleotide to induce HSC apoptosis may become a potential antifibrosis treatment strategy.[53]

In research, apoptosis signal-regulating kinase 1 (ASK1) can regulate the key apoptosis pathway of HSCs and hepatocytes, as well as the inflammatory signal.[54] Selonsertib can reduce the activation of HSCs, collagen production, activation of inflammatory cytokine pathways and oxidative stress by inhibiting ASK-1.[55] There is heartening evidence that 24 weeks treatment with selonsertib (6 or 18 mg, orally once daily) leads to improvement in fibrosis in patients with NASH with stage 2 or 3 fibrosis in a phase II trial. The proportion of patients with a ≥1 stage improvement in fibrosis was 43% (13/30 patients) in the 18 mg selonsertib group and 30% (8/27 patients) in the 6 mg selonsertib group.[54] Worryingly, many patients experienced at least one or more AEs. The most common AEs were nausea, headache, nasopharyngitis, upper abdominal pain, sinusitis, back pain, and fatigue. In fact, 6.9% of patients experienced serious AEs (5/72), and 4.2% of patients discontinued treatment because of AEs (3/72).[54] However, the phase III study of selonsertib (STELLAR-3 and STELLAR-4) did not meet the primary endpoint that fibrosis stage improvement without the progression of NASH. Selonsertib also did not reduce fibrosis in NASH patients with bridging fibrosis (F3) or compensated cirrhosis (F4) versus placebo at week 48. In STELLAR-3, the primary endpoint was achieved in 12% (p=0.93) of NASH patients with F3 in the selonsertib 6 mg group and 10% of patients (p=0.49) in the selonsertib 18 mg group. In STELLAR-4, the proportion was 14% (p=0.56) and 13% (p=0.93) in patients with F4, respectively.[56] The present data showed no effect on reversing advanced fibrosis, while the serious AEs may also not be conducive to the promotion of drugs.

As an important part of innate immunity, natural killer (NK) cells can kill activated HSCs to enhance the immune surveillance ability of NK cells and activate their scavenging and killing effects, which could be an approach to scavenge activated HSCs.[57] Therefore, the expansion of NK cells may be a new method for the treatment of liver fibrosis. However, hyperactivated NK cells can also lead to the progression of fibrosis by enhancing inflammation in the liver.[58] Thus, understanding the balance of NK cells in regulating HSCs in patients with chronic liver disease can help us design novel antifibrotic therapies. The production of interferon-γ is a marker of NK cell activation and a potent antifibrogenic cytokine contributing to inhibiting fibrogenesis via NK cells. Although the systemic use of interferon-γ has no positive results and interferon-γ-related side effects are inevitable, engineered targeted interferon-γ offers new hope as it can inhibit the activation of HSCs in carbon tetrachloride-induced fibrosis in a mouse model but does not induce related side effects.[59]

Inhibition of the production of ECM and promotion of degradation

Emerging antifibrosis therapy aims to inhibit the production of ECM and/or prevent the deposition of ECM protein. ECM is a critical determinant of cell and tissue function in fibrosis.

Matrix metalloproteinases (MMPs) are a family of more than 24 zinc-dependent endopeptidases that can degrade any component of the ECM.[60] According to their ECM substrate specificity, MMPs have been divided into five categories: gelatinases, collagenases, membrane-type, matrilysins, and stromelysins.[61] MMPs can not only degrade ECM proteins but also act on non-ECM substrates, such as chemokines and cytokines, which can modulate cell inflammation.[62] In the liver fibrosis rat model, carbon tetrachloride and bile duct ligation confirmed that suppressed tissue inhibitor of metalloproteinase (TIMP)-1 expression can inhibit the formation of liver fibrosis by promoting ECM degradation.[63]

TIMP is a family of at least four physiological inhibitors (TIMPs 1-4) that can regulate proteolytic activity in tissues. Chronic inflammation and repeated repair processes lead to excessive accumulation of ECM components, such as collagen, fibronectin and proteoglycans, which are major participants in the formation of scar tissue. Both MMPs and TIMPs are considered to play central roles in the development of liver fibrosis at different time periods. Basic studies have shown that the balance between MMPs and TIMPs plays an important role in the homeostasis of ECM content. In addition, the expression and activity of MMPs and TIMPs are necessary to ensure fibrinolysis during the regression of fibrosis. These are expected to become therapeutic targets for new drugs.

Similarly, lysyl oxidase-like protein 2 (LOXL2) can promote the cross-linking and stabilization of type I collagen, which is the key to the progression or regeneration of fibrosis. Some experiments have shown that it is effective to use the inhibitory monoclonal LOXL2 antibody AB0023 for early treatment in a mouse model of mild liver fibrosis.[64] Although these studies have shown that targeted LOXL2 inhibition is one of the treatments for the prevention or regression of liver fibrosis, it still needs to be tested in clinical trials. Simtuzumab (formerly GS-6624), a monoclonal antibody directed against the LOXL2 enzyme produced by Gilead Sciences, has completed a clinical trial in human immunodeficiency virus- and/or HCV-infected adults with liver fibrosis. However, there was no significant improvement the Ishak fibrosis stage after simtuzumab treatment for 96 weeks (p=0.12 vs. placebo, in the 75 mg Arm; p=0.13 vs. placebo, in the 125 mg Arm).[65]

Studies have shown that pirfenidone can effectively reduce the expression of heat shock protein-47 (HSP-47) and reduce the abnormal accumulation of collagen I and collagen III and down-regulate the expression of collagen II, TIMP-1 and MMP2 by regulating the activity of the TGF-β signaling pathway, effectively reducing collagen deposition by 70%, inhibiting HSC proliferation and serum transaminase levels, and preventing balloon degeneration of hepatocytes. Pirfenidone treatment reduces liver inflammation and fibrosis in patients with HCV. The Ishak fibrosis stage improved two points in 67% (p<0.05) of patients with chronic hepatitis C after receipt of study drugs at the 24-month point.[66] Despite the encouraging results, there are still concerns about the potential AEs associated with pirfenidone.[67] In reports on the treatment of pulmonary fibrosis with pirfenidone, the median time to develop an AE after the use of pirfenidone was 15 days.[68] Gastrointestinal symptoms (nausea, vomiting, and diarrhea) and skin-related AEs (rash and photosensitivity) are the most common AEs caused by pirfenidone. Liver function AEs and fatigue associated with the treatment of pirfenidone also need to be monitored.[69] Thus, further clinical trials are needed to confirm the safety in patients with fibrosis. But more worrying is that pirfenidone exhibited less of an antifibrotic effect in advanced liver fibrosis.[70,71]

HSP-47 plays a conclusive role in the secretion and maturation of collagen and other ECM. BMS-986263 is a targeted lipid nanoparticle delivering HSP-47 small interference RNA. Recently, the efficacy of BMS-986263 was announced at the 2019 meeting of the American Association for the Study of Liver Diseases. Although with a limited number of participants in this trial, the Ishak fibrosis stage was improved in the patients with advanced fibrosis after cure of HCV at week 12 (NCT03420768).

It is critical to recognize that preventing the inhibition of ECM and promoting its degradation can help the treatment of patients with advanced fibrosis or cirrhosis. Continued experimental advances are flourishing, yet most of these studies have not been carried out in humans. Therefore, there may be a long way to go to develop effective antifibrotic drugs by halting the progression or inducing the regression of ECM proteins.

Summary and perspective

Pharmacotherapy for hepatic antifibrotic continues to represent major unmet medical needs. We have summarized the major targets for the most promising pharmacological agents in clinical trials in Fig. 3. Although with different shortcomings, a number of drugs have been investigated in phase III clinical trials and provide great hope for antifibrosis therapy in the future. The research and development of newly emerging pharmaceutics targeting different signaling pathways and targets will be helpful to reduce the burden of chronic liver disease and will reduce the number of hepatic decompensations or HCC. In addition, some traditional Chinese medicines, such as Fuzheng Huayu,[72] Biejia Ruangan[73] and Ganshuang granules,[74] have gratifying antifibrosis effects in China. The effect of these traditional Chinese medicines on reducing liver fibrosis has also been confirmed.[75] However, the treatment of advanced fibrosis and liver cirrhosis may still take longer to complete reversal, and drug research for patients with irreversible liver cirrhosis is also a challenge.

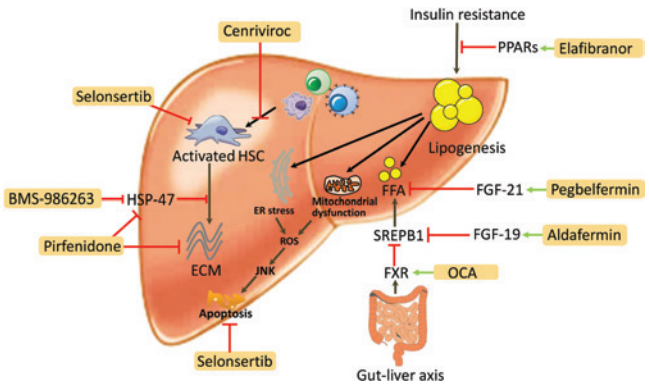

Fig. 3. Major targets for the most promising pharmacological agents in clinical trials.

Abbreviations: CCR, CC chemokine receptor; ECM, extracellular matrix; ER, endoplasmic reticulum; FGF, fibroblast growth factor; FFA, free fatty acid; FXR, farnesoid X receptor; HSC, hepatic stellate cell; HSP, heat shock protein; JNK, c-Jun N-terminal kinase; PPARs, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SREPB1, sterol regulatory element binding protein 1.

The emergence and development of fibrosis is a multifactor, multistep complex process, so it may be difficult to make a breakthrough in the treatment of a single target, a pathway, or a single link. Thus, it is an ideal option to develop a combination therapeutic strategy on multiple pathways. The combination of drugs should involve therapy strategies for curing or controlling the primary disease along with direct as well as indirect antifibrotic approaches. Anyhow, it can be expected that research on antifibrotic drugs will continue to be popular for a long time in the future. We will hopefully witness the success of the strategy of hepatic antifibrotic therapy for further improving the effectiveness and safety of treatment to improve outcomes in the near future.

Funding

This work was supported by the Tianjin Science and Technology Project (15ZXLCSY00040).

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Review of the literature and drafting of the manuscript (YC), critical revision of the manuscript (HL).

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Journal of Clinical and Translational Hepatology [Print ISSN: 2225-0719; Online ISSN: 2310-8819] is owned by the Second Affiliated Hospital of Chongqing Medical University and published by XIA & HE Publishing Inc.

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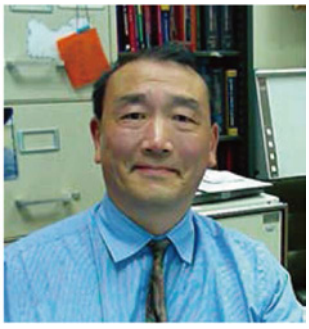
Prof. George Y. Wu (Comprehensive Editor-in-Chief)



Prof. Wu obtained his MD and PhD degree at Albert Einstein College of Medicine in 1976. He was a resident at Harlem Hospital Center from 1976 to 1979. He worked as a postdoctoral fellow at Albert Einstein College of Medicine from 1979 to 1982. From 1983, he worked as Assistant Professor and then Professor at University of Connecticut School of Medicine. He is now the Director of Hepatology Section, Division of Gastroenterology-Hepatology. Dr. Wu's awards include the following: Research Prize awarded by the American Liver Foundation in 1982; Industry Research Scholar Award from the American Gastroenterological Association for 1985 to 1988; Gastroenterology Research Group Young Scientist Award from the American Gastroenterological Association in 1990; Herman Lopata Chair in Hepatitis Research from 1992 to date; Scientific Award from the Chinese American Medical Society in 1992; He was elected to membership in exclusive societies: American Society for Clinical Investigation in 1989; Association of American Physicians in 1995; and Top Doctor in the U.S. awarded by U.S. News and World Report in 2011. He has published about 180 peer-reviewed academic articles, 11 books, and is series editor for Clinical Gastroenterology book series by Springer-Nature, and is Senior Associate editor of J. Digestive Diseases.

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Updated on June 25, 2020

Xia & He Publishing

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E-mail: service@xiahepublishing.com
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